Microglial sculpting of prefrontal cortical synapses during development and the reduced neuropil hypothesis of schizophrenia

By

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To my loving family,

Karyn, Lynne, and Prakash Mallya,

for their unwavering and unconditional support

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ABBREVIATIONS

6-OHDA	6-hydroxydopamine
Aldh1L1	aldehyde dehydrogenase 1 family, member L1
AMPA-R	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
APD	antipsychotic drug
BBB	blood-brain barrier
C1q	complement component 1q
C3	complement component 3
C4	complement component 4
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CD47	cluster of differentiation 47
CNS	central nervous system
CR3	complement receptor 3
CSF1	colony-stimulating factor 1
CSF1R	colony-stimulating factor 1 receptor
CX_3CL1	fractalkine
CX_3CR1	fractalkine receptor
DAP12	DNAX activation protein of 12 kDa
dLGN	dorsal lateral geniculate nucleus
DT	diphtheria toxin

DTR	diphtheria toxin receptor
E	embryonic day
EMPs	erythromyeloid progenitors
GABA	γ-aminobutyric acid
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GLT-1	glutamate transporter 1
HRP	horseradish peroxidase
-ir	immunoreactive
Iba1	ionized calcium binding adaptor molecule 1
IFN	interferon
IL	interleukin
ip	intraperitoneal
Irf8	interferon regulatory factor 8
kDa	kilodalton
L3	Layer 3
L5	Layer 5
LTP	long-term potentiation
LY	Lucifer Yellow
MD	mediodorsal nucleus of the thalamus
MEGF10	multiple epidermal growth factor-like domains protein 10

MERTK	proto-oncogene tyrosine-protein kinase MER
mGluR2/3	metabotropic glutamate receptors 2 and 3
MHC	major histocompatibility complex
mPFC	medial prefrontal cortex
NMDA-R	N-methyl-D-aspartate receptor
OD	optical density
P	postnatal day
PBS	phosphate-buffered saline
PCP	phencyclidine
PCs	pyramidal cells
PET	positron emission tomography
PFA	paraformaldehyde
PFC	prefrontal cortex
PSD-95	postsynaptic density protein 95
RGC	retinal ganglion cell
sc	subcutaneous
SDS	sodium dodecyl sulfate
Siglec-H	sialic acid-binding immunoglobulin-like lectin H
SIRPα	signal regulatory protein α
TBS	Tris-buffered saline
TBS^{++}	Tris-buffered saline + 4% normal horse serum + 0.2% Triton X-100

TBST	TBS + 0.2% Tween-20
TGF-β	transforming growth factor beta
Tmem119	transmembrane protein 119
TNF-α	tumor necrosis factor-α
Trem2	triggering receptor expressed on myeloid cells 2
TSPO	translocator protein
VGAT	vesicular GABA transporter
VGluT	vesicular glutamate transporter
VTA	ventral tegmental area

CHAPTER 1

SCHIZOPHRENIA

Schizophrenia is a chronic and debilitating disease that affects $\sim$1% of the population worldwide (Lewis and Lieberman, 2000). Although schizophrenia has a strong genetic load, studies of monozygotic twins, including those reared apart, consistently reveal concordance rates of $\sim$50% (Gottesman, 1991). A very large number of susceptibility genes for schizophrenia have been identified, but the relative contribution of each confers only a small degree of risk, and most are associated with psychosis rather than schizophrenia specifically (Escudero and Johnstone, 2014; Giusti-Rodríguez and Sullivan, 2013; International Schizophrenia Consortium et al., 2009; Kavanagh et al., 2015; Purcell et al., 2014). Thus, schizophrenia is not fully explained by genetic susceptibility, but depends on an interaction between genetic (or epigenetic (Kozlenkov et al., 2018; Rhie et al., 2018)) and environmental factors.

The symptoms of schizophrenia have been grouped into three categories: positive, negative, and cognitive. Positive symptoms are behavioral changes that are expressed or overt, such as hallucinations and delusions. Conversely, negative symptoms are noteworthy for the absence of expression, and include anhedonia, blunted affect, and social withdrawal. The cognitive symptoms, which include deficits in working memory, attention, and executive function, are a core feature of the illness and have increasingly garnered attention. Cognitive deficits are relatively stable across time, persist during the remission of positive symptoms, and are correlated with poor functional outcome (Green, 1996; Nuechterlein et al., 2011, 2014); they are also largely non-responsive to pharmacological treatment (Keefe et al., 2016). Accordingly, there is an urgent need to better understand the neurobiological bases of these cognitive deficits. Importantly, a variety of anatomical, biochemical, and functional changes in the prefrontal cortex (PFC) are present in schizophrenia, with many of these changes proposed to explain the cognitive processes

that are disrupted in schizophrenia (Berman and Weinberger, 1991; Davis et al., 1991; Deutch, 1992; Goldman-Rakic, 1999; Goldstein and Deutch, 1992; Keefe and Harvey, 2012; Lewis and Moghaddam, 2006; Woodward and Heckers, 2015). Because cognitive deficits do not respond or respond minimally to pharmacotherapy, considerable effort has been devoted to elucidating their underlying causes.

The cognitive deficits and negative symptoms of schizophrenia share certain characteristics (such as prevalence and course) and their independence has been the subject of considerable debate (see Harvey et al., 2006). Although primary negative symptoms can influence the outcome of assessments of cognitive dysfunction, current thought is that they are separable domains (Harvey et al., 2006). However, most studies of cognition in schizophrenia do not determine the degree to which such changes may be secondary to negative symptoms or other domains (Chan et al., 2015). Moreover, although there is a large body of data suggesting that dysfunction in the prefrontal cortices underlies several of the cognitive symptoms in schizophrenia, localization of the source of negative symptoms tentatively suggests different origins (Brady et al., 2019; Chuang et al., 2014; Menon et al., 2001; Padmanabhan et al., 2015).

1.1 Neuropathology of Schizophrenia

Attempts to define structural brain alterations in schizophrenia during much of the 20th century failed to reveal consistent neuropathological changes. The state of affairs was so bad that the neurologist Plum (1972) famously referred to the field as "the graveyard of neuropathologists," with Harrison (1999) subsequently commenting that the field was noteworthy for "generating more heat than light and being notable for memorable quotes rather than durable data." Fortunately, the last quarter of the 20th century saw the application of quantitative neuroanatomical methods to neuropathological studies and the advent of contemporary *in vivo* imaging methods. These advances allowed researchers to detect subtle but consistent anatomical

changes in the brain in schizophrenia and led to the claim that "no longer can there be doubt that there is underlying brain pathology" (Weinberger, 1995), fulfilling the view of Kraeplin (1971) that schizophrenia is a brain disorder. Although some suggest that these claims may be a bit optimistic (see Fusar-Poli and Meyer-Lindenberg 2016), meta-analyses of volumetric changes in brain as well as longitudinal imaging studies of brain and ventricular volume point to structural changes in schizophrenia, although the relative contributions of potential confounds remain to be determined (Heilbronner et al., 2016; Kambeitz et al., 2015; Olabi et al., 2011; Vita et al., 2015).

Neuroanatomical Changes

Early *in vivo* imaging studies using pneumoencephalography were a headache for investigators as well as subjects, yielding inconsistent results (see Haug, 1982). The development of new methods that allowed *in vivo* imaging of the brain in living subjects was soon followed by a seminal computed tomographic imaging study by Eve Johnstone and colleagues (1976), which reported clear evidence of ventricular enlargement in schizophrenia. Although it was initially suspected that the increase in ventricular size might reflect disease progression (see Weinberger, 1987), subsequent studies in first-episode patients firmly established that ventricular enlargement is present in schizophrenia (Shenton et al., 2001; Vita et al., 2006).

If the ventricles are enlarged, yet the brain is encased in an unyielding skull, what "gives"? Volumetric imaging studies have consistently revealed a decrease in gray matter volume in schizophrenia (Lawrie and Abukmeil, 1998; Shenton et al., 2001). These findings are corroborated by postmortem findings of reduced cortical thickness (Goldman et al., 2009). Although changes in gray matter volume or cortical thickness are not present in all afflicted subjects, group differences in ventricular enlargement, gray matter volume, and cortical thickness, particularly in the prefrontal and medial temporal cortices, are consistently observed in schizophrenia, including in first-episode and antipsychotic drug (APD)-naïve patients (Borgwardt et al., 2008; Fusar-Poli et al., 2012a; Leung et al., 2011; Song et al., 2015). Such changes have

even been reported in subjects deemed at high risk for developing the illness, although only a minority of high-risk individuals subsequently develop schizophrenia, with up to a 36% conversion rate over 3 years (Cannon et al., 2008; Fusar-Poli et al., 2012b), consistent with enlarged ventricles not being specific to schizophrenia. Recent data strongly point to lower "conversion" rates than originally reported (Addington et al., 2017; Cornblatt et al., 2015; Seidman et al., 2016).

The loss of cortical volume and thickness suggests that there may be a loss of cortical neurons in schizophrenia. However, unbiased counts of the total neocortical neuron number (Pakkenberg, 1993), as well as determination of the number of neurons in the PFC (Thune et al., 2001), have uncovered no difference in neuron number. Instead, neuronal density is increased (Selemon et al., 1995, 1998, 2003), leading Selemon and Goldman-Rakic (1999) to propose the reduced neuropil hypothesis of schizophrenia, in which they posit that the decrease in cortical volume in the face of a normal complement of neurons occurs secondary to a decrease in neuropil, including dendrites and axons.

Early studies of cortical gene expression were consistent with this hypothesis, reporting a loss of synapse-associated genes (Mirnics et al., 2000). Also consistent with the reduced neuropil hypothesis were studies of neuronal morphology that consistently revealed dystrophic changes in dendrites (Broadbelt et al., 2002; Glausier and Lewis, 2013; Kalus et al., 2000; Konopaske et al., 2014; Moyer et al., 2015). In particular, there is a decrease in the density of dendritic spines on PFC pyramidal cells (PCs) in schizophrenia (Garey et al., 1998; Glantz and Lewis, 2000; Konopaske et al., 2014; Shelton et al., 2015), but not on PCs in a psychiatric control group primarily comprised of subjects with mood disorders who were treated with APDs (Glantz and Lewis, 2000). Some studies have reported that the decrease in pyramidal cell spine density occurs selectively in deep layer 3 (L3) cells (Glantz and Lewis, 2000; Kolluri et al., 2005), consistent with a reported decrease in soma size of L3 PCs (Glantz et al., 2000; Pierri et al., 2001; Rajkowska et al., 1998). Prefrontal cortical PCs appear to be most vulnerable to spine loss, but a less pronounced decrease in spine

density has also been reported in the primary auditory cortex (Parker and Sweet, 2018; Sweet et al., 2009). In contrast, there appears to be no significant change in spine density of PCs in the visual cortex of schizophrenic individuals (Glantz and Lewis, 2000).

Because dendritic spines are the primary site of excitatory inputs onto the PC, the loss of spines on PCs may significantly disrupt excitatory signaling to corticofugal pathways. Unfortunately, there have been few studies determining if dendritic spine density is correlated with cognitive performance (see Cahill et al., 2009; Hains et al., 2009; Kim et al., 2013).

Comparing the shapes of dendritic spines that are present on PFC PCs of control and schizophrenic individuals may offer some insight into function. Morphological parameters have long been used to categorize spines into different classes, including spines that anatomists have described as thin-, stubby-, and mushroom-shaped (Peters and Kaiserman-Abramof, 1970). These adjectives are of limited utility: various parameters of spine shape (such as spine head diameter, which should be larger in mushroom than thin spines) show considerable overlap across different types of spines (Arellano, 2007). Nonetheless, thin spines, which are relatively long and lack a wide head, have been advanced as being more likely to lack α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA-Rs), i.e., have functionally silent synapses (Kerchner and Nicoll, 2008; Nimchinsky et al., 2002; however, see also Busetto et al., 2008), while larger, mushroom-shaped spines are thought to be mature (Knott and Holtmaat, 2008). Recently MacDonald et al. (2017) suggested that thin spines are preferentially lost in the auditory cortex in schizophrenia and proposed on the basis of these observations that there is a deficit in newly-formed spines in the cortex. Similar studies of the PFC have yet to be conducted.

If dendritic spines are decreased in number, there may be corresponding decreases in the presynaptic partners of lost spines. However, postmortem studies of changes in presynaptic elements in schizophrenia, including examination of proteins involved in vesicular trafficking and release, have yielded conflicting results (Castillo et al., 2010; Eastwood et al., 2000; Fung et al., 2011; Gil-Pisa et al., 2012; Glantz et al., 2000; Glantz and Lewis, 1997; Halim et al., 2003; Honer

et al., 2002; Karson et al., 1999; Katrancha and Koleske, 2015; Mirnics et al., 2000). Several factors may contribute to this lack of consistency, including different dependent variables (mRNA vs. protein levels), APD treatment, and differences in the areas and layers of the cortex being sampled. Still another reason for the conflicting data may be that most studies examining presynaptic changes have analyzed markers of synaptic release common to all neurons, thereby capturing both inhibitory and excitatory presynaptic elements. Because presynaptic axons that synapse with spines are excitatory, more consistent results emerge when excitatory inputs are analyzed separately: there is a decrease in cortical levels of the glutamatergic marker vesicular glutamate transporter (VGluT) 1 but not of another vesicular glutamate transporter, VGluT2 (Bitanihirwe et al., 2009; Eastwood and Harrison, 2005; Oni-Orisan et al., 2008). Because VGluT1 and VGluT2 are mainly expressed by cortical and subcortical glutamatergic neurons, respectively (Fremeau et al., 2001, 2004a; Kaneko and Fujiyama, 2002), synapses formed by different afferent sources defining different circuits with distinct PCs may be compromised in schizophrenia.

Neurochemical Changes

Two neurochemical hypotheses have been dominant in guiding schizophrenia research: the dopamine and the glutamate hypotheses, which in their simplest guise posit altered dopamine and glutamate function in schizophrenia, respectively. There have been several reformulations of both hypotheses.

The dopamine hypothesis (Matthysse, 1974) has historically played the dominant role in guiding research into the pathophysiology of schizophrenia. This hypothesis, initially based on similarities between amphetamine-induced psychosis and schizophrenia and the emergence of extrapyramidal side effects in patients treated with neuroleptics (Casey, 1996; Miller et al., 1998; Shirzadi and Ghaemi, 2006), was solidified by the discovery that antipsychotic drug potency (as reflected by average daily dose or plasma drug levels) correlates with striatal dopamine receptor

affinity (Creese et al., 1976; Seeman et al., 1976). This hypothesis quickly gained widespread acceptance. However, it soon became apparent that although this correlation explained the overt (positive) symptoms of the illness (Lieberman and Stroup, 2011), it failed to explain the lack of efficacy of APDs on other symptoms of schizophrenia, including negative symptoms and cognitive disturbances (Keefe et al., 2007). Moreover, when the dopamine hypothesis was initially advanced the dopamine innervation of the cortex had yet to be discovered; this left open the possibility that the cognitive disturbances and negative symptoms of schizophrenia might be related to extra-striatal dopamine function.

Davis et al. (1991) put forth a revised dopamine hypothesis suggesting that although increased dopaminergic tone in the striatum underlies psychosis (e.g., positive symptoms), a decrease in dopaminergic function in the PFC contributed to negative and cognitive symptoms. Deutch (1992) similarly suggested that PFC dysfunction subserved cognitive and perhaps negative symptoms and posited that dopamine loss in the PFC transsynaptically increased the responsiveness of the (ventral) striatal dopamine innervation to mild perturbations, leading to increased phasic release of dopamine in the striatal complex (Deutch et al., 1990; Deutch and Roth, 1991; see Goldstein and Deutch, 1992; Rosin et al., 1992).

Anatomical data are consistent with a partial loss of the dopamine innervation of the PFC in schizophrenia (Akil et al., 1999), and functional studies have shown that decreases in prefrontal cortical dopamine disrupts working memory (Arnsten and Li, 2005; Goldman-Rakic, 1996; Sawaguchi and Goldman-Rakic, 1991; Williams and Goldman-Rakic, 1995), a core cognitive deficit of schizophrenia. Furthermore, dopamine regulates dendritic structure in the PFC: dopamine depletion in rodents results in a decrease in dendritic spine density on PFC PCs (Wang and Deutch, 2008), recapitulating a key neuropathological change of schizophrenia (Broadbelt et al., 2002; Glausier and Lewis, 2013; Kalus et al., 2000; Konopaske et al., 2014; Moyer et al., 2015).

The revised dopamine hypothesis addressed several of the challenges to the original

dopamine hypothesis, and posits a cortical deficit in dopamine that results in cognitive symptoms. Consistent with this formulation of the dopamine hypothesis is the fact that treatment with APDs, which target the dopamine D2 receptor, does not significantly reduce cognitive deficits in schizophrenia (Keefe et al., 2007). Subsequently, it was determined that prefrontal cortical dopamine D1 receptor binding is elevated in APD-naïve individuals with schizophrenia (Abi-Dargham et al., 2012). Among these cognitive changes are working memory, attention, and executive function, which are thought to arise from dysfunction of the PFC.

Glutamate is a critical determinant of dendritic spine number and morphology (Bloodgood and Sabatini, 2007; Korkotian and Segal, 1999, 2000; McKinney, 2005; Passafaro et al., 2003). Homeostatic levels of glutamate are important in maintaining spine stability, with sharp increases in extracellular glutamate causing retraction of the spine by eliciting large transient increases in intra-spinous Ca^{2+} levels (Segal et al., 2000). A role for glutamate dysfunction in the pathophysiology of schizophrenia was originally suggested on the basis of reports of decreased glutamate levels in the cerebrospinal fluid of patients with schizophrenia (Kim et al., 1980), although this finding proved difficult to replicate (Perry, 1982). This difficulty is not surprising when one considers the dual roles of glutamate in intermediary metabolism (in all cells) and as the major excitatory neurotransmitter in the central nervous system (CNS).

Subsequent studies of psychotomimetic agents resurrected the glutamate hypothesis of schizophrenia. Very early studies of the non-competitive N-methyl-D-aspartate receptor (NMDA-R) antagonist phencyclidine (PCP), developed as an anesthetic agent, revealed psychotomimetic effects of the drug (Luby et al., 1959). The introduction of PCP into clinical practice and the resulting clinical experience with the drug ultimately led to withdrawal of PCP from clinical practice. Ketamine, developed to replace PCP, also had psychotomimetic effects. The psychotomimetic effects, however, proved to be valuable with the recognition that ketamine administration could serve as a model of schizophrenia: low doses of ketamine elicited positive, negative, and cognitive symptoms of schizophrenia in healthy subjects (Adler et al., 1998, 1999;

Krystal et al., 1994; Lahti et al., 2001; Newcomer et al., 1999). Moreover, even lower doses of NMDA-R antagonists, which did not produce psychotomimetic effects in healthy control subjects, acutely exacerbated symptoms in individuals with schizophrenia (Lahti et al., 1995; Malhotra et al., 1997), suggesting that these agents may affect already vulnerable or compromised mechanisms.

In its simplest form, the glutamate hypothesis posits NMDA-R hypofunction or an increase in glutamatergic tone. However, animal studies support a model of cortical disinhibition (Moghaddam and Krystal, 2012), in which ketamine and similar NMDA-R antagonists selectively target NMDA-Rs expressed on γ-aminobutyric acid (GABA)-containing interneurons. This decrease in GABAergic firing leads to disinhibition of cortical PCs and a subsequent increase in PC firing and glutamate release, culminating in glutamate signaling through AMPA-Rs rather than NMDA-Rs, which may in turn elicit spine retraction and loss (Elsworth et al., 2011a, 2011b; Hajszan et al., 2006; Ruddy et al., 2015; Figure 1). Thus, disruption of glutamatergic transmission and neuronal signaling in the cortex likely contributes to some of the core cognitive deficits in schizophrenia (Gonzalez-Burgos and Lewis, 2012; Greene, 2001; Jackson et al., 2004; Moghaddam and Javitt, 2012; Moghaddam and Krystal, 2012).

Though the basic question of whether changes in glutamate lead to changes in dopamine in schizophrenia, or vice versa, remains unanswered, interactions between dopamine and glutamate have been extensively explored. For example, dopamine impacts both presynaptic glutamate release (Yamamoto and Davy, 1992) and postsynaptic responses to released glutamate (Gonzalez-Islas and Hablitz, 2003; Wang and O'Donnell, 2001); similarly, glutamate induces dopamine release (Usun et al., 2013; Wang, 1991). Extracellular PFC glutamate and dopamine is increased by both ketamine (Moghaddam et al., 1997) and PCP (Adams and Moghaddam, 1998). Further, ketamine elicits spine loss in rodents (Ruddy et al., 2015) and PCP has been shown to cause dendritic spine loss in both rodents (Elsworth et al., 2011b; Hajszan et al., 2006) and primates (Elsworth et al., 2011a). Interestingly, dopamine depletion of the PFC in rodents, which

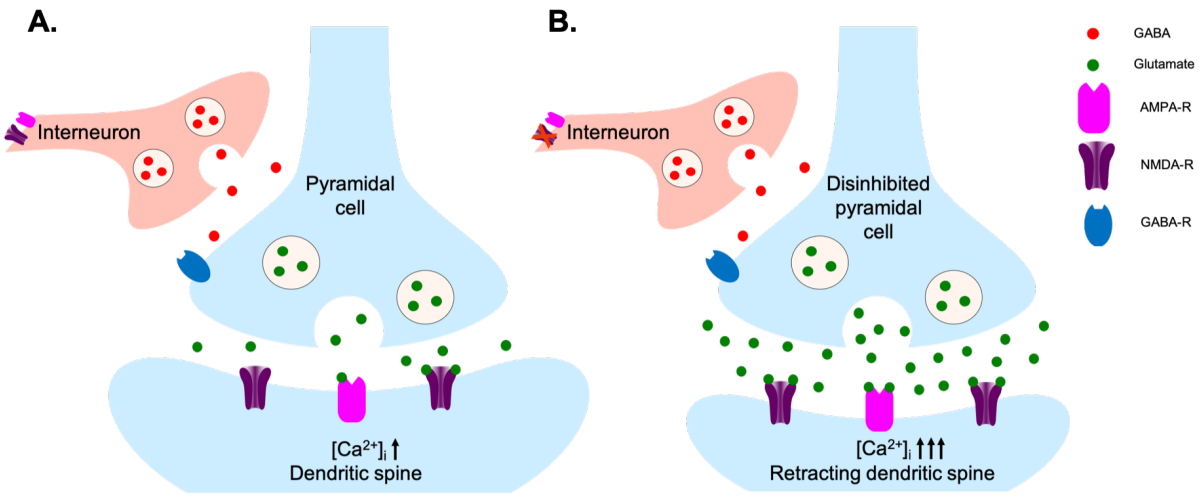


Figure 1. The glutamate hypothesis of schizophrenia. A) Under normal conditions, NMDA-R activation on GABAergic interneurons provides inhibitory control on cortical neurons to regulate glutamate signaling; moderate levels of synaptic activity and $[Ca^{2+}]_i$ maintain dendritic spine stability. (B) Hypofunction of NMDA-Rs on GABAergic interneurons results in disinhibition of pyramidal cells, an increase in extracellular glutamate, and overactivation of AMPA-Rs. $[Ca^{2+}]_i$ levels in the spine sharply increase, resulting in spine retraction and potentially spine loss.

induces spine loss on PFC PCs (Wang and Deutch, 2008), has been shown to increase levels of the astrocytic glutamate transporter 1 (GLT-1) (Vollbrecht et al., 2014), the primary cortical glutamate transporter responsible for glutamate uptake and termination of glutamate signaling (Tanaka et al., 1997). Thus, dopamine may determine spine number by modulating glutamate release, and attenuation of dopaminergic modulation of glutamatergic drive onto PFC PCs may culminate in the loss of dendritic spines in schizophrenia. Alternatively, NMDA-R hypofunction on GABAergic interneurons may result in disinhibited glutamate release onto striatal dopamine neurons and increased activation of these dopamine neurons. One point from these and other neurochemical studies is clear: schizophrenia cannot be explained solely by dopaminergic or glutamatergic dysfunction, but rather is likely the result of a complex, coordinated disruption of these (and likely additional) systems and their interactions.

While there are additional changes in neurochemically-specified PFC systems, which will not be further discussed, the dopamine and glutamate hypotheses remain the dominant hypotheses

explaining the pathophysiology of schizophrenia.

1.2 Feinberg's Neurodevelopmental Hypothesis of Schizophrenia

Schizophrenia is usually first diagnosed during late adolescence or early adulthood, with females suffering a first break later than males (Häfner et al., 1994). However, *in vivo* imaging studies of persons at high risk for developing schizophrenia suggest that changes in brain architecture antedate the first psychotic episode (Fusar-Poli et al., 2012a; Leung et al., 2011), pointing to aberrant neurodevelopmental processes as a major contributor to the pathology of schizophrenia. Though several variants of the neurodevelopmental hypothesis of schizophrenia have been advanced (see Murray et al., 2017), most hypothesize that an insult that occurs during the second or third trimester of pregnancy remains latent until symptoms emerge in adolescence. A neurodevelopmental hypothesis that is consistent with the neuropathology of schizophrenia was advanced by the sleep biologist Irwin Feinberg (1982), who posited that schizophrenia results "from a defect of synaptic elimination programmed to occur during adolescence." This hypothesis was based on the temporal pattern of postnatal development of PCs in the frontal cortex (Huttenlocher, 1979), with dendritic spines being added during development, and thereafter supernumerary spines being pruned until the PC achieves its final adult spine density (Huttenlocher, 1979; Katz and Shatz, 1996; Petanjek et al., 2011; Rakic et al., 1994).

Feinberg's hypothesis (Figure 2) has long intrigued investigators, but the mechanism that accounts for excessive spine pruning during adolescence remains unknown. Recent data suggests that microglia play an important physiological role in synaptic remodeling of neurons during development.

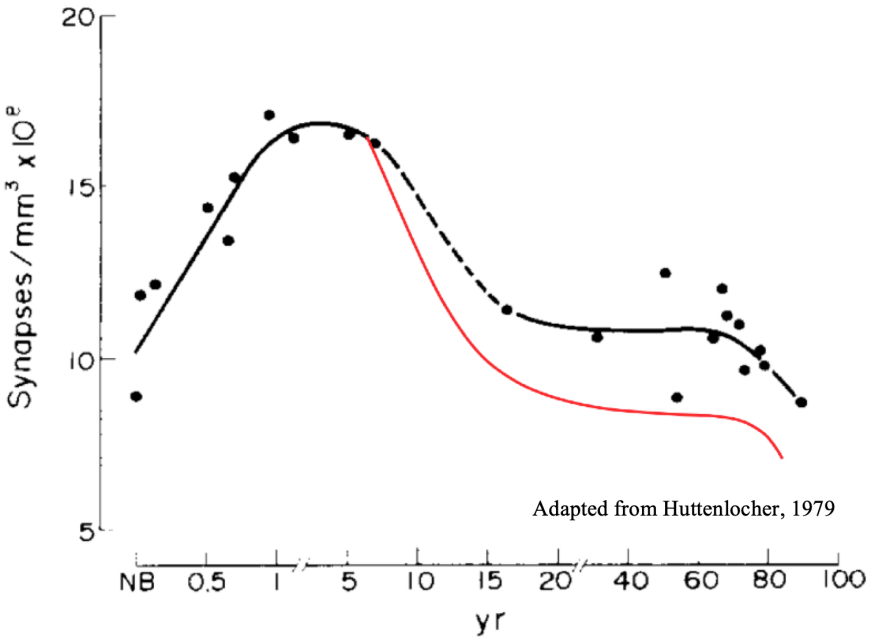


Figure 2. Feinberg's neurodevelopmental hypothesis of schizophrenia. PFC synaptic density changes through development in humans (black) and posited synaptic density changes in schizophrenia (red). Feinberg proposed aberrant synaptic elimination in schizophrenia (Reprinted from Brain Research, 163, Huttenlocher PR, Synaptic density in human frontal cortex – Developmental changes and effects of aging, 195-205, © (1979), with permission from Elsevier).

CHAPTER 2

MICROGLIA: A ROLE FOR THE IMMUNE SYSTEM IN THE PATHOGENESIS OF SCHIZOPHRENIA

Glia, named for their putative role of being the "glue" that holds elements in the brain in place, were long considered to be passive bystanders to inter-neuronal transmission in the CNS, but have belatedly been recognized as integral contributors to brain function. One index of a shift in the targets of scientific inquiry is the introduction of neologisms to denote new processes; among these new words is gliotransmission (Bezzi and Volterra, 2001).

Glia, like neurons, are heterogeneous in both morphology and function. There are three major types of glia: microglia, oligodendrocytes, and astrocytes. While there is intense interest in dissecting the evolving functions of all classes of glia, I will focus primarily on microglia.

Until very recently, microglia have mainly been discussed as a component of rapid inflammatory responses in the CNS. However, it is now clear that microglia have multifaceted roles in shaping synaptic architecture and activity. Indeed, today some speak of a "quad-partite" synapse (Schafer et al., 2013), including pre- and postsynaptic neuronal elements, astrocytes, and microglia. Microglia constantly surveil brain parenchyma for signs of insult or injury (Davalos et al., 2005; Nimmerjahn et al., 2005) and play an active role in influencing synaptic characteristics at developing and mature synapses. In particular, microglia have come to the forefront as key mediators of developmentally-specific synaptic pruning: microglia engulf synaptic elements during early postnatal development (Bialas and Stevens, 2013; Paolicelli et al., 2011; Schafer et al., 2012). Furthermore, recent data indicating significant microglial heterogeneity suggest that there are multiple roles for microglia that remain to be identified and characterized. Among the most intensely examined roles of microglia is the contribution of these cells to neuropsychiatric disorders, including schizophrenia.

2.1 Discovery of Glia: A Brief History

In the mid-nineteenth century, the German anatomist Rudolf Virchow coined the term neuroglia ("nerve cement" or "nerve glue") to describe what he took to be a connective tissue comprised of several cellular elements (see Kettenmann and Ransom 2005; Kettenmann and Verkhratsky 2008). This description was in part based on the doctoral studies of Robert Remak 20 years earlier, who noted a sheath, now recognized as Schwann cells, that encased nerve fibers (see Kettenmann and Verkhratsky, 2008). Shortly thereafter, Heinrich Müller described the eponymously named Müller (glial cell) in the retina (see Kettenmann and Verkhratsky, 2008). Subsequently, Otto Deiters commented on a star-shaped cell in white matter, which 30 years later was named astrocyte by Michael von Lenhossék and was recognized as having two subtypes (protoplasmic and fibrous) (see Kettenmann and Verkhratsky, 2008). Astrocytes are highly diverse (Durkee and Araque, 2018; Matyash and Kettenmann, 2010), and are of particular importance in maintaining the homeostasis of the extracellular milieu. How astrocytes respond to different injuries and challenges is only starting to be understood (Liddelow et al., 2017).

The separation and classification of glial cells into the classes of glia we are familiar with today was not formulated until more than 50 years after Virchow's naming of neuroglia. Santiago Ramón y Cajal (renowned for studies that culminated in the proposal of the neuron doctrine) and his student Nicolás Achúcarro refined existing methods to permit more selective (although imperfect) staining of glial cell populations, which were instrumental in furthering glial-related research (see Tremblay et al., 2015). Achúcarro, using a staining preparation involving tannin and ammoniacal silver nitrate, described cells that were localized to the site of a lesion, leading him to postulate that these cells may be involved in phagocytosis. Cajal subsequently used a sublimated gold chloride method, which ultimately allowed for better resolving of astrocytes while only scarcely staining other glial cells, leading him to notoriously label these other glial cells as the "third element" of the CNS (see Kettenmann and Verkhratsky, 2008, 2016; Tremblay et al., 2015).

It was another Cajal alumnus, Pío del Río-Hortega, who developed a silver carbonate staining method in 1919 that finally allowed for the differentiation of the distinct glial cell subtypes, culminating in del Río-Hortega's introduction in 1920 of the term microglia to describe one of these cell types (see Tremblay et al., 2015). del Río-Hortega perceptively inferred from his preparation several characteristics of microglia that were subsequently confirmed. These include the observations that microglia populate the brain early during development, are of mesodermal origin, exhibit characteristic morphologies through development, exhibit morphological transformations in pathological states, are present across all of the brain and occupy nonoverlapping territories, and have the capacity to migrate and phagocytose cellular debris (Kettenmann et al., 2011; del Río-Hortega, 1932).

In 1921, del Río-Hortega also identified and characterized oligodendrocytes as yet another "neuroglia" cell type (see Tremblay et al., 2015). These cells, which play a well-known role in axonal myelination (Simons and Lyons, 2013; Simons and Nave, 2015), are critically involved in diseases such as multiple sclerosis (Compston and Coles, 2008), but will not be discussed further. The interested reader is referred to the reviews of Bradl and Lassmann, 2010, Michalski and Kothary, 2015, and Tremblay et al., 2015 for additional information on the discovery and functions of oligodendrocytes in the CNS.

Despite these foundational studies from the leading neuroanatomists of the time, the investigation into microglia waned throughout most of the twentieth century. Tremblay and colleagues (2015) noted that until 1990 only 456 papers discussing microglia were published. However, since 1990 research on microglia has been a growth industry, with studies of microglia having revealed a more comprehensive (though unquestionably still incomplete) picture of how microglial cells function in the brain.

2.2 Microglial Form and Function

Extensive efforts have been devoted to dissecting the diverse roles of microglia in the healthy and diseased brain (for reviews see Kierdorf and Prinz, 2017; Li and Barres, 2018; Salter and Stevens, 2017; Tremblay et al., 2011; Wolf et al., 2017). These studies have completely shifted the perception of microglia from being "glue" to being active determinants of CNS structure and function.

Microglial Development, Maintenance, and CNS Colonization

Microglia are CNS macrophages derived from yolk-sac erythromyeloid progenitors (EMPs) that migrate to the neural tube early in embryonic (E) development (from E9.5 through E13.5-E14.5) (Ginhoux et al., 2010; see Salter and Beggs, 2014; Figure 3). Following microglial seeding of the CNS, the blood-brain barrier (BBB) begins to form, thereby effectively separating the CNS and the peripheral macrophage populations. The differentiation of EMPs into microglia is dependent on the transcription factors *Pu.1* (exclusively expressed by hematopoietic cells) and the myeloid cell cytokine interferon regulatory factor 8 (*Irf8*) (Kierdorf et al., 2013), as well as the colony-stimulating factor 1 (CSF1) receptor (CSF1R), which is expressed by both microglia and macrophages (Patel and Player, 2009). Transforming growth factor beta (TGF-β) is also critical for microglial differentiation: TGF-β1-deficient mice lack CNS microglia (Butovsky et al., 2014). For a comprehensive overview of microglial ontogeny across embryonic and early postnatal development the reader is referred to the review by Li and Barres (2018).

Once microglial precursors enter the CNS they "disseminate relatively homogeneously throughout the neural tissue and acquire a specific phenotype, which clearly distinguish them from their precursors, the blood-derived monocytes" (Kettenmann and Verkhratsky, 2016). Although the processes of microglia and astrocytes can overlap, each individual microglial and astrocytic cell minimally overlaps in space, a phenomenon known as tiling (see Kettenmann et al., 2013). The

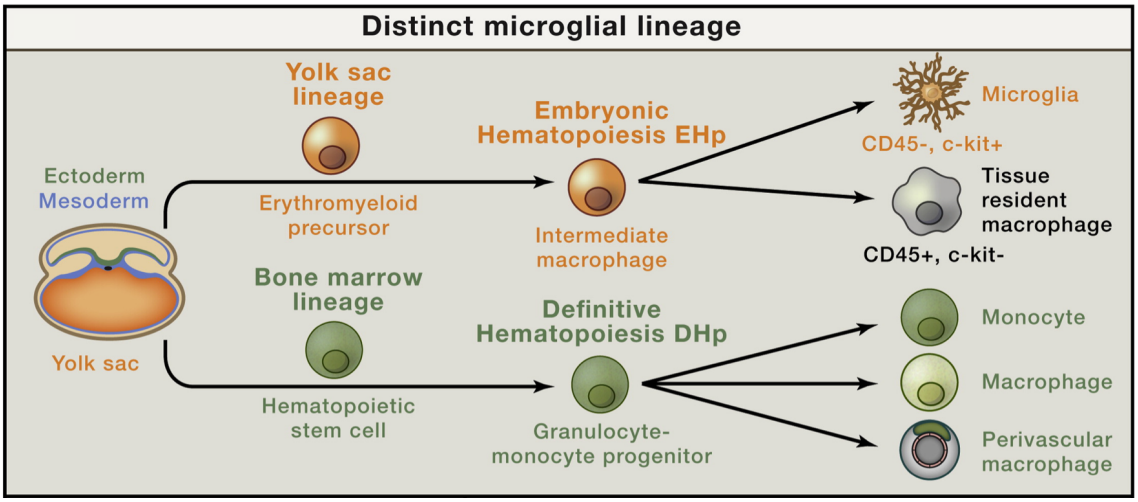


Figure 3. Microglia and macrophages are derived via distinct pathways. Microglia evolve from EMPs in a PU.1- and IRF8-dependent manner from cells that have low CD45 and high c-kit expression. The majority of macrophages are formed from stem cells through definitive hematopoiesis (Reprinted from Cell, 158, Salter MW, Beggs S, Sublime microglia: expanding roles for the guardians of the CNS, 15-24, © (2014), with permission from Elsevier).

density and distribution of microglia vary across different parts of the brain (Lawson et al., 1990), with the density ranging from 5% in the cortex to 12% in the substantia nigra, and microglia are typically more abundant in gray matter than white matter (Lawson et al., 1990; Perry et al., 1985). Subtle differences in microglial density at different times in postnatal development and in different brain regions are now appreciated (De Biase et al., 2017). The cues and mechanisms that guide the migration and distribution of microglial cells during development is an active area of investigation.

Bulk infiltration of peripheral macrophages into the CNS does not occur homeostatically (Ginhoux et al., 2010; Mildner et al., 2007); peripheral macrophages are mainly restricted to perivascular spaces, meninges, and the choroid plexus (Prinz et al., 2011; Prinz and Priller, 2014), and myeloid cells are thought to penetrate into the brain only under pathological conditions (see Prinz and Priller, 2017).

Brain microglia are thought to be locally maintained by self-renewal (Ajami et al., 2007; Bruttger et al., 2015). Using a novel fate mapping system, Tay et al. (2017) showed that at steady state the microglial population is largely stable but stochastically self-renew in a manner whose

dynamics are dependent on the regional microenvironment in the CNS (see Madore et al., 2017; Figure 4). The random pattern of renewal shifts to clonal expansion in pathological conditions, followed by cell egress and apoptosis to eliminate the surplus of pathology-associated microglia. In the rodent cortex, it is estimated that complete turnover of microglia requires ~41 months (Tay et al., 2017), highlighting microglial longevity. The microglial population is in part maintained by continuous activation of CSF1R (Erblich et al., 2011; Ginhoux et al., 2010), the endogenous ligands of which include CSF1 and interleukin (IL)-34 (Lin et al., 2008); pharmacological blockade of CSF1R rapidly depletes CNS microglia.

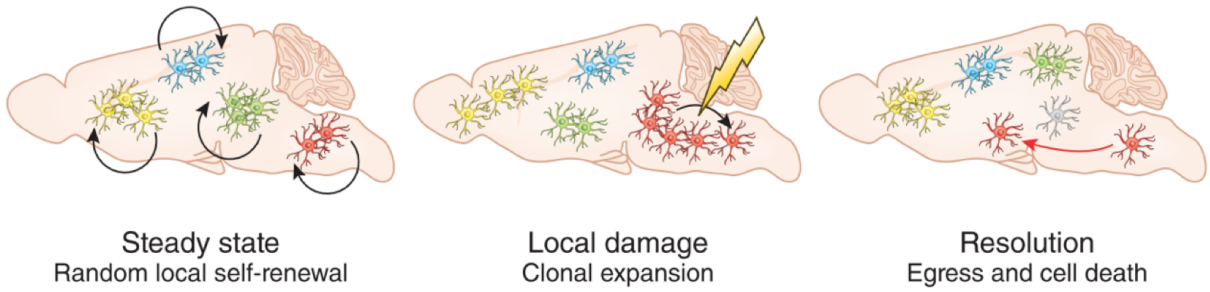


Figure 4. Microglial maintenance in steady state and injury. Left: the microglial population is maintained via stochastic self-renewal, with heterogeneous rates of turnover across different brain areas. Middle: CNS damage induces clonal microglial expansion. Right: restoration of normal microglial numbers occurs through apoptotic egress and cell death (Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature Neuroscience. Microglial confetti party, Madore C, Baufeld C, Butovsky O. © 2017).

Microglial Phenotype/States

The diversity of microglial states is increasingly been scrutinized. Microglia exhibit distinct gene expression profiles and epigenetic signatures at different phases of development, following a stepwise developmental program that synchronizes with brain development (Matcovitch-Natan et al., 2016). Microglia adopt functional profiles at different time points that allow them to effectively process developmentally-specific environmental signals while still

regulating immune responses (Matcovitch-Natan et al., 2016). Although microglia have historically been thought of as being in either an active or quiet (baseline) surveillance state, recent studies have painted a more complex picture.

Examination of the transcriptome of single microglial cells at different developmental time points and under various challenge conditions has revealed nine different microglial transcriptional profiles/states (Hammond et al., 2019), with microglia assuming distinct profiles under different inflammatory and neurodegenerative conditions (Sousa et al., 2018).

Microglial "Activation"

Injury, infection, and disease states trigger transformations in microglial shape, density, gene expression profile, and behavior (Colton and Wilcock, 2010; Graeber and Streit, 2010; Hanisch and Kettenmann, 2007; Kettenmann et al., 2011; Kreutzberg, 1996) and contribute to innate immunity (Jack et al., 2005; Lehnardt, 2010; Ransohoff and Brown, 2012; Town et al., 2005). While resting, microglia have a small somata and thin, long, and profusely branched processes. In response to injury to the brain, microglia change their shape to a so-called activated morphology, characterized by a large soma with few or no processes, which, when present, are short and stout (Karperien et al., 2013; see Kettenmann and Verkhratsky, 2016). Given how dynamic microglia are under normal conditions, it is not surprising microglia move rapidly to an injured site (Davalos et al., 2005; Nimmerjahn et al., 2005) in an ATP-dependent manner (Davalos et al., 2005), guided by chemotactic gradients (see Kettenmann et al., 2011). At the injured locus microglia remove damaged tissue, debris, or foreign elements.

In 2000, Mills and colleagues (2000) first proposed classifying activated macrophages - use of which has since extended to microglia - into two states: M1, the classical activation, proinflammatory state; and M2, the alternative activation or "acquired deactivation" repairing state (see Czeh et al., 2011; Franco and Fernández-Suárez, 2015; Tang and Le, 2016). In the M1 state, microglia produce reactive oxygen species, proteases, nitric oxide, and a number of

proinflammatory cytokines such as IFN-γ, tumor necrosis factor α (TNF-α), IL-1β, and IL-6 to induce toxic neuronal loss and defend against pathogens (Block et al., 2007; Boche et al., 2013; Wang et al., 2015).

M2 (regulatory) microglia, conversely, secrete cytokines that are involved in dampening inflammation, including IL-10 (Boche et al., 2013; Cherry et al., 2014). M2 microglia have been further subdivided into wound-healing microglia that produce neuroprotective cytokines, including IL-4, IL-10, IL-13, and TGF-β (Boche et al., 2013; Wang et al., 2015), as well as possibly producing "triggering receptor expressed on myeloid cells 2" (Trem2) (see Boche et al., 2013). These wound-healing microglia also express scavenger receptors that are involved in phagocytosis, including the mannose receptor (Stein et al., 1992), and elicit tissue and extracellular matrix repair.

Although the separation of microglia into M1 and M2 classes have long been used in studies of microglia and as means of conceptualizing microglial activation, this distinction has recently been challenged. In particular, Ransohoff (2016) has cogently argued that polarization has not been empirically validated and arose as a means of simplifying data interpretation.

Synaptic Maturation and Plasticity

Microglia are swift responders to trauma or injury in the brain. Even in their "quiescent" resting state microglia are highly active. In 2005, two seminal papers described the dynamics and motility of microglia. Using two-photon imaging of green fluorescent protein (GFP)-labeled microglia in the mouse cortex, both groups found that microglial processes constantly extend and retract (Davalos et al., 2005; Nimmerjahn et al., 2005), resulting in the entirety of the parenchyma being surveyed once every few hours (Nimmerjahn et al., 2005). Microglia frequently interact with and appose other cells, including neurons and astrocytes (Nimmerjahn et al., 2005; Figure 5). Two photon-imaging and electron microscopic analyses in the adult mouse visual cortex under normal conditions suggest that microglial processes briefly (∼4-5 minutes) contact synapses once an hour

in an activity-dependent manner, with reduced neural activity being associated with retraction of microglial processes (Wake et al., 2009). Conversely, increased neuronal activity enhances the extension of microglial processes toward neurons (Eyo et al., 2014).

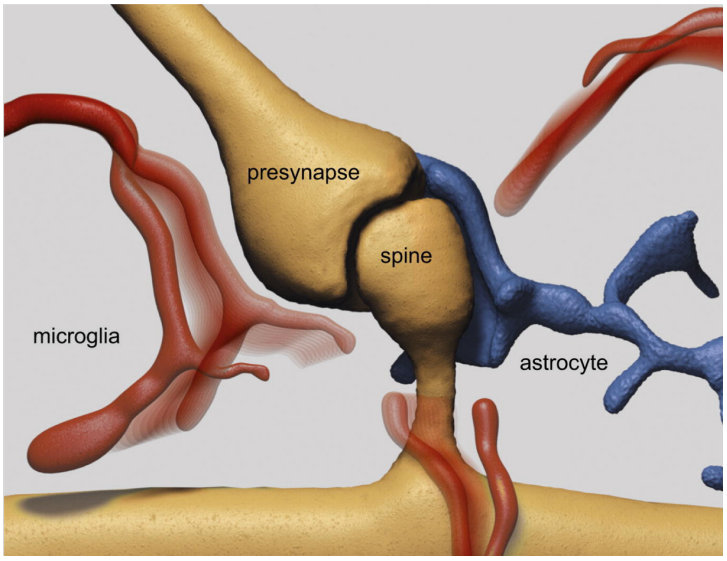


Figure 5. The "quad-partite" synapse. Microglia constantly survey the extracellular space and interact with elements at the synapse (Reprinted from Cell, 77, Kettenmann H, Kirchhoff F, Verkhratsky A, Microglia: new roles for the synaptic stripper, 10-18, © (2013), with permission from Elsevier).

Ultrastructural studies have also shown microglial cell bodies and processes in close proximity to synapse-associated elements and synaptic clefts, and in contact with dendritic spines, axon terminals, and astrocytic processes (Tremblay et al., 2010). Interestingly, microglia seem to preferentially appose smaller spines, which at first transiently expand but over the course of 2 days retract and ultimately disappear, hinting that microglia may preferentially migrate toward specific subsets of spines. Sensory experience alters these microglial behaviors such that light deprivation, which decreases synaptic strength, leads microglia to migrate toward larger dendritic spines that subsequently shrink in size (Tremblay et al., 2010).

Microglia also regulate long-term potentiation (LTP) and synaptic scaling (see Kettenmann et al., 2011; Schafer et al., 2013). For example, microglial-conditioned medium,

when added to organotypic and acute cortical slice cultures (Moriguchi et al., 2003) or dissociated hippocampal CA1 neurons (Hayashi et al., 2006), potentiates NMDA-R-mediated responses. In hippocampal slices from mice lacking CX_3CR1, the microglial fractalkine receptor (Maciejewski-Lenoir et al., 1999; Nishiyori et al., 1998; Schwaeble et al., 1998) that in part regulates microglial recruitment to neurons via interaction with the neuronal chemokine fractalkine (CX_3CL1), LTP is impaired (Rogers et al., 2011). Furthermore, these mice show learning and memory deficits, with both consequences reversed by administration of an IL-1β antagonist (Rogers et al., 2011).

Microglia also play a role in synapse formation. In the developing somatosensory cortex, microglial contact with dendritic shafts initiates filopodia formation, some of which become functional excitatory synapses; this formation occurs in part via an increase in Ca^{2+} transients and actin recruitment (Miyamoto et al., 2016). Microglia can also stimulate synaptogenesis via secretion of different factors, including thrombospondins (Chamak et al., 1995).

Microglial Phagocytosis of Apoptotic Neurons

During development, a large number of newly formed neurons – up to 50% in vertebrates (Oppenheim, 1991; Raff et al., 1993) – undergo programmed cell death (Dekkers et al., 2013; Dekkers and Barde, 2013). Apoptosis is triggered by secretion of microglial factors such as TNF-α (Bessis et al., 2007); microglia subsequently phagocytose the dying cells (Marín-Teva et al., 2004; Rigato et al., 2011; Witting et al., 2000). The interaction between Trem2 and its signaling adaptor "DNAX activation protein of 12 kilodalton (kDa)" (DAP12), which are co-expressed in microglial and other myeloid cells (Kiialainen et al., 2005; Schmid et al., 2002), is important in apoptotic processes. The Trem2-DAP12 interaction prompts microglial recognition of apoptotic neurons during programmed cell death, the stimulation of phagocytosis, and the down-regulation of inflammatory signals - thereby inducing apoptosis without prompting an inflammatory response (Takahashi et al., 2005).

2.3 Evolving Functions of Microglia

Synapses are created in excess during early development and then pruned until the adult architecture is achieved (Huttenlocher, 1979; Innocenti and Price, 2005; Katz and Shatz, 1996; Petanjek et al., 2011; Rakic et al., 1994). Where these lost synapses "go" has, until recently, been a mystery.

A major hint of the identity of the mechanism(s) by which synapses are removed came in 2007, when the involvement of the classical complement cascade in the developmental elimination of supernumerary synapses in the retinogeniculate system was reported (Stevens et al., 2007). The complement cascade (Figure 6) is a pathway in the innate immune system that tags ("opsonizes") and removes harmful pathogens and debris via phagocytosis by macrophages or by complement-mediated cell lysis (see Stephan et al., 2012). Complement component 1q (C1q), the initiating component in the complement cascade, opsonizes unwanted pathogens, cells, or debris, which in turn triggers a series of proteolytic cleavages of downstream complement components and ultimately cleavage and deposition of complement component 3 (C3), which presents the unwanted debris to a macrophage (Gasque, 2004). C1q is localized to synapses during development, and genetic deletion of C1q or C3 results in aberrant synapse elimination (Stevens et al., 2007). Based on these findings, Stevens and colleagues posited that microglia, which are the only CNS cells known to express the C3 receptor (CR3) (Gasque et al., 1998), may phagocytose excess synapses during development. Subsequently, it was shown that C1q knockout mice exhibit defects in synapse elimination in the neocortex as well, leading to enhanced synaptic connectivity (Chu et al., 2010).

Based in part on the known role of microglia in engulfing cellular debris following injury and the proposal that microglia may be involved in physiologic (developmental) synaptic pruning (Schafer and Stevens, 2010), Paolicelli et al. (2011) examined if microglia phagocytose excess synapses during critical periods. Using a transgenic mouse that expresses GFP in microglia, the

authors established that postsynaptic (postsynaptic density protein 95 (PSD-95)-immunoreactive (-ir)) and presynaptic (SNAP25-ir) elements of neurons were intracellularly localized in mouse hippocampal neurons at P15. Genetic ablation of CX_3CR1 disrupted developmental synaptic engulfment, presumably due to reduced microglial surveillance of neurons (Paolicelli et al., 2011). These data were among the first to directly implicate microglia in the developmentally-mediated elimination of synapses.

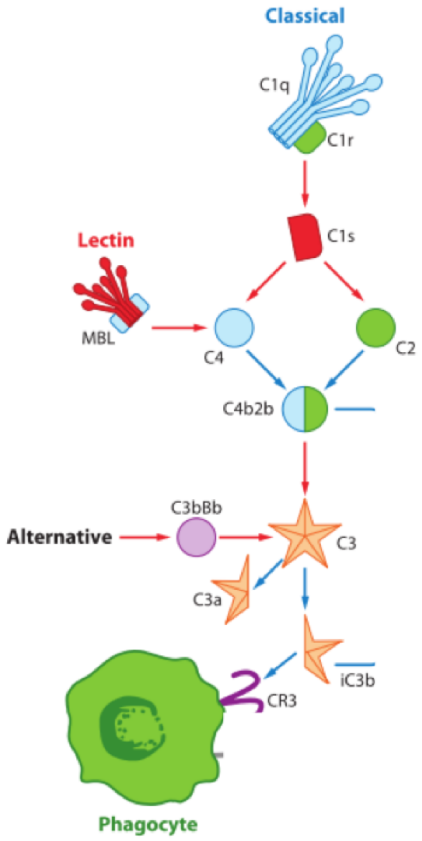


Figure 6. The classical complement cascade. C1q, the initiating component in the complement cascade, undergoes proteolytic cleavages to produce intermediary products and ultimately C3, which is in turn recognized by phagocytes that express the cognate receptor CR3 (from Stephan et al., 2012; Annual review of neuroscience by ANNUAL REVIEWS. Reproduced with permission of ANNUAL REVIEWS in the format Thesis/Dissertation via Copyright Clearance Center).

In 2012 the link between microglia, the complement cascade, and developmental synaptic pruning (Figure 7) became evident: Schafer et al. (2012) elegantly used the developing mouse retinogeniculate system to examine whether microglia prune supernumerary retinal ganglion cell (RGC) synapses. Microglia phagocytosed fluorescently-labeled RGC inputs at P5, an age of robust synaptic pruning. By P9, when pruning of excess RGC synapses is largely complete, engulfment of RGC synapses was significantly decreased, indicating that this process is developmentally regulated. Blocking neural activity led to enhanced microglial phagocytosis, pointing to pruning being an activity-dependent process. Knockout of both C3 and CR3 resulted in decreased engulfment of RGC inputs by microglia (Schafer et al., 2012). Importantly, engulfment was only reduced by ~50% in these knockout models, suggesting that other cues (or cells) also instruct developmental pruning.

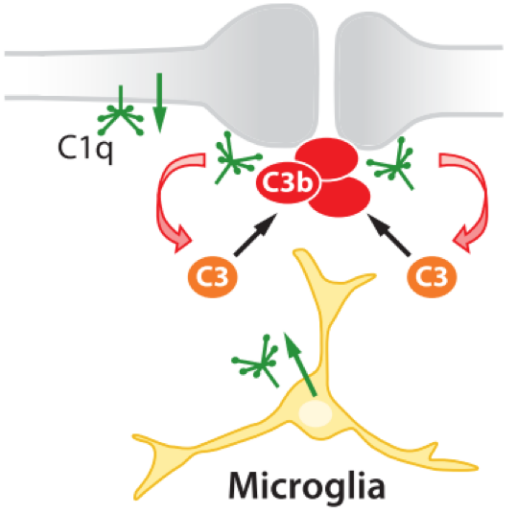


Figure 7. Microglia prune developing synapses in a complement-dependent manner. C1q opsonizes supernumerary synapses in the developing brain, resulting in the production of C3, recognition of the synapse via CR3 on microglia, and ultimately phagocytosis of the excess synapse (from Stephan et al., 2012; Annual review of neuroscience by ANNUAL REVIEWS. Reproduced with permission of ANNUAL REVIEWS in the format Thesis/Dissertation via Copyright Clearance Center).

These data together suggest that, in addition to the established role of mediating the CNS immune response, microglia influence synapse formation, maturation, and plasticity. Critically, microglia play a prominent role in shaping the architecture and function of the brain via the developmental pruning of supernumerary synapses. Microglia prune both pre- and postsynaptic elements, thereby determining the optimal number of excitatory synapses on projection neurons. While microglial pruning appears grossly similar to the phagocytosis of cellular debris in injured or damaged tissue, we are only just starting to understand the diversity and complexity of microglia, and there are clues suggesting that physiological pruning may differ from pathological pruning.

Microglial contributions to physiological pruning during development have primarily been examined in systems that mature relatively early in development. The degree to which the same processes contribute to very late-developing areas of brain, such as the prefrontal cortex, have not been examined.

2.4 Microglial Changes in Schizophrenia

Interest in the possible role of the immune system and/or neuroinflammation in promoting neuropathological changes in schizophrenia has surged over the past several years (Laskaris et al., 2016; Müller, 2018; Nimgaonkar et al., 2017; Trépanier et al., 2016). The potential involvement of microglia in schizophrenia became clear because genetic studies consistently uncovered associations of schizophrenia with the major histocompatibility complex (MHC) locus (Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014). More recently, specific variations in complement component 4 (C4) have been strongly linked to the risk for developing schizophrenia (Sekar et al., 2016). I focus here predominantly on the link between the immune system and schizophrenia; for comprehensive reviews of neuroinflammation the reader is referred to Laskaris et al. (2016) and Trépanier et al. (2016).

Unfortunately, the literature on microglial involvement in schizophrenia is littered with inconsistent results. Some studies have reported increased density of microglia (Busse et al., 2012; Fillman et al., 2013; Radewicz et al., 2000; Wierzba-Bobrowicz et al., 2005), increased microglial activation (Bayer et al., 1999; Wierzba-Bobrowicz et al., 2005), and degenerating microglial cells (Wierzba-Bobrowicz et al., 2004, 2005), as determined by cytoplasmic shrinkage and reduction and fragmentation of microglial processes. In contrast, other studies have found no change in various parameters of microglial number and function (Arnold et al., 1998; Connor et al., 2009; Falke et al., 2000; Hercher et al., 2014; Steiner et al., 2006, 2008). A recent meta-analysis of studies examining microglial density in postmortem tissue concluded that the preponderance of evidence is consistent with a significant increase in brain microglial density and a corresponding upregulation of microglial-related proinflammatory genes in schizophrenia (Van Kesteren et al., 2017), although there was substantial heterogeneity across the various studies.

Studies of glia in schizophrenia have in part been confounded by issues common to postmortem studies, ranging from the use of APDs or other drugs to agonal state. However, there is another concern specific to microglia: monocytes and macrophages, as well as microglia, express the same markers. For example, ionized calcium binding adaptor molecule 1 (Iba1), the most commonly used marker of microglia in recent time, is also found in peripherally-derived macrophages. However, the recent identification of proteins expressed by microglia but not other myeloid cells, including transmembrane protein 119 (Tmem119), sialic acid-binding immunoglobulin-like lectin H (Siglec-H), and others (see Butovsky and Weiner 2018) should open the door for more accurate studies of microglial number and density in schizophrenia.

An indirect approach to identifying changes in microglia and inflammatory processes in schizophrenia has been through the development of positron emission tomography (PET) radioligands for *in vivo* assessment of microglia. Radioligands for the 18 kDa translocator protein (TSPO), a protein thought to be involved in steroidogenesis (Papadopoulos et al., 2006, 2018), were proposed to be useful in monitoring inflammatory processes and microglial activation in

various disorders (Banati, 2002; Banati et al., 2000), including schizophrenia, based on studies indicating that TSPO expression is upregulated in inflammatory states and diseases (Batarseh and Papadopoulos, 2010; McNeela et al., 2018; Messmer and Reynolds, 1998; Turner et al., 2004) and during microglial activation (Banati, 2002; Vowinckel et al., 1997). Early imaging studies with TSPO tracers generated conflicting results because the contributions of allelic variants in TSPO binding was not appreciated (Owen et al., 2012). However, subsequent studies of subjects who were genotyped to distinguish between TSPO variants were also inconsistent (van Berckel et al., 2008; Di Biase et al., 2017; Bloomfield et al., 2016; Coughlin et al., 2016; van der Doef et al., 2016; Doorduin et al., 2009; Hafizi et al., 2017; Ottoy et al., 2018; Selvaraj et al., 2017; Takano et al., 2010).

Unfortunately, over the past several years it has become clear that TSPO is not a specific marker of microglia: the protein is also expressed in peripheral (and CNS-infiltrating) macrophages and monocytes. Moreover, TSPO binds to astrocytes, endothelial cells, and perhaps even neurons (Cosenza-Nashat et al., 2009; Lavisse et al., 2012; Notter et al., 2018; Varga et al., 2009). Finally, Owen et al. (2017) recently reported that TSPO expression is increased substantially in response to a proinflammatory challenge in rodent, but not in human, microglia. These data indicate that TSPO is not a specific marker of microglial activation and inflammation (Lockhart et al., 2003; Michell-Robinson et al., 2015; Notter et al., 2018; O'Donnell, 2017; Owen et al., 2012; De Picker et al., 2017; Turkheimer et al., 2015), and that *in vivo* studies of microglia in patient populations awaits the development of novel (sensitive and reliable) methods.

2.5 Hypothesis

The past thirty years have seen a resurgence of interest in the neuropathology of schizophrenia. The application of quantitative methods to postmortem tissue has revealed decreases in cortical gray matter volume, particularly in the PFC. However, PFC volume loss is not accompanied by a decrease in the number of neurons. Instead, the decrease in PFC volume appears to be attributable to the loss of dendritic spines, the primary site of excitatory inputs to pyramidal cells, and a corresponding decrease in the presynaptic partners of these dendritic spines. The mechanisms that account for spine loss are unclear but have been suggested to involve an exaggerated pruning of synapses during adolescence (Feinberg, 1982). Microglial pruning of spines on neurons in brain areas that mature early in postnatal development has recently been reported (Paolicelli et al., 2011; Schafer et al., 2012), but it is not known if microglia sculpt projection neurons in late-maturing structures such as the PFC. Notably, variants in genes mediating this homeostatic process have now been linked to schizophrenia (Sekar et al., 2016).

I hypothesize that microglia, the innate immune cells of the central nervous system, determine the structure of prefrontal cortical pyramidal cells by pruning supernumerary dendritic spines during adolescence (Figure 8). This is the developmental phase when symptoms and behaviors that are harbingers of psychosis (prodrome) usually first appear, culminating in the structural changes seen in prefrontal cortical neuronal morphology in schizophrenia. In the following chapters, I will present data from anatomical and biochemical techniques to support this hypothesis.

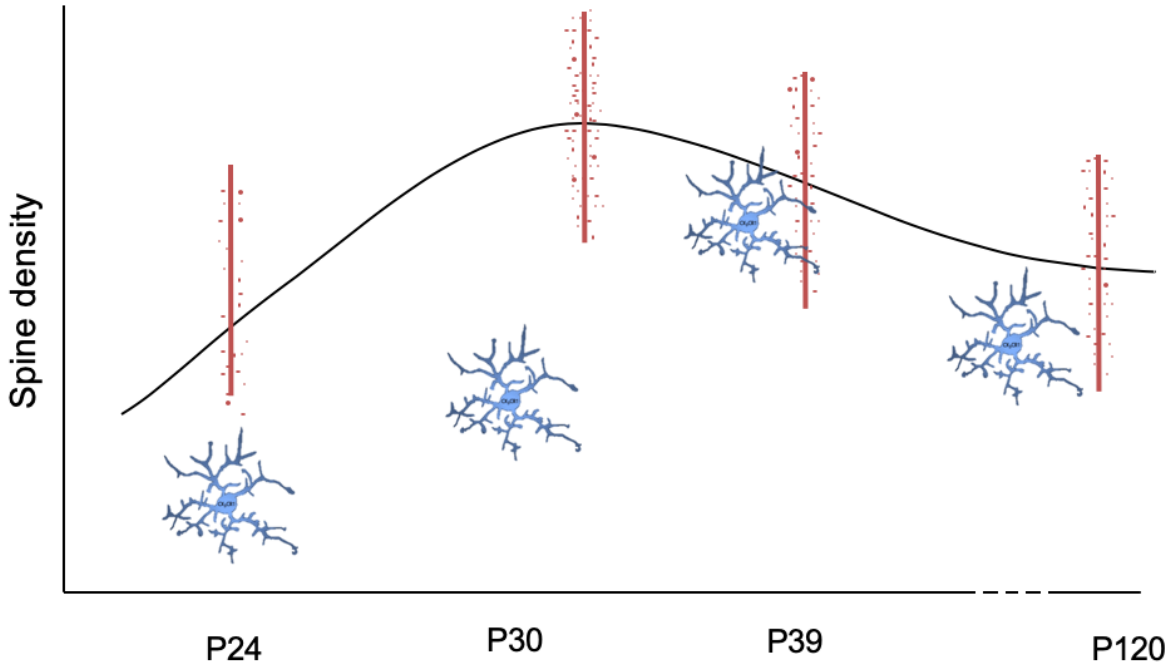


Figure 8. Hypothesis. Microglia prune supernumerary synapses in the PFC during adolescence, shortly after peak spine density is reached, and continue to do so until a mature PFC structure is achieved.

CHAPTER 3

DENDRITIC SPINE LOSS IN POSTNATAL DEVELOPMENT

During brain development the number of synapses is not constant. In early postnatal development, synapses between neurons increase in number (Innocenti and Price, 2005) until a peak number of synapses is achieved; the age at which this peak occurs varies across brain areas. A substantial number of (supernumerary) synapses are subsequently removed ("pruned"), while a smaller contingent of synapses are maintained (Katz and Shatz, 1996), optimizing the "signal-to-noise" ratio. The age at which the mature neuron structure is achieved also varies across brain regions. The PFC is the last area to mature, with PFC volume (Sowell, 2004; Sowell et al., 2001; Thompson et al., 2000) and pyramidal cell dendritic spine number (Petanjek et al., 2011) finally stabilizing in the third decade of life in humans. In rodents, data tracking the trajectory of dendritic spines through development is sparse, with poor temporal resolution. In the following experiment, I investigated the postnatal ontogeny of dendritic spine density on pyramidal cells, focusing on the periadolescent and adolescent periods.

3.1 Methods

Animals. Timed-pregnant Sprague-Dawley dams (Envigo; Indianapolis, IN) were housed on a 12:12 light-dark cycle with food and water freely available. The offspring were weaned on postnatal day (P) 21. Animals were sacrificed at P24, P30, P35, P39, P50, or P70 (±1 day) by isoflurane overdose and transcardially perfused with 9.25% sucrose followed by 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS); rats of both sexes were used.

The brains were removed and bisected along the midline, with the PFC of one hemisphere used for dendritic spine measurements and the other for microglial studies (see chapters 4 - 6). All studies were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and the Vanderbilt University Institutional Animal Care and Use Committee.

Intracellular dye injections of pyramidal cells. One hemisphere was post-fixed in 4% PFA in PBS for 30 minutes at 4°C, after which 200 µm coronal sections through the PFC were cut on a vibrating microtome.

Intracellular dye injections were performed at the following ages: P24 (n = 5; 2 males (m), 3 females (f)); P30 (n = 8; 4m, 4f); P35 (n = 8; 3m, 5f); P39 (n = 9; 7m, 2f); P50 (n = 8; 3m, 5f); and P70 (n = 8; 4m, 4f). Five randomly selected PCs in Layer 5 (L5) of the prelimbic cortex (area 32) in the medial PFC (mPFC) were filled intracellularly with 8% Lucifer Yellow (LY; L0259, Sigma; St. Louis, MO) in 0.05M Tris buffer, using a constant negative current of 3-5 nA for 8-10 minutes (Figure 9, left). Sections were fixed in 4% PFA in PBS overnight at 4°C, and then mounted and coverslipped using ProLong Antifade (P36970, Thermo-Fisher Scientific Inc.; Waltham, MA) mounting medium.

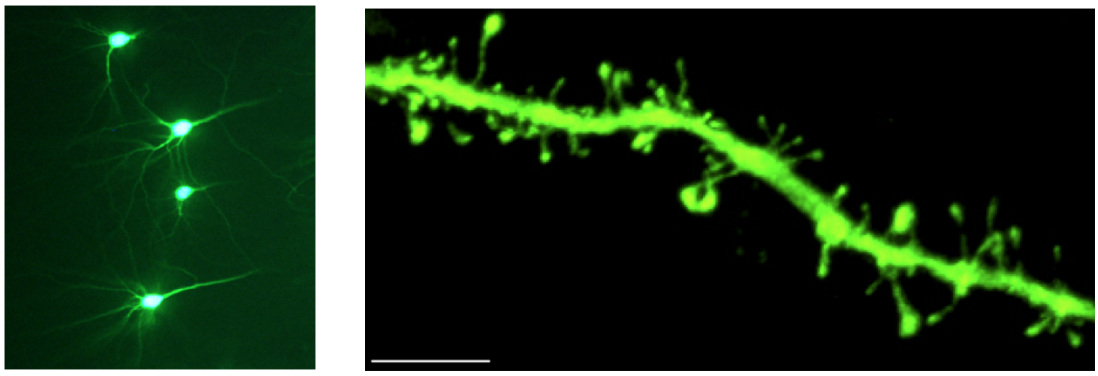


Figure 9. Intracellular dye injections. Left: L5 PFC PCs intracellularly filled with Lucifer Yellow. Right: Representative projection image of an intracellularly filled basal dendritic segment. Scale bar, 5 µm.

Spine measurements. Dendritic segments were imaged by confocal microscopy (Zeiss LSM 710) using a 1.40 NA 63x objective and 3x digital zoom.

Images of three oblique dendritic segments emanating from three different primary basal dendrites as well as the apical dendrite both located 120 - 150 μm distal to the soma on each LY-filled PC were acquired. Z stacks were deconvolved (Huygen's Essential, Scientific Volume Imaging; Hilversum, Netherlands) and the projection image of each segment generated (Figure 9, right). The number of spines/10 μm of dendritic length was determined. The mean spine densities of the three distal basal dendritic segments were collapsed to obtain an average distal basal spine density value for each individual PC, and in turn collapsed across the five PCs to yield a "per animal" basal dendrite spine density value. Apical dendrite spine densities were also averaged across each animal for a "per animal" apical spine density value.

Changes in spine density reflect the sum of spines pruned from the dendrite and any newly-generated spines. Mature and young spines have been suggested to have different shapes (shorter, mushroom-shaped and longer, thin spines, respectively). I therefore measured spine length, maximal spine head diameter, and spine head volume on basal dendritic segments of L5 PFC PCs of P24 and P39 rats to determine if there were changes in the distribution of spine type during PFC development. Spine morphology parameters were determined using Imaris (Bitplane USA; Concord, MA) from two randomly selected dendritic segments of five PCs/animal. Filopodia (spines $\geq$ 4.1 μm in length) made up a very small percentage of the total spines ($\leq$ 0.4%) and were not included in spine morphology analyses.

Data analysis. All studies were conducted under blind conditions, from acquisition of microscopic images to statistical analysis; data were coded and maintained by a person in the lab not affiliated with this particular study. Spine density was analyzed by one-way ANOVA, and Bonferroni post-hoc tests were used to define significant differences. Frequency distributions of spine length, spine head diameter, and spine head volume were compared using the Kolmogorov-Smirnov test.

3.2 Results

Basal spine density changes in the PFC through development. An ANOVA uncovered an overall age effect in L5 PFC PC spine density ($F(5,40) = 4.054$, $p = 0.0045$; Figure 10). Bonferroni post-hoc tests revealed that spine density was significantly increased at all ages at or after P35 relative to P24; spine density did not change over the time period from P50-P70.

I initially analyzed spine density data from the entire cohort of animals without distinguishing sex of the animals. Subsequently, I did a post-hoc analysis of spine density in male and female rats, although the sample sizes were much lower (see methods). I did not uncover a significant difference in spine density in male and female rats. The overall trends appeared grossly similar in males and females, with spine density changing in parallel across the sexes.

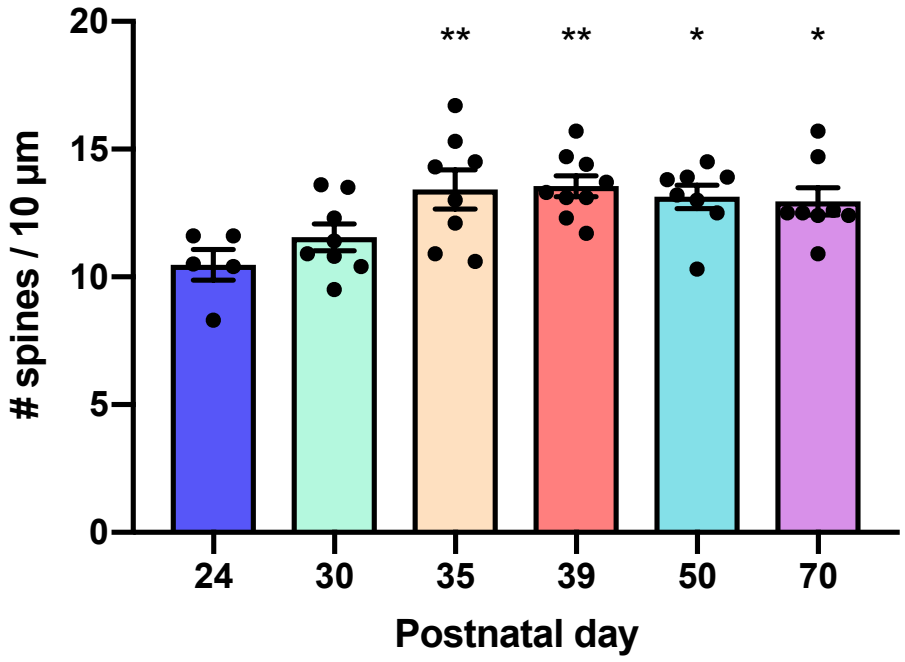


Figure 10. Basal spine density changes in L5 prefrontal cortical pyramidal cells as a function of age. Mean (±SEM) basal spine density differed significantly as a function of age. Spine density peaked at P35 and remained significantly higher than P24 at all ages thereafter. *$p \leq 0.05$; **$p \leq 0.01$.

Basal spine morphology shifts through development. Spine length distribution was shifted to the left at P39 relative to P24 ($D = 0.1686$, $p \leq 0.0001$), i.e. dendritic spines on PFC PCs of P39 (adolescent rats) were shorter than those of P24 (preadolescent) animals (Figure 11A). The frequency distributions of spine head diameter ($D = 0.0953$, $p \leq 0.01$; Figure 11B) and spine head volume ($D = 0.1134$, $p = 0.0001$; Figure 11C) were both significantly shifted to the right at P39 compared to P24, i.e. the spine heads were larger in the adolescent rats.

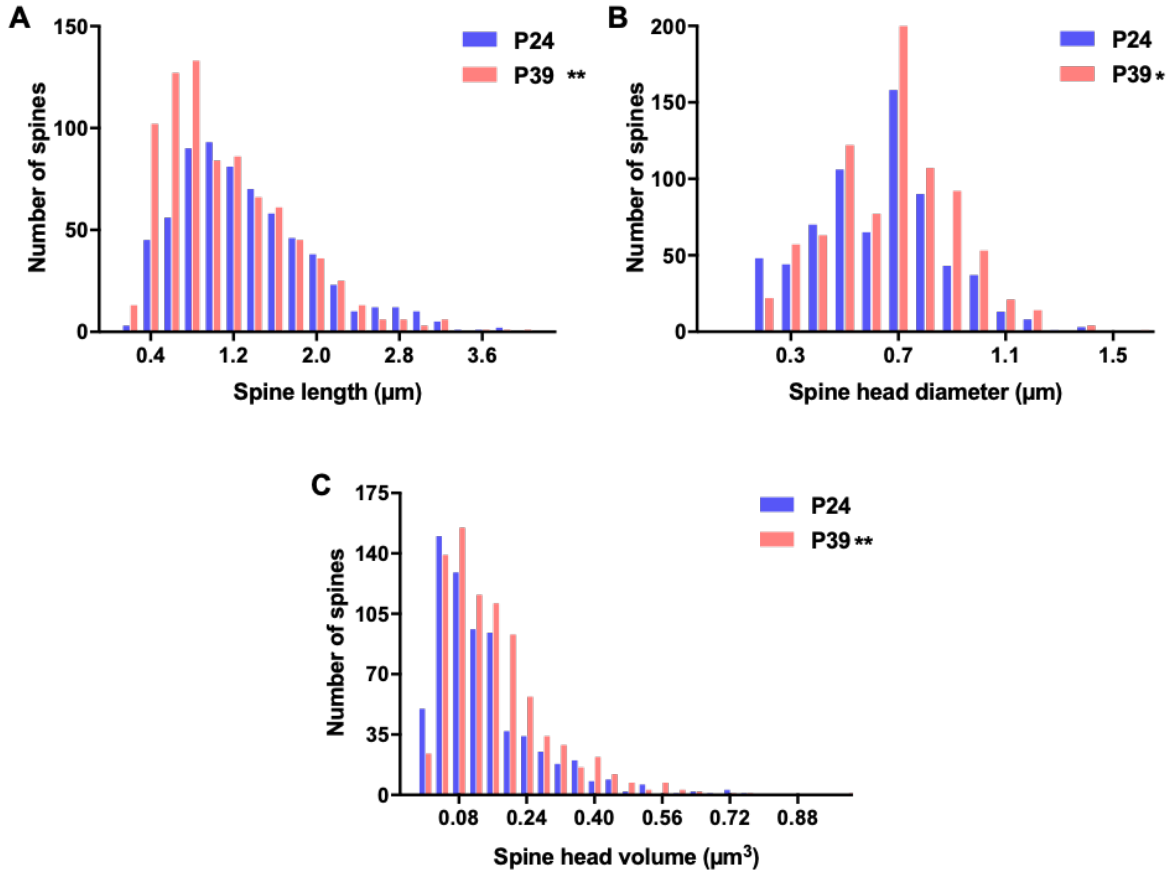


Figure 11. Dendritic spine morphology in prefrontal cortical L5 pyramidal cells across development. The frequency distributions of (A) spine length, (B) maximal spine head diameter, and (C) spine head volume were compared at P24 and P39. The distribution of spine length at P39 was significantly different from that at P24 and was shifted to the left. The frequency distributions of spine head diameter and volume were shifted to the right at P39. *$p \leq 0.01$; **$p \leq 0.0001$.

Prefrontal cortical apical spine density changes through development. There was an overall age effect on apical spine density of L5 PFC PCs ($F(5,14) = 4.187$, $p = 0.0155$; Figure 12). Bonferroni post-hoc analysis showed that spine density was significantly increased at P35, P39, and P50 but not at P70 relative to P24, i.e., apical spine density paralleled the pattern of spine density changes seen in the basal dendritic tree over the first 50 postnatal days.

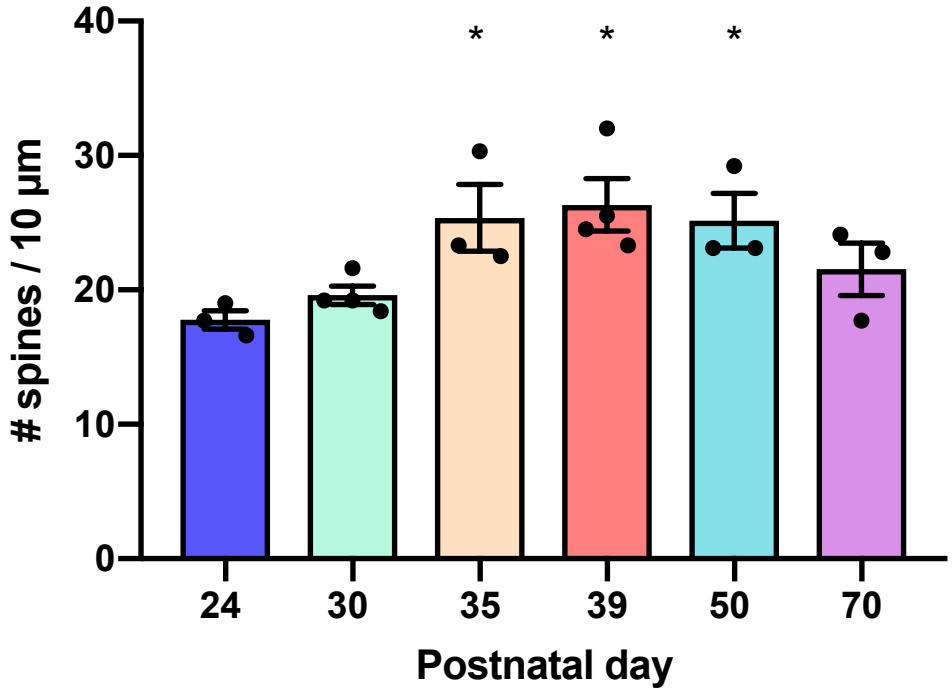


Figure 12. Trajectory of apical spine density changes in L5 prefrontal cortical pyramidal cells across adolescence. Mean (±SEM) apical spine density differed significantly as a function of age. Apical spine density peaked at P35 and remained elevated relative to P24 only through P50. *$p \leq 0.05$.

3.3 Discussion

Dendritic spine density on both basal and apical dendrites of L5 PCs in the PFC peaked at P35. Over the next five weeks basal spine density plateaued; apical spine density also remained level through P50, when levels seemed to start to decrease to reach a mature PC morphology.

Surprisingly, there have been relatively few previous studies of the ontogeny of dendritic spines on PFC PCs. Koss et al. (2014), using Golgi impregnation, reported that basal spine density on L5 PCs of the rat PFC increased from P20 to P35 and decreased at P90 relative to P35. Markham et al. (2013), studying L3 PCs in the rat prelimbic cortex, found that basal and apical spine density increased from P20 to P30, and then decreased from P30 to P56 on the basilar dendrites of female but not male rats. Drzewiecki et al. (2016) observed a similar sex-specific temporal pattern in the rat PFC, with females but not males showing an increase from P25 to P35, and a decrease from P35 to P45. Gourley et al. (2012), using mice in which GFP was expressed in PCs under a Thy1 promoter, concluded that spine density in the orbitofrontal cortex peaked at P31 and declined by P56-60. A decrease in apical dendritic spine density of L5 PFC PCs between P31 and P42 has also recently been reported (Shapiro et al., 2017), although density was analyzed at a distance ranging from 25 - 150 μm from the soma.

My data and these reports agree that basal and apical spine density on frontal cortical PCs peaks between P30 and P35, but the results of studies on spine density in animals older than P35 diverge. I did not observe a decrease in basal dendritic spine density at P70, the latest time point at which I assessed basal spine density. Previous studies that reported a decrease in spine or synapse density prior to P70 were performed in cohorts consisting of only male or female animals (Drzewiecki et al., 2016; Markham et al., 2013; Markham and Juraska, 2002) but not a mixed sex population, as we studied, or in orbitofrontal rather than medial prefrontal cortices different (Gourley et al., 2012). Koss et al. (2014) did not examine a time point between P35 and P90. Although my apical spine density data differ from Shapiro et al. (2017) in the timing of apical

density decrease, this group monitored spine density in the apical dendrites over a much greater distance range from the soma.

The lack of consistency across studies is not surprising because PC dendritic spine density varies across brain regions and laminar position of the cells examined, and is influenced by technical factors, such as the methods used to reveal and analyze spines (Van Aerde and Feldmeyer, 2015; Hattox and Nelson, 2007; Wang et al., 2006). I focused my efforts on pyramidal cells in L5 of the prelimbic PFC for several reasons: (1) the prelimbic PFC has been suggested to be homologous to the dorsolateral PFC of primates, including humans (see Uylings et al., 2003; however see also Preuss, 1995); (2) L5 of the prelimbic cortex in the rat contains the highest density of dopaminergic terminals in the medial PFC (Descarries et al., 1987; Van Eden et al., 1987); (3) dopamine depletion of the PFC induces dendritic spine loss in L5 but not L2/3 PCs in the prelimbic cortex (Wang and Deutch, 2008); and (4) L5 is the primary layer for integrated output from the prelimbic PFC to other brain regions.

Exogenous factors, such as housing conditions and other environmental conditions, can also influence spine density (see Rosenzweig, 2003). Because of the large number of endogenous and exogenous factors that can modify spine density and morphology, it is necessary that each study attempting to relate developmental changes in spine density or other dendritic parameters define the ontogeny of dendritic changes experimentally, rather than rely on published data.

As noted previously, the same gross temporal pattern in spine density on PFC PCs was seen in male and female subjects. However, my experiment was underpowered to detect a significant sex effect on secondary analysis. Future studies will be required to determine if there are significant differences between males and females in dendritic spine density during postnatal development, particularly because the age of first diagnosis of schizophrenia is later in females than males.

Dendritic spines have been grouped into different classes based on morphological features (Jones and Powell, 1969; Peters and Kaiserman-Abramof, 1970). This spurred attempts

to relate different morphological types of spines to different functions. However, subsequent careful assessments revealed that measures of spine structure, such as spine length and spine head diameter, fall along a continuum and do not form non-overlapping distributions (Arellano, 2007). Nonetheless, it has remained in vogue to monitor different morphological "types" of spines because of perceived relationships between the structure of a spine (long and thin vs. short and possessing a spine head) and physiological (functional) measures. In early postnatal development, filopodia-shaped or thin spines are common; as development progresses, spines transition to a larger, mushroom shape with a well-defined head and neck (Lin and Koleske, 2010). "Thus, spine density and morphology reflect neuronal development, as well as plasticity and connectivity within a given region" (Glausier and Lewis, 2013).

My data indicate that the overall distribution of spine length is shifted to the left and the distributions of spine head diameter and spine head volume are shifted to the right. In other words, spines at P39 are shorter and have larger heads than spines on PCs of animals two weeks older. These observations suggest that longer, immature spines are preferentially targeted for elimination, while the population of spines with large heads and short necks is presumably enriched (see Lin and Koleske, 2010). This notion is consistent with a large body of data indicating that spine maturation and stability is activity dependent, operating under a use-it-or-lose it rule (Holtmaat and Svoboda, 2009; Xu et al., 2009; Zuo et al., 2005).

Most studies of dendritic maturation on PFC PCs lack the temporal resolution necessary to discern structural changes during the periadolescent and adolescent period, when PFC neurons undergo relatively rapid changes in morphology. To that end, I examined spines at relatively high temporal resolution across these developmental periods. Nonetheless, my data represent static snapshots of dendritic trees over this period of active change, including both pruning of spines and the potential contribution of spinogenesis. Longitudinal multiphoton "cortical window" studies will be required to unravel the relative contribution of spinogenesis and spine pruning during periadolescence and adolescence.

There is no universally accepted definition of adolescence in rodents or humans. The periadolescent period in rats has been defined by Spear and Brake (1983) as the time between the onset of diurnal gonadotropin cycling ($\sim$P28) and the age at which reproductive capacity is achieved ($\sim$P38-P42). A variety of data suggest that adolescence then extends until $\sim$P50. For example, based on recordings of PFC interneurons, Tseng and O'Donnell (2007) suggest that the physiological transition from adolescence to young adulthood in the PFC occurs at $\sim$P50. Consistent with this suggestion, dopamine D1 and D2 receptor binding in the rat PFC peaks at P40 and declines significantly by P60 (Andersen et al., 2000). Moreover, the dopamine innervation of the rat PFC doesnt achieve its stable adult density until $\sim$P60 (Kalsbeek et al., 1988). These considerations collectively lead me to henceforth refer to the period between P35 and P50 as adolescence.

CHAPTER 4

MICROGLIAL PHAGOCYTOSIS OF SYNAPSES: DEVELOPMENTAL POSTSYNAPTIC PRUNING

The connections of neurons are achieved by dynamic processes that refine and sculpt synapses. During early postnatal development neurons form exuberant synaptic connections (Innocenti and Price, 2005). However, many synapses are subsequently pruned (Katz and Shatz, 1996). As discussed in Chapter 2, studies have recently identified a critical role for microglia in synaptic pruning during early postnatal developmental windows (Paolicelli et al., 2011; Schafer et al., 2012). Targeting of microglia to synapses appears to be mediated in part by a neuronal chemokine, fractalkine (Schafer et al., 2013). Fractalkine regulates microglial recruitment, activation, and number by acting on its canonical receptor (Schafer et al., 2013), CX_3CR1, the expression of which is largely restricted to microglia (Harrison et al., 1998).

Microglia prune synapses to achieve mature neuronal connections. This occurs at different postnatal times, depending on brain area involved. For example, in the dorsal lateral geniculate nucleus (dLGN) of the murine thalamus, the first synapse of the retinal projection to central visual areas, synaptic pruning occurs at P5 (Schafer et al., 2012). This corresponds to the time during which eye-specific segregation, which involves retinal ganglion cell (RGC) inputs competing for territory in the dLGN, occurs. In contrast, maturation of the PFC is protracted, and involves a refinement process in which there is an increase in spine density during the first month of age (Koss et al., 2014; Markham et al., 2013), after which spines are eliminated until the PCs of the PFC reach their adult maturity. It is not known if microglia sculpt synapses on projection neurons in late-maturing structures such as the PFC, in which the projection neurons suffer dendritic spine loss in schizophrenia. I therefore determined if microglia are responsible for pruning supernumerary dendritic spines in the PFC during development (Figure 13), focusing on

preadolescence and adolescence, when symptoms and behaviors that are harbingers of psychosis usually first appear.

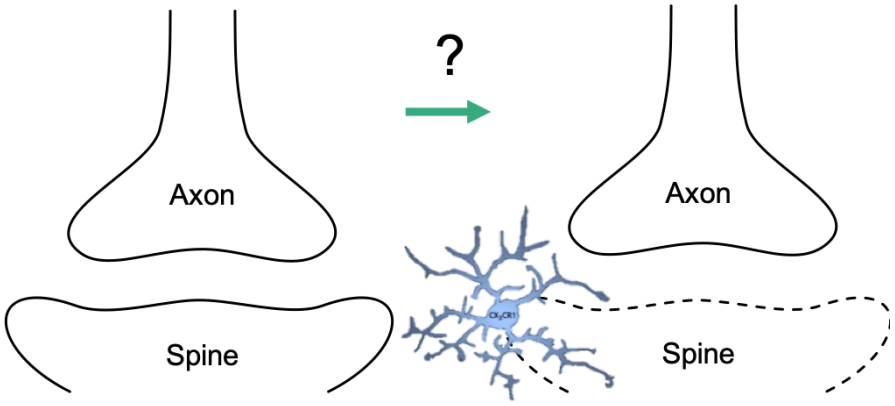


Figure 13. Do microglia prune dendritic spines of prefrontal cortical pyramidal cells during development?

4.1 Methods

Immunohistochemistry. One hemisphere of the perfused brain (see Chapter 3) was post-fixed in 4% PFA in PBS overnight at 4°C, and then cryoprotected in 30% sucrose. Sets of coronal sections (42 μm) through the PFC were cut on a sliding microtome. Different sets were used for engulfment analyses (below and Chapter 5) and microglial activation assessments (Chapter 6).

An immunofluorescent approach was used to assess microglial engulfment of dendritic spines. Sections were blocked in Tris-buffered saline (TBS) containing 4% normal horse serum (16050114, Thermo Fisher Scientific Inc.) and 0.2% Triton X-100 (TBS^{++}) before being incubated for 36-48 hours at 4°C in a cocktail of an antibody directed against the microglial marker Iba1 and an antibody that recognizes the postsynaptic (spine) marker PSD-95 (Table 1). Sections were then placed in the appropriate fluorescently-conjugated secondary antibodies from Jackson ImmunoResearch (Table 1) for 2 hours, washed in TBS, mounted, and coverslipped with ProLong Antifade.

Antibody	Host	Company	Catalog Number	Dilution
Iba1	Rabbit, polyclonal	Wako Richmond, VA	019-19741 RRID: AB_839504	0.167 µg/mL
PSD-95	Mouse, monoclonal	Millipore Sigma Burlington, MA	73-066 RRID: AB_2092365	0.733 µg/mL
VGluT1	Mouse, monoclonal supernatant	UC Davis/NIH NeuroMab Davis, CA	73-066 RRID: AB_10673111	0.483 µg/mL*
Alexa Fluor 488 AffiniPure Donkey Anti-Rabbit IgG (H+L)	Donkey, polyclonal	Jackson ImmunoResearch West Grove, PA	711-545-152 RRID: AB_2313584	1.25 µg/mL
Cy3 AffiniPure Donkey Anti-Mouse IgG (H+L)	Donkey, polyclonal	Jackson ImmunoResearch West Grove, PA	715-165-150 RRID: AB_2340813	1.25 µg/mL

Table 1. Source information and dilutions for antibodies used for assessment of microglial engulfment.
*Concentration varies by lot. The concentration of lot number 463-1DH-55 was 29 µg/mL.

Microglial engulfment. Z stacks of eight randomly-selected microglial cells per animal in L5 of the prelimbic cortex were obtained using a 1.40 NA 63x objective, with a digital 3x zoom on a Zeiss LSM 880 confocal microscope. The Z stacks were deconvolved (Huygen's Essential, Scientific Volume Imaging; Hilversum, Netherlands), and projection images generated (Figure 14). Microglial engulfment of synaptic elements was assessed by obtaining the ratio of the area of "yellow" pixels (colocalization of microglia and synaptic elements), identified by the overlap of defined threshold ranges for the red and green channels, to the area occupied by the red channel (microglia), yielding a "colocalization index." Orthogonal views of confocal images were used to verify colocalization (Figure 15).

Data analysis. Measures of microglial engulfment of postsynaptic dendritic spines were analyzed with the Kruskal-Wallis non-parametric ANOVA followed by Dunn's multiple comparison test when indicated.

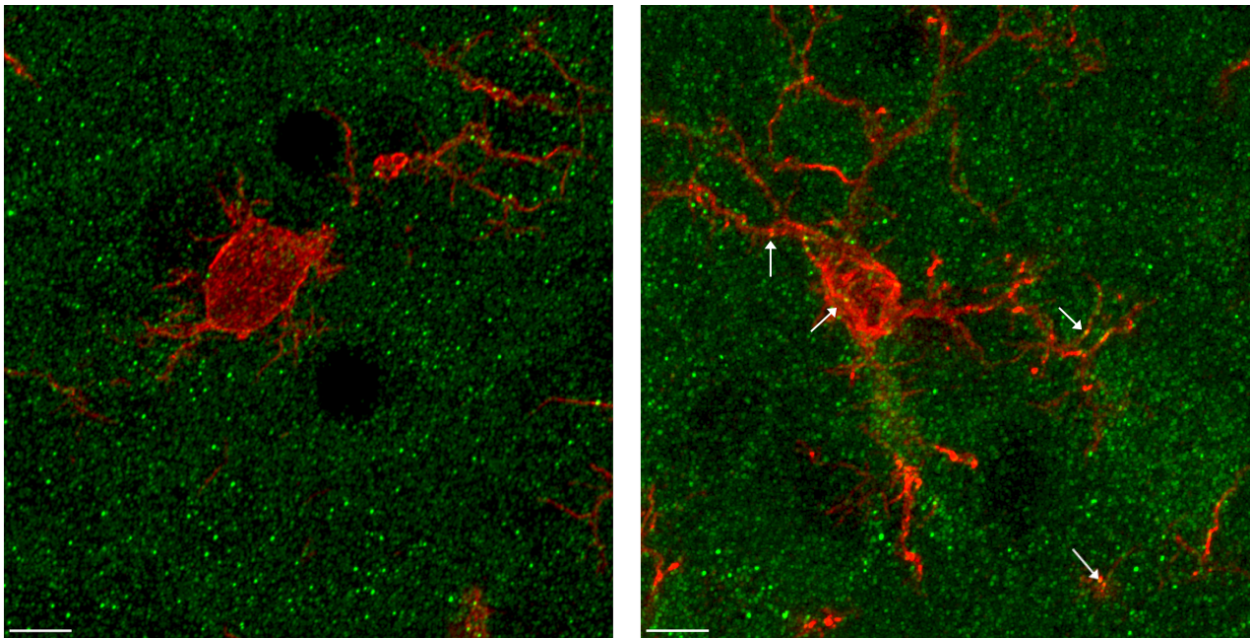


Figure 14. Representative immunofluorescence staining of microglial engulfment of spines. Examples of projection images of microglia with low (left) and high (right) levels of colocalization (yellow puncta) of Iba1-labeled microglia (red) and PSD-95-immunoreactive dendritic spines (green). PSD-95-ir puncta were visible in the soma and in the processes (white arrows). Scale bars, 5 μm.

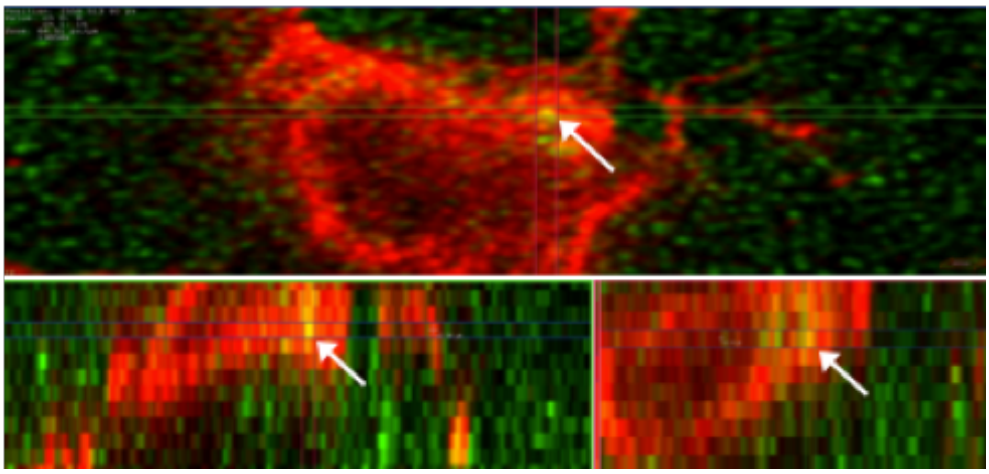


Figure 15. Orthogonal views showing the microglia and the dendritic spine in the same plane (white arrows) were used to verify colocalization.

4.2 Results

Microglial engulfment of dendritic spines. The ANOVA revealed a significant age effect for microglial engulfment of dendritic spines ($H = 36.9$, $p \leq 0.0001$; Figure 16). Post-hoc tests showed a significant increase in microglial phagocytosis of spines at P39 relative to P24 ($p \leq 0.05$). By P50 microglial engulfment of spines had decreased compared to both P24 ($p \leq 0.01$) and P39 ($p \leq 0.0001$).

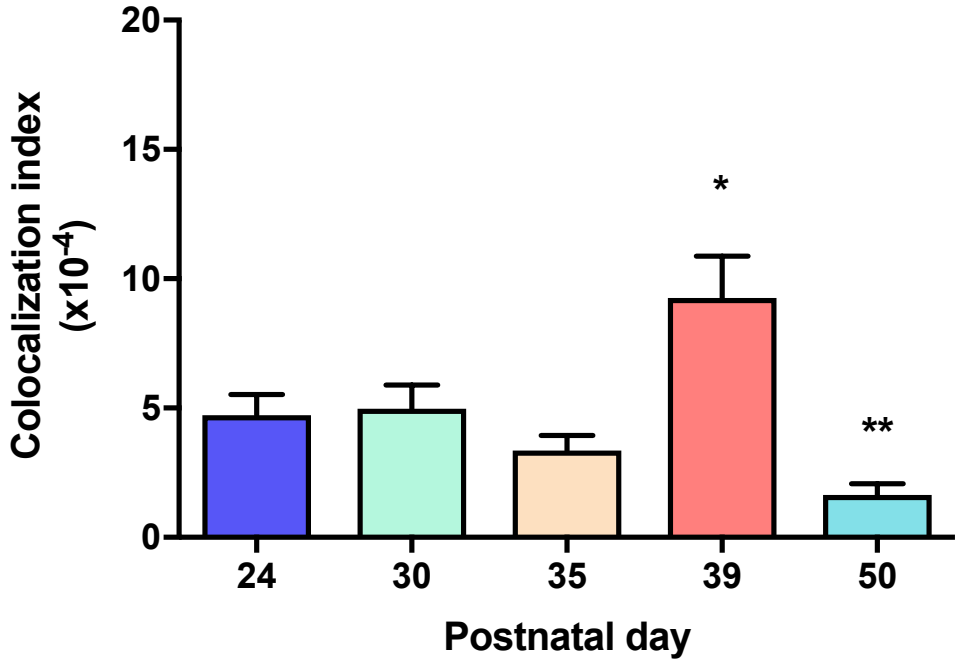


Figure 16. Microglial engulfment of dendritic spines during development in the prefrontal cortex. A sharp increase in microglial phagocytosis of dendritic spines was observed at P39 relative to earlier time points. By P50 microglial engulfment had subsided and was significantly lower than observed at P24. *$p \leq 0.05$; **$p \leq 0.0001$.

4.3 Discussion

Microglia age-dependently phagocytose dendritic spines, the postsynaptic elements of excitatory synapses on L5 PFC PCs. Microglia actively engulfed dendritic spines of PFC PCs at P39, shortly after peak spine density (P35) is reached; engulfment abated and was decreased at P50.

The past several years has seen a major reconsideration of the role of microglia in shaping the mature architecture of neurons. Most studies of microglial engulfment of synapses during postnatal development have focused on brain regions that mature relatively early in postnatal life (Paolicelli et al., 2011; Schafer et al., 2012). There have been no studies examining developmentally-mediated microglial engulfment in the PFC, a late-maturing structure (Petanjek et al., 2011).

I observed a sharp increase in microglial engulfment of PSD-95-ir dendritic spines in P39 rats, shortly after peak spine density was achieved. PSD-95 is a well-validated marker of dendritic spines that is localized to the post-junctional spine head, with minimal expression in the spine neck (Harris and Weinberg, 2012; Hunt et al., 1996).

PSD-95-ir elements subjectively appeared to be observed more frequently in the soma and proximal processes of microglia rather than the distal processes, a finding consistent with previous reports of proximal microglial processes containing cellular elements (Tremblay et al., 2010).

PSD-95-ir puncta were not seen in every PFC microglia of P39 animals. In some cases, puncta were seen throughout the soma and processes of microglial cells, but in other microglia no PSD-95 spine head particles were observed; this may suggest that microglia are functionally heterogeneous. Lawson et al. (1990) noted subtle differences in the distribution of microglia across brain regions, suggesting different populations of microglia. More recently, De Biase et al. (2017) reported that local cues determine divergent structural and functional properties of microglia. It is not clear if these local cues include “eat me” and “find me” signals (and their

negative counterparts), as described for apoptotic cells (see Hochreiter-Hufford and Ravichandran 2013) and more recently for microglia (Lehrman et al., 2018; Schafer et al., 2012). Recently, Tay et al. (2017) reported differences in regional self-renewal and turnover dynamics of microglia across several brain regions, and recent single microglia transcriptome data have uncovered multiple (9) distinct classes that microglia adopt that vary across development and inflammatory states (Hammond et al., 2019), further underscoring the heterogeneity of microglia across the brain. The diversity of microglia, which were previously considered to be a homogeneous population of cells, is increasingly appreciated and has been reviewed in detail (see reviews by Gertig and Hanisch, 2014; Olah et al., 2011; Silvin and Ginhoux, 2018). Understanding the homeostatic temporal and spatial states of microglia and how they might be influenced by different factors is critical for our understanding of how perturbations might drive disease. This is particularly of interest when considering the two-hit model of schizophrenia (Keshavan, 1999; Keshavan and Hogarty, 1999), in which a stressor or maldevelopment early in life primes microglia such that a second stressor results in an exaggerated response by microglia - such as overactive synaptic pruning.

The presence of PSD-95 puncta in microglia is interpreted as being a consequence of active phagocytosis of spines attached to the dendritic shaft. It is possible that microglia phagocytose spines that have already been severed from the parent neuron. Running counter to this possibility, however, are light and electron microscopic studies that indicate that there is direct contact between microglia and both pre- and postsynaptic elements of neurons (Tremblay et al., 2010; Wake et al., 2009). While not conclusive, these studies suggest that microglia remove spines from neurons rather than engulfing “free” spines, particularly when coupled with data indicating that microglia contain phagocytic structures under normal conditions, with smaller dendritic spines being more frequently contacted by microglia (Tremblay et al., 2010) and *CX_3CR1* knockout mice exhibiting a transient increase in spine density during the second and third postnatal weeks (Paolicelli et al., 2011). These data suggest that microglia remove spines

from their structural foundations (dendritic shafts) rather than phagocytosing material already shed by a neuron.

I focused my attention on the involvement of microglia in the pruning of synapses during development. However, the synaptic stripping that accompanies postnatal maturation of the cortex may not be solely attributable to microglia. Although there is not an overt astrocytosis in schizophrenia (Falkai et al., 1999; Garey, 2010), it is possible that astrocytes may also be involved in synaptic stripping in the developing prefrontal cortex. In other brain areas, astrocytes have been shown (at other postnatal times) to contribute to synapse elimination both directly (via recognition of an "eat me" signal and subsequent phagocytosis of synaptic elements) and indirectly (by inducing the deposition of complement proteins at synapses, which are then recognized and eliminated by microglia) (see Chung et al., 2015). Future studies will be required to determine if astrocytes contribute to PFC PC spine pruning during development

I have discussed potential roles for astrocytes as well as microglia in pruning synapses during postnatal development. Processes other than phagocytosis may also contribute to decreasing synapse number. Simple retraction of the spine into its parent dendritic shaft may lead to reduced spine number (Nitsch and Riesenberg, 1995).

Autophagy, which involves removal of unnecessary cellular machinery and is independent of phagocytosis of another cell (for review see Glick et al., 2010), plays an important role in regulating synapse number during development. Disruption of autophagic processes in mice leads to an increase in basal spine density on L5 PCs in the A1/S2 temporal cortex (Tang et al., 2014). The relative contribution of spine retraction, autophagy, and engulfment by other glial cells during these developmental periods remains to be determined.

CHAPTER 5

MICROGLIAL PHAGOCYTOSIS OF SYNAPSES: DEVELOPMENTAL PRESYNAPTIC PRUNING

In Chapter 4 I identified a role for microglia in the pruning of dendritic spines during adolescence. Multiple independent studies have reported a decreased density of dendritic spines on PFC PCs in schizophrenia (see Glausier and Lewis, 2013; Moyer et al., 2015). If dendritic spines are decreased in number, there may be a parallel decrease in presynaptic elements. I therefore determined if microglia phagocytose excitatory presynaptic elements of PFC PCs (Figure 17) during the preadolescent and adolescent periods.

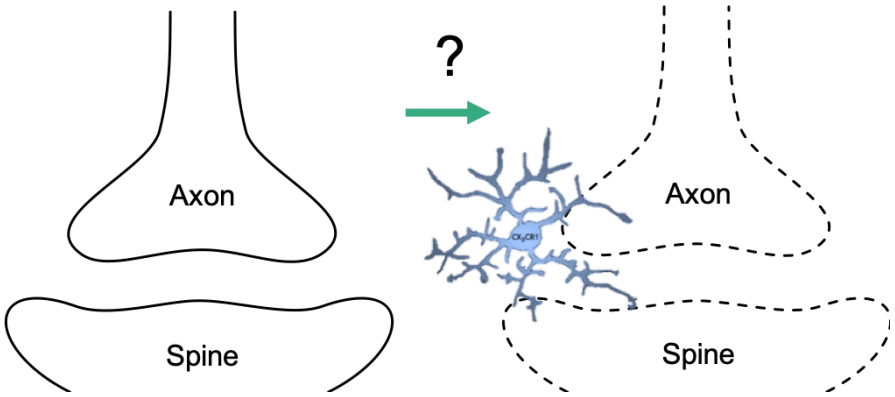


Figure 17. Do microglia prune excitatory presynaptic elements as well as dendritic spines during preadolescence or adolescence?

5.1 Methods

The ages and sexes of the animals at the time of sacrifice, methods, measurements, and analysis used in this experiment are the same as described in Chapters 3 and 4, with the exception that sections were immunofluorescently labeled with Iba1 and the presynaptic glutamatergic protein VGluT1 (Figure 18) in place of Iba1–PSD-95. VGluT1 was selected as a marker of

excitatory presynaptic elements at the asymmetric axospinous synapse on PCs in the PFC (Fremeau et al., 2004a). Antibody information for primary and secondary antibodies is outlined in Table 1 (Chapter 4).

Because spine density peaked at P35 and phagocytosis of spines was maximal at P39, I restricted assessment of microglial pruning of presynaptic elements to four time points: P24, P35, P39, and P50.

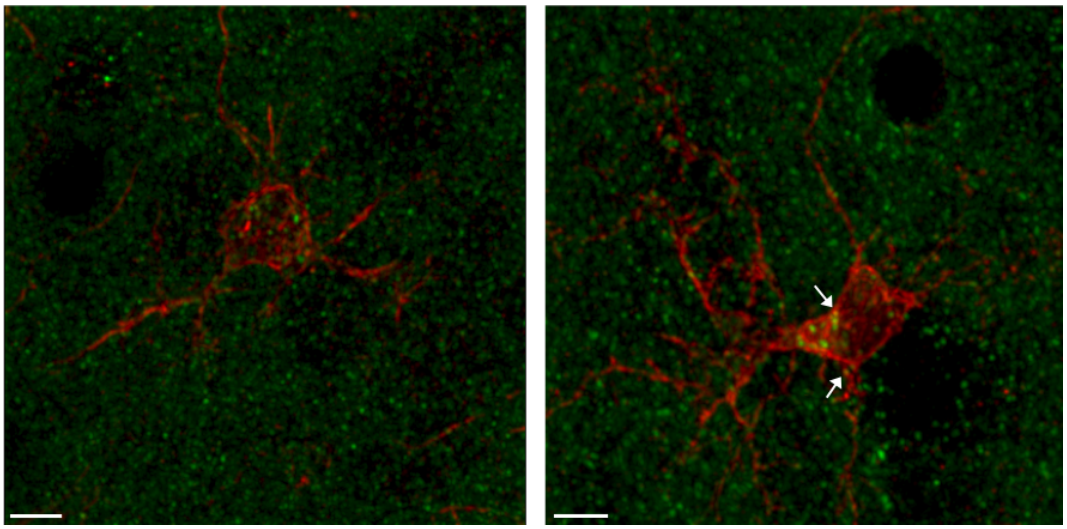


Figure 18. Representative images of microglial engulfment of presynaptic elements. Examples of projection images of microglia with low (left) and high (right) levels of colocalization (yellow puncta; white arrows) of Iba1-ir microglia (red) and VGluT1-ir presynaptic elements (green). Scale bars, 5 μm.

5.2 Results

Microglial engulfment of presynaptic elements. There was an overall age effect on microglial phagocytosis of presynaptic glutamatergic elements in the PFC ($H = 30.1$, $p \leq 0.0001$; Figure 19). Post-hoc tests found an increase in microglial engulfment of VGluT1-ir glutamatergic terminals at both P39 ($p \leq 0.05$) and P50 ($p \leq 0.0001$) relative to P24. In addition, the engulfment of presynaptic elements at P50 was significantly greater than observed at P39 ($p \leq 0.001$).

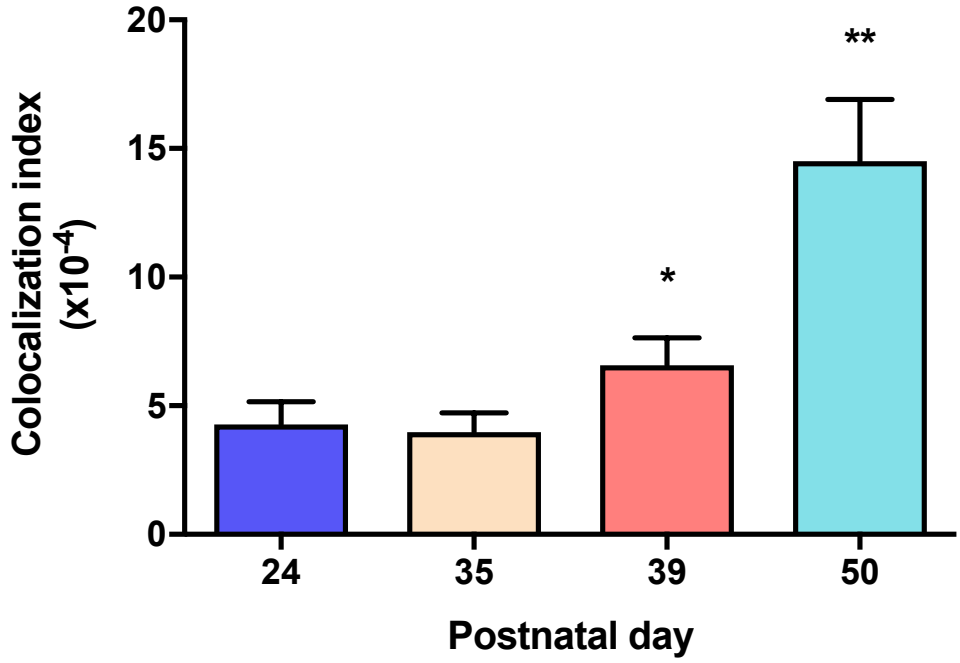


Figure 19. Microglia engulf excitatory presynaptic elements in the prefrontal cortex during adolescence. The engulfment of glutamatergic (VGluT1-ir) presynaptic elements by microglia was significantly increased at both P39 and P50 relative to P24. *$p \leq 0.05$; **$p \leq 0.0001$.

5.3 Discussion

Microglial engulfment of glutamatergic presynaptic elements was increased at P39, when microglial phagocytosis of spines occurred, but also at P50, when spine pruning by microglia had ceased. To date, most studies of microglial involvement in neuronal sculpting have directed their attention to presynaptic elements (Bialas and Stevens, 2013; Schafer et al., 2012). My findings are consistent with microglia developmentally pruning both pre- and postsynaptic elements of axodendritic PC synapses, as has been reported in the hippocampus at P15 (Paolicelli et al., 2011). The observation that microglial engulfment of presynaptic elements began at the same time as microglial engulfment of spines but persisted until spine pruning had ceased agrees with the model of synapse development in which spine growth precedes synapse formation (Knott et al., 2006; Miller and Peters, 1981; Yuste and Bonhoeffer, 2004), and suggests that synapse elimination is spine instructive.

Most axons that synapse onto dendritic spines are glutamatergic. I therefore used an antibody directed against VGluT1, which defines a subset of glutamatergic neurons (Fremeau et al., 2001, 2004a; Kaneko and Fujiyama, 2002), to mark presynaptic axons. I examined VGluT1-ir axons because postmortem studies of vesicular glutamatergic transporter levels in schizophrenia suggested that VGluT1-positive neurons may be preferentially affected in schizophrenia. Several studies have reported decreased VGluT1 (Bitanihirwe et al., 2009; Eastwood and Harrison, 2005; Oni-Orisan et al., 2008) but not VGluT2 (Oni-Orisan et al., 2008; Shan et al., 2013) levels in samples from patients with schizophrenia. Because VGluT1-ir axon terminals are predominantly derived from cortical neurons, with VGluT2-ir mainly expressed by subcortical neurons (Fremeau et al., 2001; Kaneko and Fujiyama, 2002), it will be of interest to determine if there is selective microglial pruning of synapses of specific cortical circuits. Though some cortical axons appear to co-express both VGluT1 and VGluT2, this coexistence is seen primarily in L4 (Graziano et al., 2008; Nakamura et al., 2005) – which the PFC lacks.

Given the difference in the timing of pre- and postsynaptic engulfment, it is possible that different "eat me"/"don't eat me" signals, or a difference in the timing of the expression patterns of these instructive signals, coordinate or subserve pruning of the different sides of the synapse. Recently, Lehrman et al. (2018) proposed that cluster of differentiation 47 (CD47), via interaction with the macrophage (microglial) receptor signal regulatory protein α (SIRPα), serves as a "don't eat me" signal that functions in parallel to the C1q/CR3 "eat me" signal during developmentally-mediated pruning in the dLGN (see Rivest, 2018; Figure 20). In the CNS, these signaling molecules have largely been explored in the context of the pruning of the axon terminal; similar analyses have not been conducted for spine pruning, nor have they been performed in late-maturing structures such as the PFC. Hinting toward the possibility of different mediators of pre- and postsynaptic pruning is my observation that microglial engulfment of spines is significantly decreased at P50 when presynaptic pruning is high, suggesting that there is a switch in localization of the signal or that a different signal is elaborated by presynaptic elements. Further, disruption of the complement "eat me" signals does not abolish pruning altogether but leads to engulfment deficits of $\sim$50% (Schafer et al., 2012). There may thus be (possibly functional) redundancy in signals mediating pruning. Future studies examining the expression patterns and localization of these proteins, and the exploration of additional signals, during PFC development are warranted.

Pruning of presynaptic elements in part depends on the complement cascade (Schafer et al., 2012; Sekar et al., 2016; Stevens et al., 2007). Complement-dependent pruning has been demonstrated using C1q, C3, CR3, and C4 rodent deletion models. I am precluded from directly examining in this way the effects of complement disruption on spine and axonal pruning because C1q knockout mice are prone to seizures (Chu et al., 2010), which affects spine density and morphology (Ma et al., 2013). Given the linearity of the complement cascade, it is likely that disruption of downstream complement components would similarly cause seizures and confound the analyses.

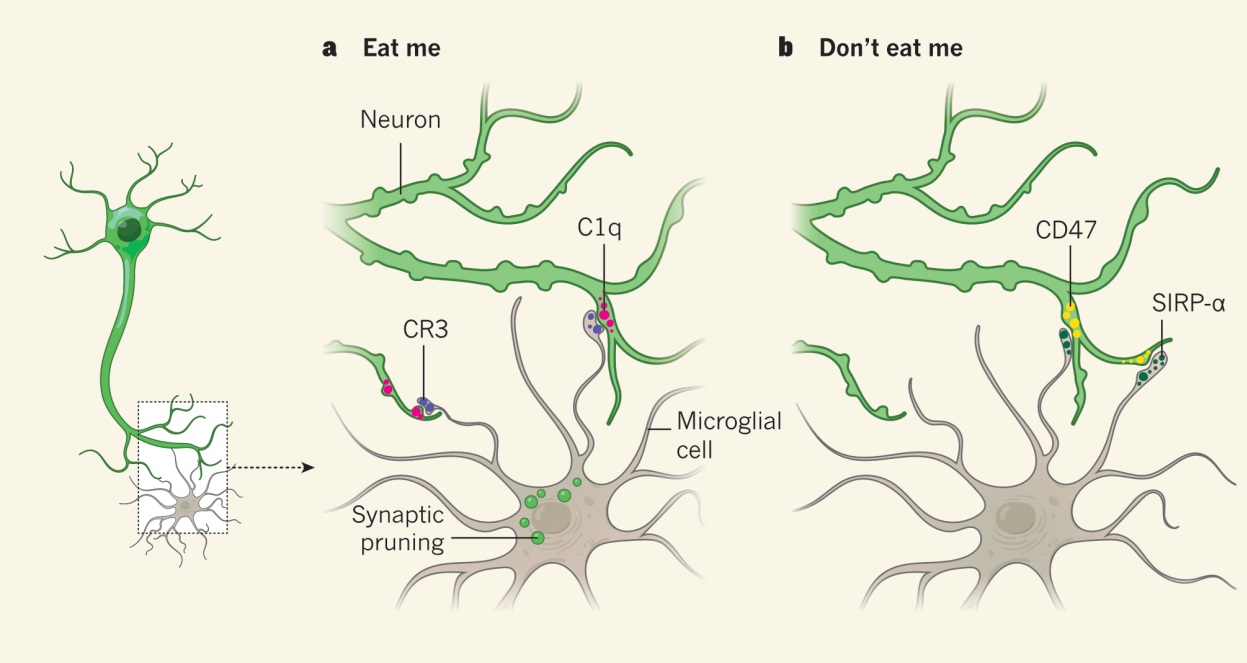


Figure 20. Established "eat me" and "don't eat me" signals mediating synapse pruning. Complement proteins serve as "eat me" signals for synapses, while CD47-SIRPα serve as a protective, "don't eat me" signal (Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature, Nature. A 'dont eat me' immune signal protects neuronal connections, Rivest S. © 2019).

C1q and C3, which localize to synapses, are upregulated in the dLGN during RGC synaptic refinement (P5) and are subsequently downregulated (Schafer et al., 2012; Stevens et al., 2007). I thus tried to determine the prefrontal cortical expression pattern of C1q (Abcam, ab71940) and C3 (MP Biomedicals, 55730) as a prelude to determining if they are to see if they are upregulated during PFC synaptic pruning. However, staining with both of these antibodies was problematic: C1q displayed differential expression patterns under acidic vs. basic antigen retrieval conditions, while C3 yielded inconsistent and sparse staining. Further, when I characterized the antibodies by immunoblotting, both the C1q and C3 antibodies labeled multiple bands (data not shown). I did not have access to C1q or C3 knockout tissue to validate the antibodies, and I therefore did not pursue further studies aimed at evaluating complement protein levels across adolescence. Additional work will be required to determine if microglial-mediated

pruning of synapses in the PFC is complement-dependent.

Schafer et al. (2012) have reported findings that suggest that microglial pruning of presynaptic elements in the thalamus occurs in an activity-dependent manner, with "weaker" synapses being targeted for pruning. At the time this experiment was performed, there was no way to label microglia but not peripheral macrophages that infiltrate the CNS. I was therefore unable to directly assess if influencing neural activity alters pruning of synapses in the PFC. The recent identification of Tmem119 as a protein expressed by microglia but not by peripherally-derived cells (Bennett et al., 2016) now makes it possible to determine if modulating neural activity alters pruning of synapses during postnatal development.

CHAPTER 6

MICROGLIAL "ACTIVATION" DURING PHAGOCYTOSIS OF SYNAPSES

Microglial form and function have long been considered to be linked. Specifically, microglial shape is thought to reflect the activation state of microglia. Under normal physiological conditions microglia are highly branched with a small soma, and shift to a simple, round form with few processes in response to injury or inflammation (see Hanisch Kettenmann, 2007). This distinction into two shapes belies the fact that microglial morphology exists on a continuum (see Karperien et al., 2013; Figure 21). In this experiment, parameters commonly associated with microglial "activation" were examined to determine if microglia involved in developmental pruning adopt the same activation profile as those involved in inflammatory processes.

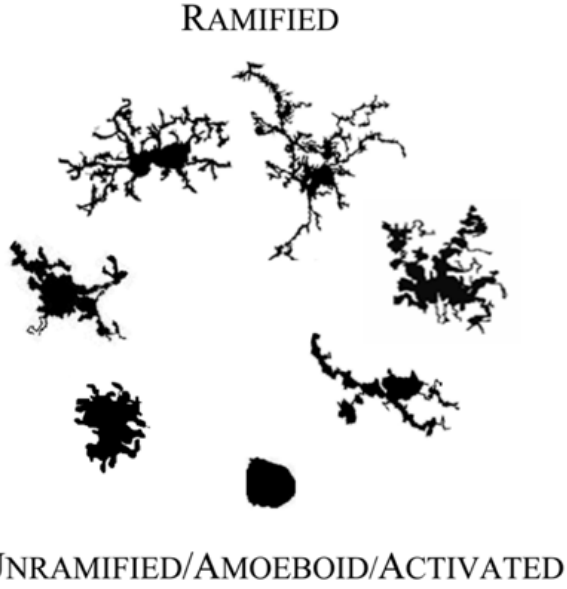


Figure 21. Classical microglia structures in the adult CNS. Microglia reversibly transition from a "ramified resting" shape to an intermediate shape to an "activated amoeboid" shape (from Karperien et al., 2013).

6.1 Methods

Immunohistochemistry. An immunoperoxidase method was used to reveal Iba1-ir microglia for determinations of microglial soma area and density. Sections through the PFC (see Chapter 4) from P24, 35, 39, and 50 animals were incubated in 10% methanol and 0.6% hydrogen peroxide in 50 mM TBS for 10 min, blocked in TBS^{++}, and incubated in rabbit anti-Iba1 (Table 2) for 18-24 hours at room temperature. Sections were then incubated in a biotinylated secondary antibody, followed by horseradish peroxidase (HRP)-conjugated streptavidin (Table 2). Sections were developed in 0.05% 3,3'-diaminobenzidine (32750, Sigma; St. Louis, MO) in TBS containing 0.009% hydrogen peroxide. Slide-mounted sections were dehydrated through increasing concentrations of ethanol followed by Histo-Clear (HS-200, National Diagnostics; Atlanta, GA) and coverslipped with DPX (44581, Sigma; St. Louis, MO).

Antibody	Host	Company	Catalog Number	Dilution
Iba1	Rabbit, polyclonal	Wako Richmond, VA	019-19741 RRID: AB_839504	0.25 µg/mL
Biotin-SP AffiniPure Donkey Anti-Rabbit IgG (H+L)	Donkey, polyclonal	Jackson ImmunoResearch West Grove, PA	711-065-152 RRID: AB_2340593	1.0 µg/mL
Peroxidase-Streptavidin		Jackson ImmunoResearch West Grove, PA	016-030-084 RRID: AB_2337238	0.625 µg/mL

Table 2. Source information and dilutions for antibodies used for determination of microglial soma area and density.

Microglial density and soma area. Three images from L5 of the prelimbic cortex from P24, P35, P39, and P50 rats were captured using a Nikon Eclipse Ni-U microscope (see Figure 22). Microglia soma area was determined by measuring the area of three microglial cells from each of the images, for a total of 9 microglia/animal. In addition, the density of microglia (number of microglia/15,500 μm^2) was determined. The area of the microglia somata and density of microglia were averaged to yield a "per animal" value for each measure.

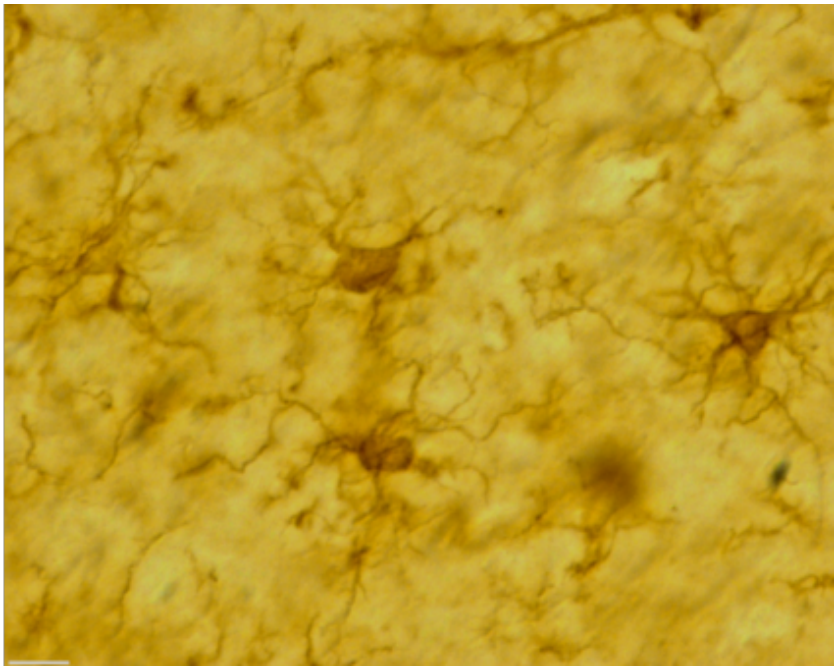


Figure 22. Microglia in L5 of the prefrontal cortex. Representative image of immunoperoxidase staining of Iba1-labeled microglia from which microglial density and soma area were determined. Scale bar, 10 μm.

Immunoblotting. The offspring of timed-pregnant Sprague-Dawley dams (Envigo; Indianapolis, IN) were used as subjects; housing conditions and ages at which animals were sacrificed (up to P50) were the same as described in Chapter 3.

Animals were sacrificed by isoflurane overdose. The brains were removed and the medial mPFC, including both the infralimbic (area 25) and prelimbic (area 32) cortices were dissected from 1.0 mm thick coronal slices (see Figure 23), frozen on dry ice, and stored at -80°C until assayed.

The tissue samples were homogenized in a 2% sodium dodecyl sulfate (SDS) solution with 1:100 protease inhibitor (5892791001, Millipore Sigma; Burlington, MA) using a sonicator, and an equal volume of 2X sample buffer with β-mercaptoethanol was added to the homogenate. Samples were heated at 70°C for 10 minutes and separated on a 12.5% SDS-polyacrylamide gel by electrophoresis. Proteins were transferred to nitrocellulose membranes, stained with Ponceau-S,

and the membrane was scanned in order to compare total protein levels in each lane (see Aldridge et al., 2008 and Romero-Calvo et al., 2010).

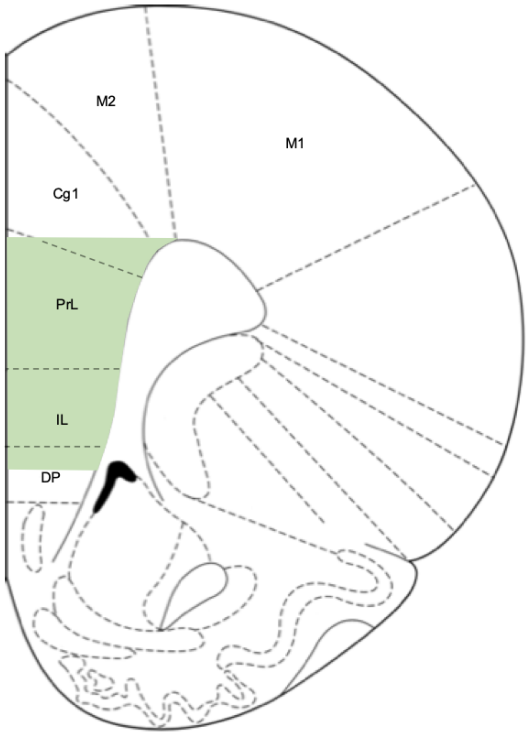


Figure 23. Coronal section at the level of the medial prefrontal cortex. The prelimbic (PrL) and infralimbic (IL) cortices (green) were dissected out.

Membranes were blocked in 4% nonfat dry milk in TBS with 0.2% Tween-20 (TBST) and then incubated for 1 hour at room temperature (RT) or overnight at 4°C in 4% milk in TBST with a primary antibody targeting the astrocytic marker glial fibrillary acidic protein (GFAP; Figure 25, right). Membranes were washed with TBST, incubated in the appropriate peroxidase-conjugated donkey antibody (Table 3), and developed using chemiluminescence (Plus-ECL; Perkin-Elmer; Waltham, MA). The optical densities (OD) of bands of interest were densitometrically analyzed using ImageJ, and normalized to total protein levels from the Ponceau-stained membranes (Romero-Calvo et al., 2010).

Antibody	Host	Company	Catalog Number	Dilution
GFAP	Chicken, polyclonal	Neuromics Edina, MN	CH22102 RRID: AB_10014322	0.857 µg/mL
Peroxidase AffiniPure Donkey Anti-Chicken IgG (H+L)	Donkey, polyclonal	Jackson ImmunoResearch West Grove, PA	703-035-155 RRID: AB_10015283	0.08 µg/mL

Table 3. Source information and dilutions for antibodies used to examine protein levels of GFAP.

Data analysis. Microglia density and soma area from immunohistochemically-stained material were analyzed by one-way ANOVAs, and Bonferroni post-hoc tests were used to define significant differences when indicated by a significant main effect in the ANOVA.

For the immunoblotting experiment, the ratio of the OD of the band of interest to that of the total protein of the associated lane was obtained (ratio = OD protein of interest/OD total protein lane). The ratio was then expressed as a percent of the mean of P24 for each individual gel. Protein level changes were analyzed by one-way ANOVA; Bonferroni post-hoc tests were used to determine significant differences.

6.2 Results

Microglia soma area and density. The ANOVA uncovered a significant age-dependent difference in soma area ($F(3,19) = 4.273$, $p = 0.0182$; Figure 24A). Post-hoc tests revealed that microglial soma area was larger in P50 than P39 subjects ($p \leq 0.05$). However, the density of microglia did not differ as a function of age ($F(3,18) = 0.839$, NS; Figure 24B).

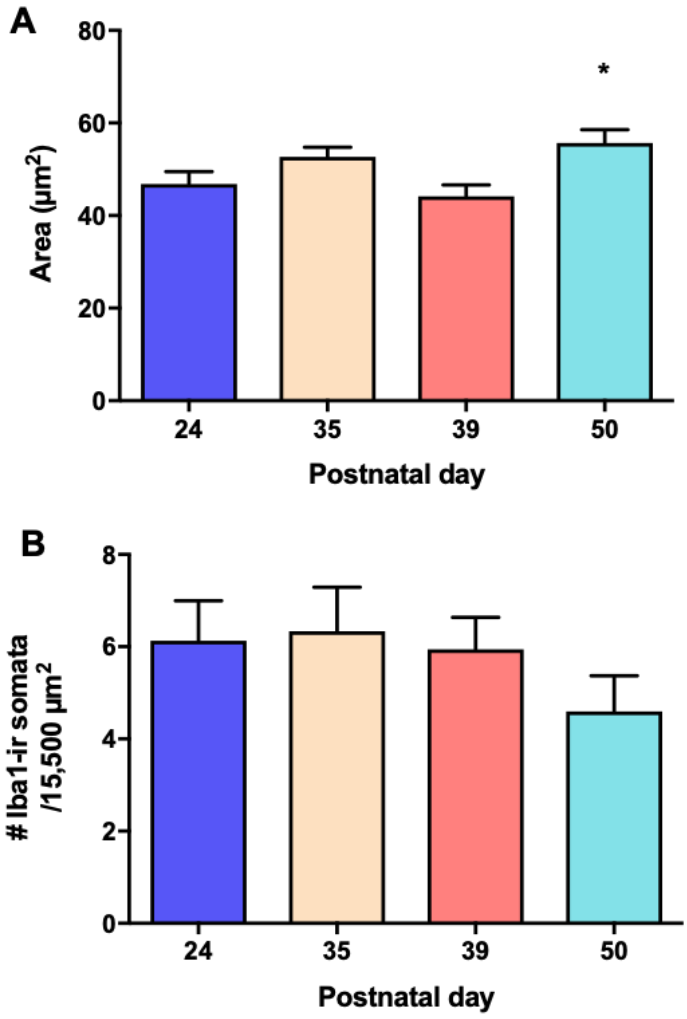


Figure 24. Microglia soma size and density in the prefrontal cortex through adolescence. (A) The mean (±SEM) area (in μm^2) of PFC microglia somata at P39 was significantly less than at P50. *$p \leq 0.05$. (B) Microglia density in L5 was unchanged across development.

GFAP protein level changes across PFC development. GFAP levels significantly differed across adolescence ($F(4,25) = 3.829$, $p = 0.015$; Figure 25, left). Post-hoc analyses revealed that levels at P39 ($p \leq 0.05$) and P50 ($p \leq 0.05$) were significantly higher than at P24.

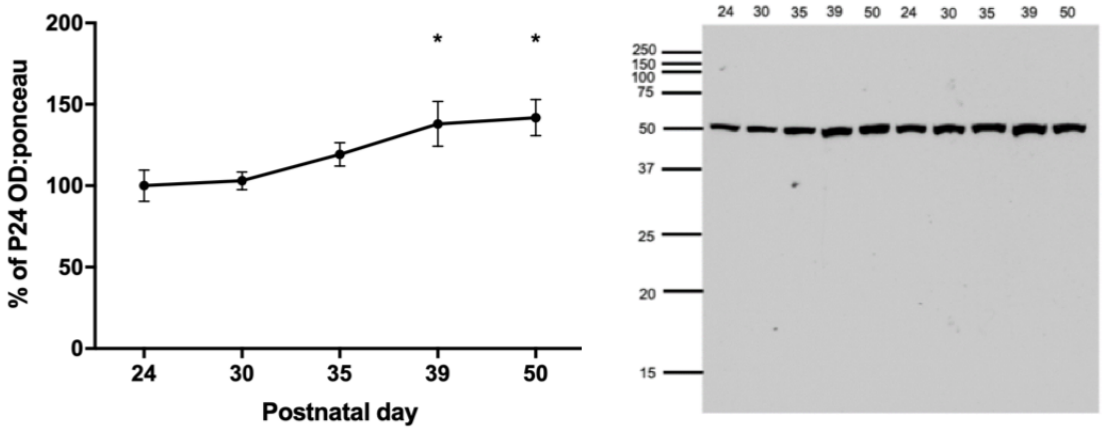


Figure 25. GFAP protein changes across development. Left: GFAP protein levels are significantly higher at P39 and P50 than P24. *$p \leq 0.05$. Right: representative immunoblot of GFAP from mPFC tissue of animals sacrificed at P24, 30, 35, 39, and 50. A single band at the appropriate molecular weight ($\sim$55kDa) was detected.

6.3 Discussion

As noted earlier, highly ramified microglia with extensively branched processes and smaller somata are thought to surveil the brain for injury or pathogens, while microglia with large soma and few or no processes are thought to be in an activated state that phagocytose the pathogens or injured or dead cells (see Hanisch and Kettenmann, 2007; Ransohoff and Perry, 2009). Despite the fact that there was extensive microglial engulfment of spines at P39 relative to earlier time points (see Chapter 4), the area of microglia soma, a putative marker of activated microglia, was not significantly larger at P39 than at earlier ages. We observed a difference in soma size only in P50 relative to P39 animals, i.e., at a time when evidence of microglial-mediated spine pruning had ceased. Moreover, microglia that engulfed synaptic elements often had extensively branched processes, consistent with the findings of Sierra et al. (2010) and Schafer et al. (2012). These observations suggest that microglial morphology is not an invariant marker of microglial activation, at least during normal (physiological) postnatal development.

In addition to examining the morphology of microglia in the PFC, we assessed the density of Iba1-positive cells. Iba1 is a cytoplasmic protein that is expressed by microglia in the CNS (Imai et al., 1996; Ito et al., 1998); Iba1 expression levels are thought to be increased in activated states (Ito et al., 1998, 2001). Iba1 is also expressed by myeloid cells in the periphery or in peripherally-derived myeloid cells that have entered the CNS. Increased density of Iba1-expressing cells in the CNS has also been reported following injury in the adult (Thored et al., 2009; Venkatesan et al., 2010).

There was no significant difference in the density of Iba1-positive microglia across the time points examined, suggesting that this measure does not reflect developmental microglial activation.

GFAP protein expression has been used as an approximate measure of astrocytic activity

and response to injury (Brahmachari, 2006; Eng and Ghirnikar, 1994; Liddelow and Barres, 2017; Sofroniew and Vinters, 2010). I examined GFAP to determine if astrocytes are activated during developmental synaptic pruning by microglia. There was a small but significant increase in GFAP protein levels at P39 and P50 relative to P24. This change in GFAP levels is of interest in light of the contribution of astrocytes to developmental synaptic pruning (Chung et al., 2015). However, previous studies have indicated that central GFAP levels, including in the mouse hippocampus (Kim et al., 2011), increase sharply after the first postnatal week and then stabilize. Cortical mRNA levels in the rat reach a peak at P12 and then appear to stabilize through P25. Astrocytes, like microglia, are heterogenous; studies using a different marker of astrocytes such as aldehyde dehydrogenase 1 family, member L1 (Aldh1L1), which in contrast to GFAP appears to be an invariant marker of astrocytes (Cahoy et al., 2008), might yield a different picture.

These data suggest that the processes microglia use to find and engulf synapses during normal (physiological) development may differ from the means by which microglia remove pathological (non-physiological) debris. There must be at least two different types of activated microglia (not corresponding to M1 and M2, as discussed in Chapter 2): those activated in response to a pathological challenge, and those activated to engage in physiological neuronal sculpting. In agreement, Hammond et al.'s (2019) single-cell transcriptome analysis of microglia found that demyelination (as an injury model) revealed groups of transcripts that differentiated the "injured" microglial transcriptome from the transcriptome of microglia in other (non-injured) state, suggesting that there are several reactive microglial subtypes – even in response to the same injury.

CHAPTER 7

MICROGLIAL PHAGOCYTOSIS OF SYNAPSES: POSTSYNAPTIC PRUNING IN THE ADULT

Microglial involvement in synaptic pruning occurs during postnatal development (Mallya et al., 2019; Paolicelli et al., 2011; Schafer et al., 2012) but largely abates once synaptic structure has stabilized (Schafer et al., 2012). However, microglia maintain their phagocytic capacity after these periods of robust pruning (Schafer et al., 2012). In Chapters 4 and 5, I demonstrated that microglia prune both postsynaptic and presynaptic elements in the developing prefrontal cortex. However, it is unclear if microglial also participate in the synaptic pruning that occurs in response to a challenge in the mature (adult) brain.

NMDA-R antagonists are psychotomimetic: acute administration of these drugs elicit positive and negative symptoms as well as cognitive disturbances in normal control subjects; lower doses yield comparable symptoms in remitted schizophrenic individuals (Krystal et al., 1994; Lahti et al., 1995, 2001). Subchronic treatment with NMDA-R antagonists in rodents, including MK-801 (dizocilpine), ketamine, and PCP reduces PFC PC spine density (Ruddy et al., 2015) and leads to cognitive deficits (Li et al., 2011; Mandillo et al., 2003; Mansbach and Geyer, 1989; van der Staay et al., 2011). I therefore sought to determine if subchronic MK-801 treatment reduces spine density in the adult PFC by increasing microglial-mediated pruning.

7.1 Methods

Animals. Adult male Sprague-Dawley rats (Envigo; Indianapolis, IN; 225-249g) were used as subjects; housing conditions were the same as described in Chapter 3.

Experimental design and treatments. Animals received five daily intraperitoneal (ip) injections of 0.2 mg/kg MK-801 dissolved in saline (n = 6) or an equivalent dose of the saline vehicle (n = 5). One day after the final injection, animals were transcardially perfused and brains were removed and prepared as described in Chapter 3 for spine analyses; the contralateral hemisphere was prepared for immunohistochemistry as described in Chapter 4.

Methods for spine analyses, including density and morphology, are described in Chapter 3. Immunohistochemistry and microglial engulfment analyses were performed as described in Chapter 4.

Data analysis. Differences in spine density and microglial engulfment were determined using an unpaired two-tailed t-test. Frequency distributions of spine morphology parameters were compared using the Kolmogorov-Smirnov test.

7.2 Results

MK-801 induces spine loss on L5 PFC PCs. Basal dendritic spine density on L5 PFC PCs was decreased in MK-801-treated rats ($t(9) = 2.784$, $p = 0.0213$; Figure 26). Mean basal spine density for vehicle- and MK-801-treated animals was 13.22 and 10.95 spines/10 µm, respectively.

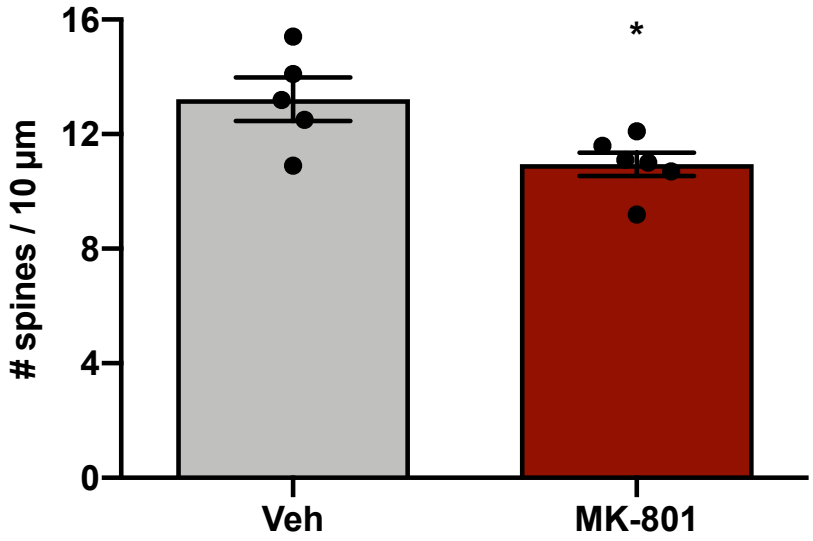


Figure 26. Subchronic treatment with MK-801 reduces cortical spine density. Spine density was significantly reduced in MK-801-treated animals. *$p \leq 0.05$.

MK-801 treatment changes spine length, but not head diameter or volume. The frequency distribution of spine length was significantly shifted to the right in MK-801-treated animals (D = 0.10, p = 0.0002; Figure 27A), i.e. remaining spines in MK-801-treated animals were longer than vehicle-treated animals. The frequency distributions of spine head diameter (D = 0.033, p = 0.676; Figure 27B) and spine head volume (D = 0.036, p = 0.60; Figure 27C) did not differ between treatment groups.

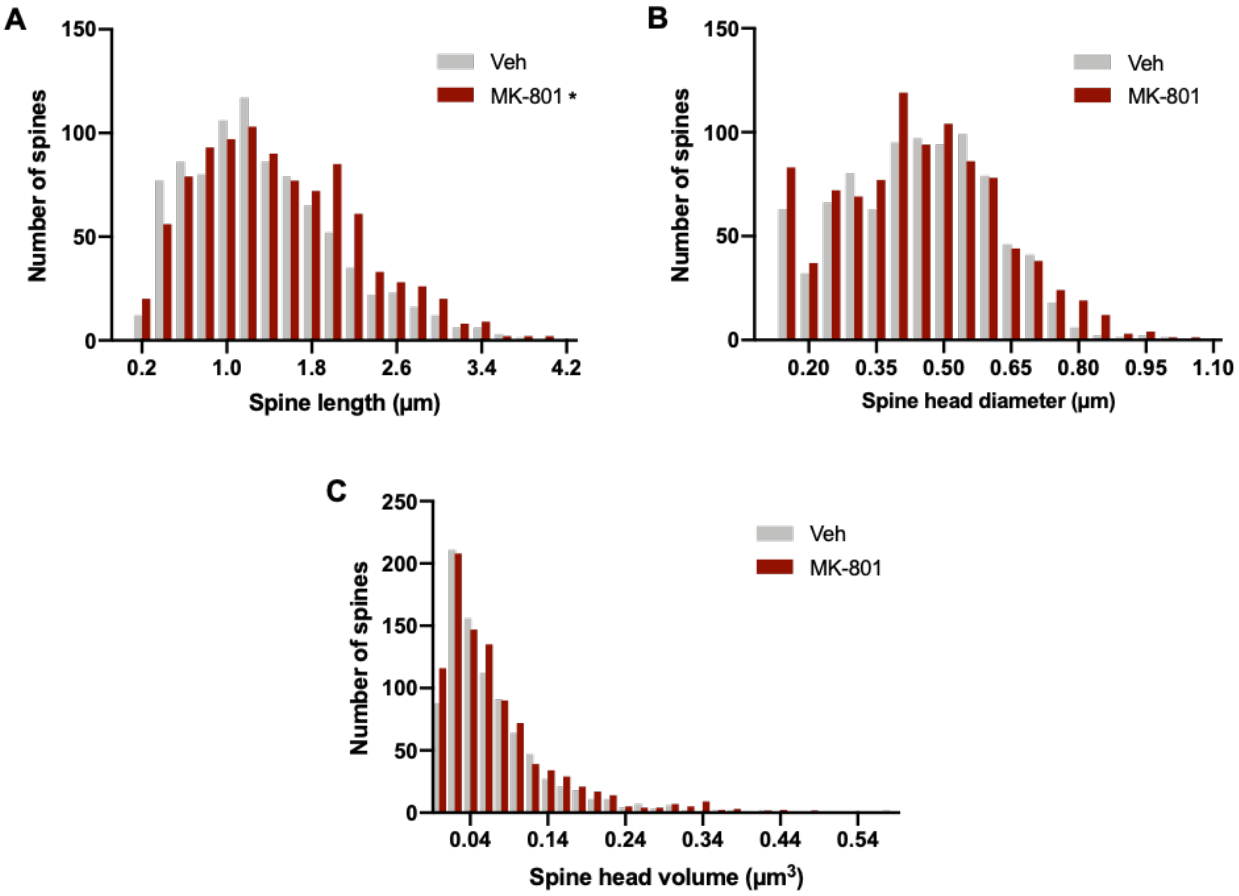


Figure 27. The frequency distribution of spine length changes as a result of subchronic MK-801 treatment. The frequency distributions of (A) spine length, (B) maximal spine head diameter, and (C) spine head volume were compared in saline-treated and MK-801-treated animals. The distribution of spine length in MK-801-treated animals was significantly shifted to the right relative to saline-treated animals. *p $\leq$ 0.001.

Possible role for microglia in engulfing spines lost by MK-801 treatment. There was no significant difference in the colocalization index between treatment conditions (Figure 28). However, there was a non-significant trend toward an increase in MK-801-treated animals ($t(87) = 1.91$, $p = 0.06$).

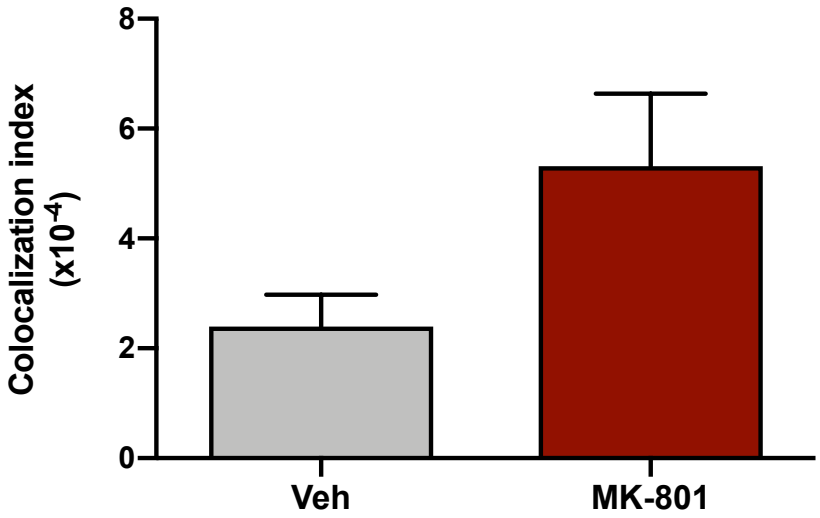


Figure 28. Microglia may contribute to spine loss following MK-801 treatment. The colocalization index was not significantly increased in MK-801-treated animals. However, there was a trend ($p = 0.06$) toward increased engulfment of spines in MK-801-treated rats.

7.3 Discussion

Subchronic MK-801 treatment induced dendritic spine loss in L5 PFC PCs; this was accompanied by the remaining spines being longer. I did not uncover a significant increase in microglial pruning, suggesting that some different mechanism from that operative during developmental (physiological) pruning of synapses is responsible. However, there was a clear trend toward an increase in microglial engulfment of spines, raising the possibility that the study was underpowered.

I was particularly interested in whether an NMDA-R antagonist decreased dendritic spines through microglial phagocytosis because of the glutamate hypothesis of schizophrenia (see Chapter 1) and because a low dose of the NMDA-R antagonist ketamine results in behavioral changes in healthy human volunteers that are strikingly similar to those seen in schizophrenia (Adler et al., 1998, 1999; Krystal et al., 1994; Lahti et al., 2001; Newcomer et al., 1999). Several reports have suggested that NMDA-R antagonists other than MK-801, including ketamine in rodents (Ruddy et al., 2015) and PCP in both rodents (Elsworth et al., 2011b; Hajszan et al., 2006) and non-human primates (Elsworth et al., 2011a), also reduce spine density in the PFC. NMDA-Rs also elicit pathophysiological and behavioral changes relevant for schizophrenia (Becker and Grecksch, 2004; see Frohlich and Van Horn, 2014; Moghaddam et al., 1997). However, there is no animal model of schizophrenia; we can model certain aspects of the illness, but not the full spectrum of changes.

MK-801, like ketamine and PCP, is an uncompetitive NMDA-R antagonist, but has some distinct characteristics relative to ketamine. MK-801 is approximately 200 times more potent than ketamine (Sircar et al., 1987) and has a slower off-rate than other NMDA-R antagonists (MacDonald et al., 1991), leading to a more sustained effect on the receptor.

A previous examination of spine morphology in rodents treated subchronically with MK-801 did not uncover changes in spine length, head diameter, or head:neck ratio compared to saline-

treated animals (Ruddy et al., 2015). I also did not find any significant effect of MK-801 treatment on spine head diameter. However, in contrast to Ruddy et al. (2015), I did observe an increase in the length of dendritic spines in MK-801-treated rats, suggesting that spines remaining after MK-801 treatment have a thinner morphology than observed in control subjects. Because shorter spines were lost following MK-801 treatment, but longer spines were pruned during development (see Chapter 3), this suggests that there are differences in the subtypes of spines targeted for elimination following a challenge in the adult brain as compared to development. Additional work will need to be conducted to elaborate these differences.

Microglia appear to play a role in synapse elimination in disorders such as Rett Syndrome (Schafer et al., 2016) and Alzheimer's Disease (Shi et al., 2015, 2017). I assessed whether microglia similarly engulf synaptic elements in MK-801-treated rats. I failed to observe a significant increase in microglial engulfment of PSD-95-ir spines in MK-801-treated rats relative to saline-treated control subjects. However, I did note a trend toward an increase in microglial engulfment ($p = 0.06$). In light of this result, I can neither confirm nor rule out a role for microglia in the engulfment of spines following MK-801 treatment. Replication and expansion of this study, both with additional animals as well as examining engulfment at different MK-801 treatment intervals, will be required to conclusively define a role for microglia in this process. Further, examining presynaptic element engulfment and microglial "activation" would be of interest.

CHAPTER 8

DISCUSSION

I have demonstrated that dendritic spine density on pyramidal cells peaks in the prelimbic cortex (area 32) of the medial prefrontal cortex at postnatal day 35. The morphology of spines transitioned from a long and thin shape to a shorter spine with a larger head diameter and volume as development progressed from P24-39. I also showed that microglia phagocytose post- (dendritic spines) and presynaptic (axonal elements) in the prefrontal cortex shortly after peak spine density, during early and late adolescence, respectively. However, during microglial engulfment of synapses at P39, the microglia did not display the typical morphological features associated with "activation": they did not become cells with smaller somata and short, stubby processes, nor was there an increase in the density of microglia. Finally, because the pruning of synapses during development was not accompanied by a change in the morphology of microglia to the so-called "activated" state, I wondered if challenges that elicit a decrease in dendritic spines in the adult PFC removed spines by microglial engulfment of synapses. Using the NMDA-R antagonist MK-801 to elicit dendritic spine loss in the PFC, changes suggestive of microglial phagocytosis of spines were observed.

8.1 Conclusions and Future Studies

These findings have broad implications for microglial structure and function, for developmental studies (particularly those that investigate changes during adolescence), and the neurobiology of schizophrenia. In this chapter I will discuss more broadly several issues raised by the studies, and I discuss below the conclusions of my studies and how the results of my inquiries suggest future empirical work.

What (and When) is Adolescence?

Adolescence is thought of as a critical time period over which there are "notable morphological and functional transformations in the brain that, along with increasing hormone levels and other biological changes, interact with cultural, economic, and psychosocial forces to shape how adolescents think, feel, and behave" (Spear, 2013). Schizophrenia and related psychotic disorders typically emerge in late adolescence or early adulthood, suggesting that this period may be particularly sensitive to perturbations from typical development (Keshavan et al., 2014; Lee et al., 2014; Paus et al., 2008). Indeed, the two-hit model of schizophrenia (Keshavan, 1999; Keshavan and Hogarty, 1999) posits that a combination of an insult during prenatal or early life stages primes the nervous system such that a later developmental insult, such as one during adolescence, disrupts neurological processes (for example, microglial pruning) and leads to disease onset (Feigenson et al., 2014; Maynard et al., 2001).

The definition of adolescence in rodents is controversial, with many reports defining the period operationally rather than on the basis of extant data; this leads to some groups referring to a period as adolescence that others designate adult or childhood. For example, Reynolds et al. (2018) defined early adolescence as ∼P22-31, mid-adolescence as ∼P35-44, and adulthood as ∼P75-84 based on "1) PFC dopamine fiber development and receptor expression, 2) differential regulation of proteins involved in axon growth and plasticity in response to stimulant drug exposure, and 3) vulnerability to the long-term effects of abused drugs or dopamine receptor activation." In Chapter 3, I introduced Spear and Brake's (1983) rationale for defining periadolescence as ∼P28 through ∼P38-42, corresponding with the onset of diurnal gonadotropin onset and reproductive capabilities, respectively. However, gonadal adolescence, defined on the basis of sex hormone production, differs from brain adolescence - in which maturation of brainstem neurons and neuronal systems neurons involved in pain sensation may differ from those involved in pain perception, which in turn differ from those in the PFC.

Because the field has not reached a consensus on what constitutes rodent adolescence,

each study must delineate the period they consider to be adolescence based on a rationale that is both articulated and defended. In the rodent, the processes homologous to those in human development occur over a far more compressed time period. I defined rodent adolescence earlier (in chapter 3) as the period between P35-50 based on a confluence of data, including 1) consideration of the rationale of Spear and Brake (1983), 2) the PFC dopamine innervation reaching its adult density maturation at ∼P60 (Kalsbeek et al., 1988), 3) the peak of dopamine D1 and D2 binding in the PFC occurring at P40 and significantly declining by P60 (Andersen et al., 2000), and 4) prefrontal cortical interneuron function reaching steady state at ∼P50 (Tseng and O'Donnell, 2007).

These specifications are region-specific. For example, dopamine D1 and D2 receptor density in the nucleus accumbens increases between 25 and 40 days of age, thereafter plateauing through 120 days of age (Teicher et al., 1995). In the dorsal striatum the peak in D1 and D2 binding maximum occurs at ∼P40 and declines by up to 75% by P120 (Gelbard et al., 1989). Therefore, it is essential that one takes into account the relevant data for each particular area of the brain when defining adolescence.

I quickly discovered that there was limited data investigating the trajectory of spine development in the rodent prefrontal cortex across the relevant developmental epochs, let alone in specific layers of the PFC. I therefore performed a temporally fine-grained and detailed analysis of PFC spine density in the rat across the general period of preadolescence (P24-34), adolescence (P35-50), and early adulthood (P51-70).

Basal and apical spine density peaked at P35; microglia engulfed pre- and postsynaptic elements shortly thereafter, early in adolescence (P39). While basal spine density levels persisted through P50, the time during which presynaptic elements were phagocytosed, and into early adulthood at P70, apical spine density showed a non-significant trend toward a decrease at P70 relative to P50; unfortunately, the study was underpowered to detect a significant difference between P50 and P70 because of the large number of post-hoc comparisons.

Even in humans, the term adolescence, and the age period over which it spans, is ill-defined. The World Health Organization has defined adolescence as the ages between 10 and 19, but, based on the evolving understanding of the neurobiology of frontal cortical development (Petanjek et al., 2011; Sowell, 2004), also defines "young people" as individuals between the ages of 10 and 24. Some have cogently argued that earlier onset of puberty together with sustained growth and shifting perceptions necessitates expanding this definition to 10-24 years of age (Sawyer et al., 2018). Still others believe that there is already enough confusion in the field, and any deviations from defining the adolescent period as 10-19 years of age will only add to the confusion, instead calling for the demarking of ages 20-24 as young or early adulthood (McDonagh et al., 2018).

While the exact time of adolescence varies across definitions, there is a consensus that adolescence exists on a continuum that includes 1) early adolescence, during which time hormonal and reproductive maturation is achieved, 2) middle adolescence, which corresponds with psychosocial maturation, and 3) late adolescence, when height has stabilized and brain size and volume has (mostly) finished changing. It is over this gross time period during which there is also significant synaptic pruning.

This lack of agreement in the time period that adolescence encompasses is particularly important when interpreting the available data showing normal PFC development in humans and querying when the behavioral pathology of schizophrenia begins. Studies of postnatal prefrontal cortical synapse development include cases that range from childhood to later life – with the notable exception of adolescence, which is represented in the relevant studies by very few individuals (Huttenlocher, 1979; Petanjek et al., 2011). This requires that one extrapolate over the period of missing (adolescent) data. Regression and extrapolation analyses taking the earlier and later trajectories of development are suggestive of synapse decline occurring sometime during adolescence (and in fact for the PFC extending into the third decade of life (Petanjek et al., 2011)), but full analyses of this gap in time have not yet been conducted.

Importantly, it is during the period of adolescence when individuals at clinical high-risk of psychosis first display attenuated symptoms of the illness (Mollon and Reichenberg, 2018). This phenotype includes cognitive symptoms (Addington et al., 2017; Brewer et al., 2005; Fusar-Poli et al., 2012c; Giuliano et al., 2012; Jones et al., 1994b; Lam et al., 2018), such as working memory deficits (Seidman et al., 2016). Despite the presence of these prodromal symptoms, only a minority of individuals at high risk go on to develop schizophrenia (Addington et al., 2017; Cannon et al., 2008; Cornblatt et al., 2015; Fusar-Poli et al., 2012b; Seidman et al., 2016). It is therefore critical that adolescence be appropriately defined in both rodents and humans in order to characterize the homeostatic processes that take place during this period, understand the processes that contribute to prodromal symptoms and the trigger of an individual to full-blown disease onset, develop therapeutics, and administer the therapeutics at the appropriate time.

Is Developmental Pruning by Microglia a Brain-Wide Phenomenon?

Microglia have been shown to engulf synaptic elements in the thalamus (Schafer et al., 2012, 2016) and the hippocampus (Paolicelli et al., 2011) during relatively early postnatal life. We have extended these studies to a very late maturing structure, the prefrontal cortex (Mallya and Deutch, 2018; Mallya et al., 2019).

I focused my efforts on the prefrontal cortex because of the involvement of the PFC in cognition (Goldman-Rakic et al., 1989; Levy and Goldman-Rakic, 2000; Miller, 1999, 2000; Ott and Nieder, 2019; Sakurai and Gamo, 2018; Snow, 2016), particularly those aspects of cognition that are impaired in schizophrenia (Berman and Weinberger, 1991; Davis et al., 1991; Deutch, 1992; Goldman-Rakic, 1999; Goldstein and Deutch, 1992; Keefe and Harvey, 2012; Lewis and Moghaddam, 2006; Sakurai and Gamo, 2018; Woodward and Heckers, 2015).

Detailed analyses of spine ontogeny similar to those conducted in this dissertation are lacking in other areas of the brain, including other cortices. This paucity of data is not surprising given the time- and labor-intensive nature of the experiments, particularly when analyzing both

apical and basal dendrites in a region- and lamina-specific manner. Consequently, it is difficult to guess when microglia might be phagocytosing synaptic material in brain regions with lesser defined developmental patterns, especially when considering the relatively short time periods over which microglia sculpt neurons. For example, in the dLGN, microglia engulf RGCs at P5, but engulfment has virtually ceased by P9 (Schafer et al., 2012). I found that microglia engulf spines at P39 but not P35. Microglial engulfment of spines ceased by P50. I did not examine time points between P39 and P50; it is possible that spine engulfment is over well before P50.

Is Microglial Phagocytosis a Circuit-Specific Phenomenon?

In addition to determining whether microglial pruning of synapses is region-specific, it is unclear if microglia phagocytose synapses in a circuit-specific manner. In Chapter 1, I discussed that levels of VGluT1 but not VGluT2 seems to be decreased in schizophrenia (Bitanihirwe et al., 2009; Eastwood and Harrison, 2005; Oni-Orisan et al., 2008), and that these proteins define cortical and subcortical glutamatergic neurons, respectively (Fremeau et al., 2001, 2004b; Kaneko and Fujiyama, 2002). This suggests that circuits emanating from VGluT1- and VGluT2-positive neurons may be differentially affected in schizophrenia.

I demonstrated a role for microglia in pruning VGluT1-ir elements during early and late adolescence (P35 and P50). An intriguing hypothesis is that microglia may preferentially target specific VGluT1-expressing excitatory neurons and their axons, possibly due to aberrant expression of an "eat me" or "don't eat me" signal.

Further, we have previously shown that dopaminergic denervation of the PFC induces spine loss on PFC PCs that project to the mediodorsal nucleus of the thalamus (MD) but not on PCs projecting to other targets, including to the basolateral amygdala and ventral tegmental area (VTA) (In Preparation). This is particularly relevant given the known role of the MD-PFC circuit in contributing to working memory (see Parnaudeau et al., 2018). For example, do microglia engulf spines on PFC PCs that project to the MD, but not those that project to the VTA?

Mechanisms Underlying PFC Microglial Phagocytosis.

I demonstrated that developmental microglial pruning of PFC synapses occurs at a much later age than has previously been shown in other brain areas (Paolicelli et al., 2011; Schafer et al., 2012), and have shown that microglia engulf synapses of PFC PCs during adolescence. Yet to be defined are the mechanisms mediating this process in the PFC.

In the thalamus, phagocytosis of RGC synapses is in part complement component-dependent (Schafer et al., 2012): genetic deletion of C3 and CR3 disrupts pruning in the dLGN by $\sim$50%. Furthermore, mice deficient in C4 also display thalamic synaptic pruning deficits (Sekar et al., 2016). Complement may play a similar role in PFC microglial phagocytosis. Unfortunately, my efforts to explore this possibility were hindered by C1q and C3 antibodies that were nonspecific and either did not stain consistently or did not recognize the antigens in rat tissue (see Chapter 5). I also could not use animals in which complement components were genetically deleted due to the seizure phenotype induced by knockout of C1q (Chu et al., 2010), which in turn affects spines (Ma et al., 2013). It will not only be of interest to define whether complement is involved in phagocytosis, but whether thin (presumably immature) spines are preferentially opsonized for removal. Because pruning is only reduced by half, this raises the likelihood of other complement proteins as well as other independent phagocytic mechanisms being involved. Although the complement pathways are in general well conserved, there are a few noticeable differences in complement pathways between humans and rodents. For example, while humans have two C4 isotypes, C4A and C4B, the mouse genome contains a single C4 gene that shares features with both isotypes (see Sekar et al., 2016).

Do Other Cells in the PFC Phagocytose PFC Synapses?

I have focused on microglial-mediated removal of synapses from PFC pyramidal cells. However, other cell types may also play a role. Chung and colleagues (2013) showed that astrocytes are directly involved in developmental (in the dLGN) as well as adult elimination (in

the somatosensory cortex) of excitatory and inhibitory synapses via the phagocytic multiple epidermal growth factor-like domains protein 10 (MEGF10) and proto-oncogene tyrosine-protein kinase MER (MERTK) pathways. The findings of Bialas et al. (2013) also indicated an indirect role for astrocytes, with astrocyte-derived TGF-β regulating C1q expression in the developing visual system. Defining the role of astrocytes in performing these functions in the PFC, as well as whether microglia and astrocytes are also eliminating inhibitory PFC synapses, will be critical for understanding the homeostatic processes mediating PFC structure and function.

What is Microglial "Activation?"

Microglia have historically been thought to adopt different morphologies based on their functional state. Surveilling microglia are extensively branched and have a smaller somata than activated microglia, which assume a large ball-like shape with few or no processes (see Hanisch and Kettenmann, 2007; Ransohoff and Perry, 2009).

In contrast to this traditional view, recent data, including my data (see Chapter 6), indicate that microglia do not assume the classic "activated" morphology during developmental synapse pruning, instead displaying many profusely branched processes (Lehrman et al., 2018; Mallya et al., 2019; Schafer et al., 2012; Sierra et al., 2010). These findings suggest that there are at least two different types of activated microglia: those triggered in response to a pathological challenge and those activated to engage in physiological neuronal sculpting. Furthermore, microglial phenotype is governed by as yet unidentified local cues (De Biase et al., 2017). Recent studies have provided evidence that microglia can be classified into one of up to nine different phenotypes under different conditions and at different points in development (Hammond et al., 2019; Matcovitch-Natan et al., 2016). It is not yet known if microglia release cytokines during non-pathological pruning of synapses, and if so, which cytokines (see Boche et al., 2013; Czeh et al., 2011; Hanisch and Kettenmann, 2007; Wang et al., 2015). Disentangling the processes and signals that dictate the functional state of microglia during developmental phagocytosis as

opposed to those mediating inflammation- and pathology-based phagocytosis will be critical for future understanding of microglial function in health and disease.

Defining Central vs. Peripheral Macrophages.

An issue not often addressed but critical to understanding microglial function is how to assess the contribution of microglia that are intrinsic to the CNS without contamination from infiltrating macrophages generated in the periphery.

Studies of developmental pruning, including the data presented here, have used markers common to both peripheral or central cells, such as Iba1 and the fractalkine receptor CX_3CR1. In the healthy CNS, microglia are the resident macrophages, while peripheral macrophages are mainly restricted to perivascular spaces, meninges, and the choroid plexus (Prinz et al., 2011; Prinz and Priller, 2014). The extent to which the bloodbrain or cerebrospinal fluidbrain barriers may be porous under conditions such as inflammation or in illnesses such as schizophrenia is not known; however, the permeability of the BBB seems to increase in disease states such as obesity (Gustafson et al., 2007; Stranahan et al., 2016), Alzheimer's disease (see Zlokovic, 2005), and aging (Montagne et al., 2015). It is not clear to what degree peripherally derived monocytes enter the CNS at circumventricular sites (Pineau and Lacroix, 2007) and from there migrate to other areas. The recent discovery of microglia- but not peripheral macrophage-specific markers, including Tmem119 (Bennett et al., 2016) and others (Butovsky and Weiner, 2018), will be essential in defining the functions performed by the different macrophage populations. Moreover, markers that unambiguously distinguish microglia from peripherally-generated cells markers will permit experimental manipulations that disrupt the BBB while allowing one to distinguish between resident microglia and other cells.

8.2 Microglial Pruning and Schizophrenia

Schizophrenia is associated with cognitive impairments, including deficits in executive function, working memory, and attention. Cognitive impairment can predict psychosocial integration of patients with schizophrenia (Green, 2006; Nuechterlein et al., 2011), but current treatments do little to alleviate these symptoms; this has led to a push to investigate both the underpinnings of these symptoms and new therapeutics that improve the cognitive symptoms in addition to the positive and negative symptoms.

The prefrontal cortex appears to subserve, in part, several of these cognitive functions (Brutowski, 1965; Goldman-Rakic, 1995; Jacobs et al., 2007; Ott and Nieder, 2019). Structural and functional changes in the prefrontal cortex are thought to in part underlie the cognitive deficits in schizophrenia (see Chapter 1). The loss of dendritic spines on PFC PCs is among the most replicated postmortem finding in schizophrenia (see Glausier and Lewis, 2013; Moyer et al., 2015). Because cortical volume loss, which is not attributable to a decrease in neurons (Selemon and Goldman-Rakic, 1999), may presage the diagnosis of schizophrenia (Borgwardt et al., 2008; Fusar-Poli et al., 2012a) and is present at the time of the first psychotic episode (Borgwardt et al., 2008; Leung et al., 2011), some event involving the neuropil that occurs prior to diagnosis is thought to be a primary factor in the pathogenesis of schizophrenia. The mechanisms that account for spine loss are unclear but have been suggested to involve an exaggerated dendritic spine pruning process during adolescence (Feinberg, 1982; Forrest et al., 2018). Individuals who are at high risk for developing schizophrenia, based in part on attenuated symptoms, exhibit cognitive as well as psychotic symptoms during this time period (Addington et al., 2017; Brewer et al., 2005; Fusar-Poli et al., 2012c; Giuliano et al., 2012; Jones et al., 1994b; Lam et al., 2018; Seidman et al., 2016). I suggest here that microglia may be an effector of this excess prefrontal cortical synapse loss during adolescence, thereby contributing to the cognitive symptoms of schizophrenia.

8.3 Translating Microglial (Dys)Function to Clinical Strategies

Among the reasons for the current excitement in research on microglial function as it relates to schizophrenia is that the work may open new avenues for therapeutic intervention. As commented on several times, current pharmacological treatments, while being effective in reducing psychosis, do little to mitigate cognitive dysfunction; other approaches to cognitive dysfunction, such as cognitive remediation, are in their infancy and their broad effectiveness unknown. Part of the problem with current pharmacological treatments is that they are widely derivative of earlier drugs, simply slightly modifying the same basic mechanisms, such as dopamine or serotonin receptor antagonism.

In contrast, drugs that might reduce cognitive dysfunction (and do so early in the course of the illness) by targeting microglial processes would offer a completely novel approach to pharmacotherapy. Neuronal elements destined for elimination elaborate complement-related "find me" and "eat me" signals that direct microglia to the neuron and cue the microglia to phagocytose a particular spine or axonal element (Schafer et al., 2012). Very recently the discovery of an association of complement C4 and risk for developing schizophrenia (Sekar et al., 2016) has fueled further interest in drugs targeting phagocytosis. Among the potential targets are not only "find me" and "eat me" signals, but also "don't find me" and "don't eat me" signals, such as CD47-SIRPα, that help a spine evade detection and pruning (Lehrman et al., 2018; see Rivest, 2018), similar to those seen in apoptotic cells (Hochreiter-Hufford and Ravichandran, 2013). The potential for such treatments has become clear because of pharmacological interventions designed to target phagocytosis of apoptotic cells; such drugs are now in early trials for certain cancers (see Weiskopf, 2017).

Pharmacological or molecular suppression of the "find me"/"eat me" signals or amplification of the "don't find me"/"don't eat me" signals, particularly during adolescence when physiological spine pruning is active, might diminish excess pruning of spines on PFC PCs,

thereby averting some of the behavioral pathology of schizophrenia. However, it is possible that too much suppression will result in an excess of spines, as seen in Autism Spectrum Disorder (ASD) and Fragile X syndrome. Of interest, in both ASD and schizophrenia, social cognition is impaired, suggesting that there may be an optimal spine number above or below which negative consequences occur. Alternatively, the specific deficits in social cognition may differ, or the learned consequences may differ because of the different ages at which spine number changes occur. Specific targeting of these signals in a regionally-specific and temporally-specific manner will be required.

In Chapter 2, I discussed the use of TSPO as an *in vivo* marker of microglial activation. TSPO is the latest in a series of potential radioligands for PET studies of inflammation (see Janssen et al., 2018). A number of PET studies examined TSPO binding in individuals with recent-onset schizophrenia. The results were inconsistent (van Berckel et al., 2008; Di Biase et al., 2017; Bloomfield et al., 2016; Coughlin et al., 2016; van der Doef et al., 2016; Doorduin et al., 2009; Hafizi et al., 2017; Ottoy et al., 2018; Selvaraj et al., 2017; Takano et al., 2010). Further work showed that there are allelic variations that define low and high binders of TSPO, but even more recently it has become clear that TSPO does not bind only to microglia, but also to a number of other CNS cells (Cosenza-Nashat et al., 2009; Lavisse et al., 2012; Notter et al., 2018; Varga et al., 2009). This has culminated in some questioning the use of TSPO as an index of microglial activation but also whether there is increased activation of microglia in schizophrenia at all. In this dissertation, and discussed above, I have shown that microglia that engulf synaptic elements during development do not display the typical morphological characteristics of microglia activated under pathological instances. It follows that microglia involved in synapse pruning in schizophrenia may not evince the typical "activated" parameters expected in microglia – including with respect to TSPO. The field, and more importantly families and those individuals at risk, eagerly and anxiously await the identification of measures and biomarkers that, with fidelity, can predict risk for developing schizophrenia.

8.4 Summary

In summary, my data suggest that microglia may be an effector of excessive synaptic pruning during adolescence in schizophrenia (Mallya and Deutch, 2018; Mallya et al., 2019; Figure 29) posited by Feinberg (1982). Similar microglial involvement at other postnatal ages could contribute to the "spinopathies" of Autism Spectrum Disorder and even Parkinson's Disease. Understanding the processes subserving synaptic remodeling, including dendritic spine loss, may lead to the development of new microglial-based pharmacological targets aimed at early intervention to attenuate or prevent the cortical pathology and emergence of cognitive dysfunction in schizophrenia.

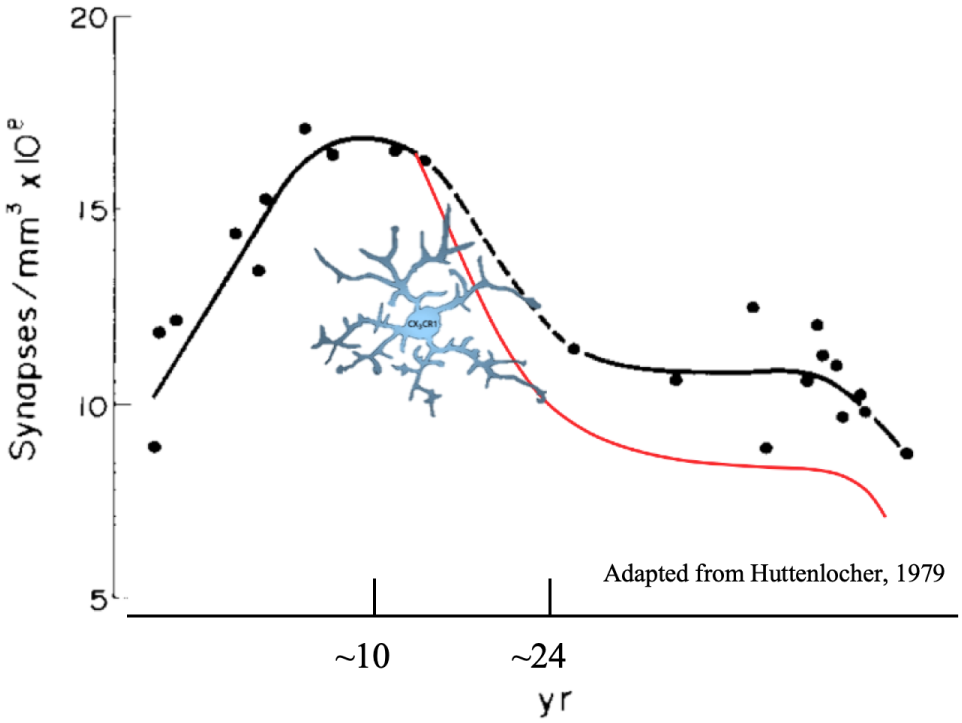


Figure 29. Summary. Microglia may be effectors of excessive spine loss during adolescence in schizophrenia (Reprinted from Brain Research, 163, Huttenlocher PR, Synaptic density in human frontal cortex – Developmental changes and effects of aging, 195-205, © (1979), with permission from Elsevier).

APPENDIX A

SYNAPSE-ASSOCIATED PROTEIN LEVELS IN POSTNATAL DEVELOPMENT

Although there have been studies examining levels of select synaptic-related proteins in the prefrontal cortex during discrete periods of development, a comprehensive examination across periadolescence and adolescence in the PFC is lacking. Understanding the normal developmental trajectory of such proteins is critical for determining whether alterations in schizophrenia are reflective of aberrant postnatal developmental processes or of disease onset and/or progression. In the following experiment I investigated if synapse-associated protein levels vary across age.

A.1 Methods

Immunoblotting. Immunoblotting was performed on mPFC tissue at P24, 30, 35, 39, and 50 and analyzed as described in Chapter 6.

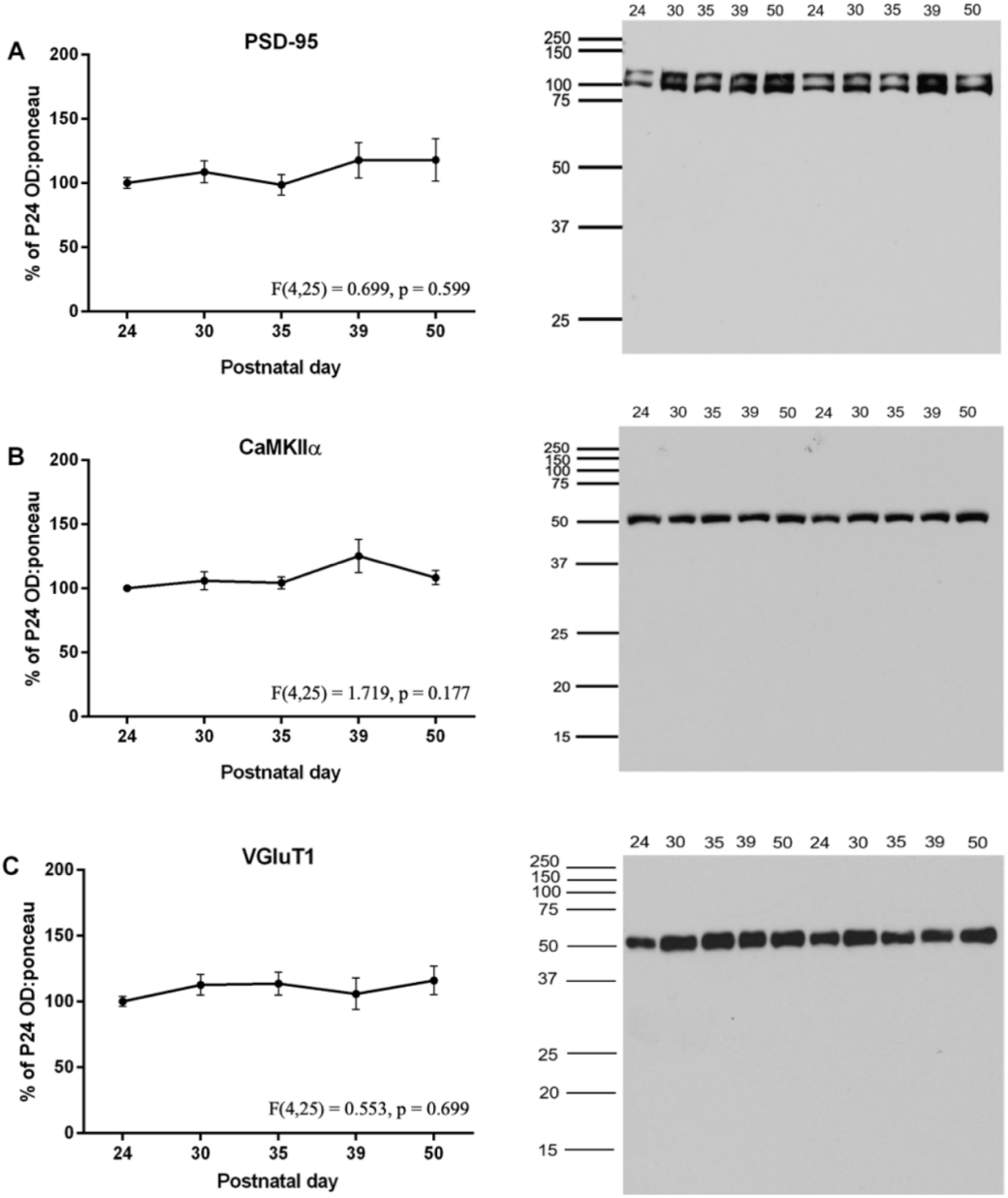
Blots were probed with a primary antibody (Table 4) targeting a postsynaptic marker (PSD-95), a pyramidal cell marker (Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) α), presynaptic markers (VGluT1 and VGluT2), an inhibitory neuron marker (vesicular GABA transporter (VGAT)), and a macrophage receptor (SIRPα) that interacts with the synapse-localized CD47 to protect synapses during development (Lehrman et al., 2018; see Chapter 5).

Antibody	Host	Company	Catalog Number	Dilution
PSD-95	Mouse, monoclonal	Millipore Sigma Burlington, MA	MAB1596 RRID: AB_2092365	2.2 µg/mL
CaMKIIα	Mouse, monoclonal	Thermo Fisher Scientific Waltham, MA	MA1-048 RRID: AB_325403	0.25 µg/mL
VGluT1	Rabbit, polyclonal	MAb Technologies Stone Mountain, GA	VGT1-3 RRID: AB_2315551	1:4,000*
VGluT2	Guinea Pig, polyclonal	Millipore Sigma Burlington, MA	AB2251 RRID: AB_2665454	1:5,000*
VGAT	Mouse, monoclonal	UC Davis/NIH NeuroMab	75-457 RRID: AB_2651169	3.0 µg/mL
SIRPα	Rabbit, polyclonal	QED Bioscience Inc.	2428 RRID: AB_130075	0.5 µg/mL
Peroxidase AffiniPure Donkey Anti-Mouse IgG (H+L)	Donkey, polyclonal	Jackson ImmunoResearch West Grove, PA	715-035-150 RRID: AB_2340770	0.08 µg/mL
Peroxidase AffiniPure Donkey Anti-Rabbit IgG (H+L)	Donkey, polyclonal	Jackson ImmunoResearch West Grove, PA	711-035-152 RRID: AB_10015282	0.08 µg/mL
Peroxidase AffiniPure Donkey Anti-Guinea Pig IgG (H+L)	Donkey, polyclonal	Jackson ImmunoResearch West Grove, PA	706-035-148 RRID: AB_2340447	0.08 µg/mL

Table 4. Source information and dilutions for antibodies used in immunoblotting experiments of synapse-associated proteins.
*Concentration information is not provided for these antibodies by their respective companies.

A.2 Results

Synapse-associated protein levels across development. Levels of none of the proteins examined changed significantly from P24-50. Outcomes for each protein are embedded within the figure.



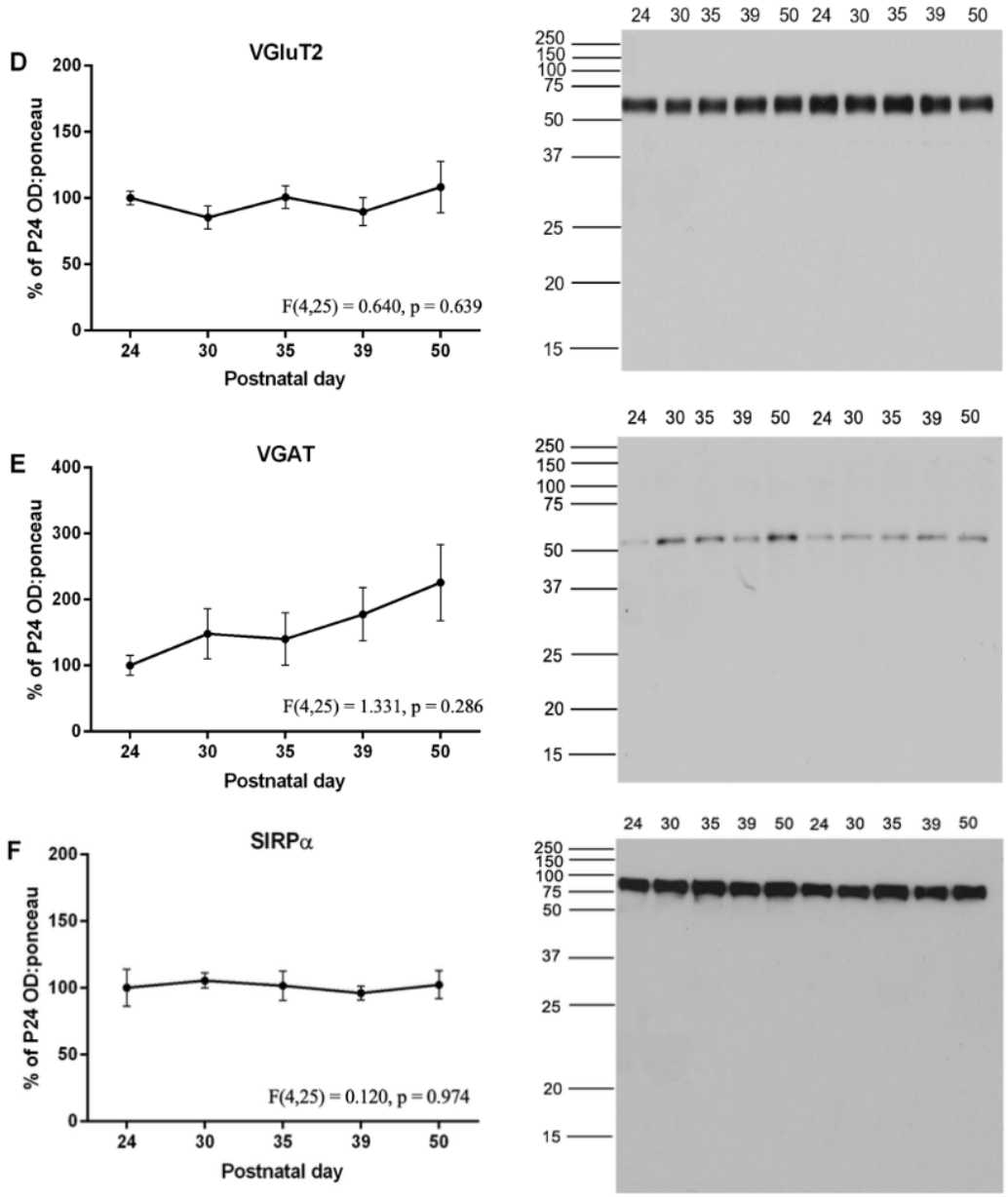


Figure 30. Synapse-associated protein levels across development. Expression of all investigated synapse-associated proteins did not differ across time points examined.

A.3 Discussion

Protein levels of all synapse-associated proteins examined, including PSD-95, CaMKIIα, VGluT1, VGluT2, VGAT, and SIRPα, did not differ as a function of age. These findings suggest that the maturation of these individual proteins precedes that of spine density (Chapter 3) and microglial engulfment of synaptic elements (Chapters 4 and 5), and that levels of these markers cannot be used to estimate spine density.

The PFC samples dissected in this experiment include most of the medial wall of the hemisphere at pregenual levels, extending from the infralimbic cortex ventrally to the medial precentral cortex dorsally. Although I did not detect any significant change in levels of the proteins examined, I cannot rule out that there are subtle but significant changes within certain cytoarchitectonic regions of the mPFC (such as the prelimbic cortex) or even by lamina. Immunohistochemical studies examining the pattern of expression in discrete regions will be required to elucidate whether there are regionally-specific changes.

PSD-95

PSD-95, a member of the membrane-associated guanylate kinase superfamily of proteins, is found in nearly all mature, excitatory glutamatergic synapses and is localized to the post-junctional spine head (Harris and Weinberg, 2012; Hunt et al., 1996). Among many other functions, PSD-95 is thought to be important for excitatory synapse function (Hunt et al., 1996; Kornau et al., 1995), interacts with, anchors, and traffics ionotropic glutamate receptors to the postsynaptic membrane (Chen et al., 2000, 2015; Kornau et al., 1995), and is essential for maintaining the molecular organization and structure of the postsynaptic density itself (Chen et al., 2011). Interestingly, PSD-95 knockout mice show an increased proportion of silent synapses on morphologically "mature" spines (defined by spine volume and spine length; Béïque et al., 2006), again underscoring the confounds of trying to correlate spine morphology and function

(see Arellano, 2007; refer to Chapter 3).

PSD-95 mRNA (Ohnuma et al., 2000) and protein levels (Catts et al., 2015) have been reported to be decreased in the PFC in schizophrenia. Because of the critical role of PSD-95 in modulating glutamatergic transmission and synaptic plasticity, PSD-95 dysfunction during development could have profound effects on synapse formation and function. Furthermore, PSD-95 dysfunction could disrupt NMDA-R function, contributing to the neurochemical changes, particularly those related to the glutamate hypothesis (see Chapter 1), observed in schizophrenia.

In the human PFC, PSD-95 protein expression levels have been reported to increase until early adolescence and plateau thereafter (Glantz et al., 2007). The ontogeny of PSD-95 protein levels in rodents shows a similar trajectory, with PSD-95 increasing fivefold between P11 and P49 (Pinto et al., 2013); the largest changes in expression levels seems to take place between P4 and ∼P30, after which time expression levels change very subtly (Pinto et al., 2013). My data in large part agree with this previous report of PSD-95 level stabilization during this time. Although these data do not fully agree with the spine density measurements, this is not surprising given the higher degree of sensitivity in the spine density analyses as compared to immunoblotting. Further, several studies have shown that up to 20% of spines do not contain PSD-95 (Cane et al., 2014; Isshiki et al., 2014; Villa et al., 2016), although some of these PSD-95-lacking spines are transient or newly-formed (Cane et al., 2014).

CaMKIIα

In the cortex, CaMKIIα appears to be expressed solely by pyramidal cells (Jones et al., 1994a; Liu and Jones, 1996, 1997; McDonald et al., 2002). Binding of calmodulin to CaMKII subunits relieves autoinhibition and prompts inter-subunit autophosphorylation of CaMKII, resulting in CaMKII activation and allowing for phosphorylation of substrates by the holoenzyme. CaMKII decodes the frequency, duration, and amplitude of Ca^{2+} influx (De Koninck and Schulman, 1998) to modulate synaptic plasticity (Colbran, 2015; Colbran and Brown, 2004;

Coultrap and Bayer, 2012) and long-term potentiation (Lisman et al., 2012; Silva et al., 1992a, 1992b). Importantly, CaMKII is abundant in dendritic spines (Feng et al., 2011; Merrill et al., 2005) and the postsynaptic density, representing 2-6% of total protein in the PSD (Chen et al., 2005). CaMKII is critical for synaptic reorganization, in part via interactions with actin-related proteins (see Borovac et al., 2018; Colbran, 2004; Khan et al., 2018; Murakoshi and Yasuda, 2012; Okamoto et al., 2009, 2004; Robison, 2014), and spine stability via interactions with the PSD (Hell, 2014). Further, CaMKIIβ is important for regulating dendritic arborization (Fink et al., 2003; Zou and Cline, 1999).

Although a direct link between CaMKII and schizophrenia has not been found, mice lacking CaMKII anchoring proteins exhibit behavioral phenotypes that have face validity with certain symptoms of schizophrenia (see Robison, 2014).

Previous studies of the ontogeny of cortical CaMKIIα have shown that levels increase more than 20-fold between P1 and P28, with the largest increase occurring between P7 and P14 (Viberg et al., 2008). Levels of CaMKIIα in particulate and soluble fractions from forebrain peak at P30 (Sugiura and Yamauchi, 1992). My data suggests that CaMKIIα levels in the mPFC have stabilized and plateaued by preadolescence and adolescence.

It will be of interest to examine levels of phospho-Thr^{286}-CaMKIIα, the autonomously activated version of CaMKIIα (see Colbran, 2004), as well as CaMKIIβ, which has a different postnatal developmental pattern than CaMKIIα and can directly interact with F-actin (see Colbran, 2004). Previous reports have suggested a peak at P20 in the forebrain, although CaMKIIβ levels were far lower than CaMKIIα (Sugiura and Yamauchi, 1992). However, detailed analyses in the rodent mPFC have not yet been conducted.

VGluT1 and VGluT2

Accumulation of glutamate into synaptic vesicles is mediated by VGluTs, including VGluT1 and VGluT2, making these proteins critical for proper glutamatergic signaling. These

transporters are dependent on a proton gradient created via ATP hydrolysis, which allows the influx of H^+ into the synaptic vesicle, thereby creating a pH gradient; the glutamate anion can then be transported into the synaptic vesicle.

VGluT1 and VGluT2 are reliable markers of glutamatergic neurons (see Liguz-Lecznar and Skangiel-Kramska, 2007a). In most cases, neurons contain a single VGluT (Fremeau et al., 2001, 2004a, 2004b; Kaneko and Fujiyama, 2002), although a few neurons express both VGluT1 and VGluT2, especially in the early postnatal period (Liguz-Lecznar and Skangiel-Kramska, 2007b; Nakamura et al., 2005). The overall expression of VGluT1 and VGluT2 is largely complementary, with VGluT1 mainly expressed by cortical neurons and VGluT2 expressed in subcortical sites.

I previously discussed the relation of these transporters to schizophrenia (refer to Chapters 1 and 5). Briefly, there is evidence of VGluT1 but not VGluT2 changes in the PFC in schizophrenia (Bitanihirwe et al., 2009; Eastwood and Harrison, 2005; Oni-Orisan et al., 2008). Furthermore, I found that microglia age-dependently phagocytose VGluT1-ir elements. I am not aware of previous studies of the ontogeny of the VGluTs in the PFC.

VGluT isoform expression is age-dependent, and the pattern of expression is regionally specific. However, there is a general trend that cortical expression of both VGluT1 (Liguz-Lecznar and Skangiel-Kramska, 2007b; Minelli et al., 2003a; Nakamura et al., 2005) and VGluT2 (Liguz-Lecznar and Skangiel-Kramska, 2007b) increase with age. Northern blot analyses of whole rodent brain suggest that VGluT1 levels are relatively constant between P14-26, with a subjectively subtle decline between P26 and P60, although this was not seen between P14 and P60 (Schäfer et al., 2002). In the barrel cortex, VGluT1 levels increase until peaking at P14, thereafter declining at P21 to reach adult levels; VGluT2 levels reach peak adult levels at P21 (Liguz-Lecznar and Skangiel-Kramska, 2007b). In the somatosensory cortex, VGluT1-ir reaches the adult expression *pattern* at P30. However, protein *levels* similarly peak at P30 but decline by $\sim$14% of peak expression levels by 60 days (Minelli et al., 2003a).

I did not detect any differences in protein expression of VGluT1 or VGluT2 across PFC postnatal development from P24-50. While a number of factors could contribute to the slight discrepancies between my data and others (e.g. the slight decline in VGluT1 expression observed by Minelli and colleagues (2003a)), it is likely that there are simply differences in VGluT ontogeny reflective of the developmental patterns and functional specificity of the area examined, and that any changes in prefrontal cortical VGluTs over the discrete time period I examined may not be large enough to detect. Further, the latest time point in my analyses was P50, whereas the decline in the somatic sensory cortex was seen at P60.

Future studies will also need to determine if levels of VGluT3, which is expressed in very few neurons (see Liguz-Lecznar and Skangiel-Kramska, 2007a) and in pericytes (Mathur and Deutch, 2008), change during development.

VGAT

Inhibitory synaptic transmission in the CNS is largely mediated by GABA (see Cherubini and Conti, 2001). VGAT is the primary transporter responsible for GABA accumulation in synaptic vesicles (Takamori et al., 2000) and is dependent on both pH and electrical gradients for activity (McIntire et al., 1997; Sagné et al., 1997).

Alterations in GABA-related genes in the PFC (see Lewis et al., 2005), including modestly decreased mRNA levels of VGAT (Hoftman et al., 2015), has been reported in schizophrenia. However, another study found no difference in VGAT mRNA levels in the dorsolateral PFC (Fung et al., 2011), suggesting there may be subtypes or subgroups of schizophrenia in which GABA-related transcripts are affected (Volk et al., 2012). Furthermore, some of these GABA-related changes are restricted to distinct subsets of interneurons and occur in a lamina-specific manner (Volk et al., 2000). Overall, the GABA-related changes are suggestive of decreased inhibitory control over PCs in the PFC.

In the PFC of rhesus monkeys, VGAT mRNA expression levels increase marginally during

postnatal development (Hoftman et al., 2015). In rodent cortical development, VGAT protein levels increase sharply between P0-15, thereafter stabilizing to adult levels (Minelli et al., 2003b). I did not observe a change in VGAT protein levels in the mPFC across PFC development, suggesting that VGAT development largely takes place prior to these periods.

SIRPα

SIRPα is a transmembrane inhibitory receptor in the signal regulatory protein family. It is expressed mainly by myeloid cells, including microglia, macrophages monocytes, granulocytes, and dendritic cells, and also by neuronal cells (Adams et al., 1998). SIRPα interacts with its endogenous ligand, CD47, which is expressed by several cell types (Reinhold et al., 1995), via its extracellular region. While levels of SIRPα on phagocytes are largely stable under inflammatory conditions (Adams et al., 1998), CD47 expression fluctuates (Van et al., 2012). Interestingly, SIRPα-CD47 interaction in neurons has been shown to induce the formation of filopodia and spines, in part through activation of Cdc42 (Murata et al., 2006).

CD47 is thought to be an anti-phagocytic, "don't eat me" signal for cells. CD47 levels are down-modulated during apoptosis to allow for cell clearance (see Hochreiter-Hufford and Ravichandran, 2013). This evasion signal has been extensively studied in cancer models: interaction of SIRPα with CD47 on tumor cells (Campbell et al., 1992) prevents the elimination of the tumor cells (Jaiswal et al., 2009; Zhao et al., 2011). This has prompted clinical trials of agents that block SIRPα in various types of cancers (Barclay and van den Berg, 2014; McCracken et al., 2015; Takahashi, 2018).

Although CD47 and SIRPα are expressed in the developing CNS (Elward and Gasque, 2003), their roles in the brain have been much less studied until very recently. Because of the known roles of complement cascade components in both the peripheral (see Elward and Gasque, 2003; Lauber et al., 2004) and CNS (Schafer et al., 2012; Stevens et al., 2007) immune systems, Lehrman and colleagues (2018) hypothesized that CD47-SIRPα may play a role in the CNS similar

to its role in peripheral tissue and help synapses evade elimination by microglia. Lehrman et al. (2018) showed that CD47 is enriched at certain thalamic synapses during peak developmental pruning periods (P5), while SIRPα is expressed by microglia at the same time; expression of SIRPα largely shifts from microglia to neurons by P30 (Lehrman et al., 2018). They further showed that CD47-SIRPα prevents excess developmental microglial pruning (see Chapter 5).

I assessed if SIRPα is developmentally regulated in the PFC. There was no change in SIRPα protein levels between P24-50. However, detailed immunohistochemical or *in situ* analyses will be required to determine if there are region- or lamina-specific alterations. Disruptions in the delicate balance of complement-mediated "eat me" signals and CD47-SIRPα-mediated "don't eat me" signals during PFC development could have profound effects on prefrontal cortical structure and could contribute to schizophrenia pathology (Glausier and Lewis, 2013; Moyer et al., 2015).

APPENDIX B

DIPHTHERIA TOXIN TO ABLATE MICROGLIA

I showed that microglia engulf pre- and postsynaptic elements during adolescence (see Chapters 4 and 5). As a follow-up experiment, I planned to use transgenic mice in which the diphtheria toxin (DT) receptor can be inducibly and selectively expressed in microglia to assess if transient depletion of microglia by DT during adolescence alters PFC PC spine density in the adult (Figure 31).

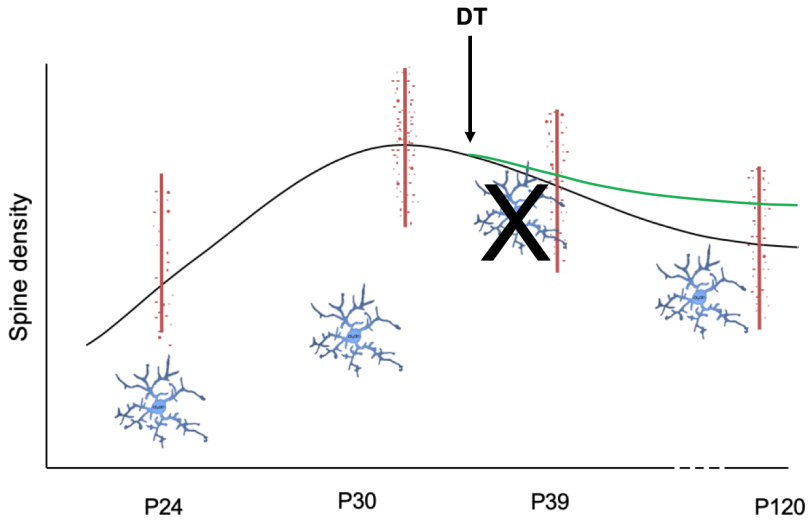


Figure 31. Effects of microglial ablation during developmental pruning on adult spine density. Diphtheria toxin will be used to ablate microglia just prior to developmental spine pruning. Spine density will be assessed in the adult animal.

As a prelude to these experiments, the extent of PFC microglial depletion and rate of microglial recovery after DT treatment needed to be determined. Microglia self-renew by proliferation, with a very slow rate of turnover (Lawson et al., 1992). In contrast, peripheral monocytes and inflammatory macrophages have a rapid turnover rate (see Parkhurst et al., 2013). Myeloid cell populations, including microglia, express the fractalkine receptor CX_3CR1, but because of their fast turnover rate, peripheral CX_3CR1^+ cells are rapidly replaced by bone

marrow progenitors (Parkhurst et al., 2013).

Taking advantage of this differential turnover rate and the absence of endogenous DT receptor (DTR) in mice, Parkhurst and colleagues (2013) generated a mouse line expressing tamoxifen-inducible Cre recombinase and DTR in microglia under the control of the CX_3CR1 promoter ($CX_3CR1^{CreER/+}$:$R26^{iDTR/+}$; Figure 32). The authors reported that the number of microglia (as assessed by fluorescent-activated cell sorting) was decreased by ~99% one day after DT treatment and only recovered by 15% at six days post-DT; there was no significant loss of peripheral macrophages (Parkhurst et al., 2013). In contrast, Bruttger and colleagues (2015) noted a ~75% recovery of microglia at 7d post-DT. Neither of these studies examined microglia in the PFC, instead monitoring microglia across whole cortex or brain. Because the number of microglia varies significantly across brain areas (De Biase et al., 2017; Bruttger et al., 2015; Lawson et al., 1990) and different mouse strains (Kezic and McMenamin, 2013), I attempted to perform a detailed time course of microglial depletion and recovery after DT treatment of tamoxifen-primed $CX_3CR1^{CreER/+}$:$R26^{iDTR/+}$ mice.

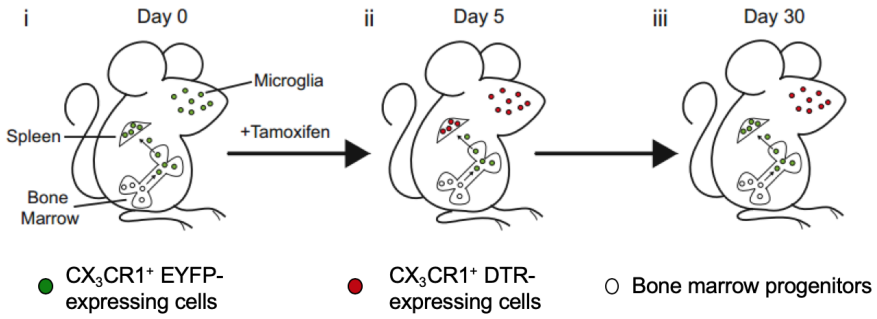


Figure 32. Expression of DTR by microglia, but not peripheral macrophages, is persistent. i. At day 0, $CX_3CR1^{CreER/+}$:$R26^{iDTR/+}$ mice express GFP in CX_3CR1^+ cells, including both microglia and peripheral macrophages. ii. Following tamoxifen administration, all CX_3CR1^+ undergo recombination and express the DTR. iii. Peripheral macrophages turn over and are replenished by CX_3CR1^+ cells that have not undergone recombination, while the microglial population does not, leading to DTR expression in only microglial cells (Reprinted from Cell, 155, Parkhurst CN, Yang G, Ninan I, Savas JN, Yates JR 3rd, Lafaille JJ, Hempstead BL, Littman DR, Gan WB, Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor, 596-609, © (2013), with permission from Elsevier).

B.1 Methods

Microglial ablation. $CX_3CR1^{CreER/+}$:$R26^{iDTR/+}$ mice received daily injections of tamoxifen (75 mg/kg, subcutaneous (sc)) for five successive days. After waiting an additional six days to allow for recombination and replacement of peripheral macrophages, mice were treated with DT (50 μg/kg, ip) every other day for three days to deplete microglia. The density of PFC microglia was examined in animals sacrificed 2, 4, 8, 10, or 16 days after the final DT injection.

Tamoxifen was initially made up at 20 mg/mL in olive oil, but subsequently made up at 10 mg/mL to increase the volume injected into the animal for increased accuracy. After six trials, corn oil was substituted for olive oil. Tamoxifen was prepared at 37°C for three or greater than three hours or overnight.

Although initial trials indicated that DT treatment led to extensive depletion of microglia (see results), I failed to obtain consistent outcomes over subsequent replications and trials. As a result, I modified various parameters in an attempt to obtain reliable and consistent depletion (and recovery) of prefrontal cortical microglia. More than ten trials were conducted, varying the number of tamoxifen injections, the vehicle for the tamoxifen, and the route of tamoxifen administration, as well as the number of DT injections and the dose of DT administered (see Tables 5 and 6).

Immunohistochemistry and microglial density. Methods for immunofluorescence and microglial density determination are as described in Chapters 4 and 6, respectively, with the following exceptions: 1) sections were incubated only with Iba1 (see Table 1 in Chapter 4), 2) microglial density was determined over a larger area (38,000 μm^2) in L5 of the PFC, 3) microglial density was determined in four separate regions of L5 of the PFC, instead of 3.

Data analysis. Microglial density for each time point examined was expressed as a percentage of the density in wild type controls.

B.2 Results

There were six trials in which tamoxifen (75 mg/kg, sc) was administered in an olive oil vehicle (prepared at 20 mg/mL; refer to Table 5). In these trials, the dose of DT was varied (from 50 ng to 50 µg to 1 µg/kg per injection, most representing the dose irrespective of body weight). In all cases, DT was administered intraperitoneally.

In the first two trials (see Table 5), I observed extensive ($>$74%) ablation of microglia at 2d post-DT. In the first of the two trials (in which tamoxifen was administered twice, spaced one day apart, with DT (50 µg DT) injected once every other day over three days starting at 3d after the final tamoxifen injection), $\sim$90% depletion of microglia was achieved (Figure 33, left). In the second trial (in which tamoxifen was administered for three consecutive days and 50 ng DT was injected once every other day for three days starting 4d post-tamoxifen), the number of PFC L5 microglia was decreased by 74.74% (Figure 33, right).

Although the extent of microglial depletion was similar across the two trials, the rate of recovery of microglia varied markedly across the two trials: Trial 1 showed substantial recovery at 4d post-DT ($\sim$65% of wildtype controls), with near full recovery by 8d post-DT (Figure 33, left). In contrast, in Trial 2, microglia did not recover by the fourth day post-DT (Figure 33, right). In summary, the results of the two trials varied only slightly in the degree to which microglia were depleted but differed markedly in the rate of recovery of PFC microglia.

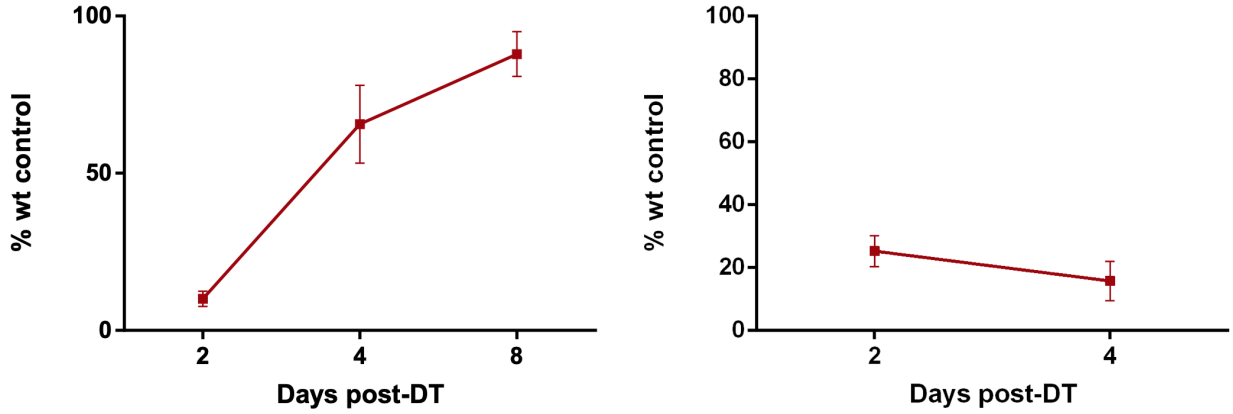


Figure 33. DT-mediated ablation of microglia, Trials 1 and 2. Left: Trial 1. Microglia were substantially ablated at 2d post-DT and recovered nearly fully by 8d post-DT. Right: Trial 2. Microglial ablation at 2d was extensive, and remained as such through 4d post-DT.

Trial	**TAM dose, # treatments**	**DT dose, *cumulative DT***	**# DT injections # days post-TAM**	**Results**
1	75 mg/kg, sc 2, every other day	50 µg *100 µg*	2 every other day 3d post-TAM	Ablation observed. See Figure 33, left.
2	75 mg/kg, sc 3 daily	50 ng *100 ng*	2 every other day 4d post-TAM	Ablation observed. See Figure 33, right.
3	75 mg/kg, sc 4 daily	50 µg *150 µg*	3 every other day 3d post-TAM	No gross evidence of ablation. Did not quantify. No spine density differences.
4	75 mg/kg, sc 4 daily	50 µg *100 µg*	2 every other day 3d post-TAM	No gross evidence of ablation. Did not quantify.
5	75 mg/kg, sc 4 daily	1 µg *3 µg*	3 daily 3d post-TAM	Deaths and poor health in mice. Lethargy, etc. Mixed results.
6 3 DT doses	75 mg/kg, sc 5 daily	250 ng/kg *750 ng/kg* 500 ng/kg *1500 ng/kg* 1 µg/kg *3 µg/kg*	3 every other day 7d post-TAM	No gross evidence of ablation. Did not quantify.

Table 5. DT-mediated microglial ablation trials with tamoxifen prepared in olive oil. Tamoxifen was administered subcutaneously

During the tamoxifen injection procedure, I noted that in some animals there appeared to be leakage of the viscous tamoxifen solution during the subcutaneous injection, with some of the solution visible on the animals backs. I therefore changed the tamoxifen vehicle from olive oil to corn oil; in addition, the concentration of the tamoxifen was changed from (20 mg/mL to 10 mg/mL). I also switched from using an insulin syringe (28g, 1/2" long) to a needle with a slightly larger bore (25g, 5/8" long) and changed the route of injection from sc to ip.

Table 6 shows the different trials using tamoxifen prepared in corn oil. Unfortunately, I again obtained inconsistent results. In an attempt to minimize any error due to the injection procedure, I took a refresher course on murine handling and drug administration offered by the Division of Animal Care. However, the course did not reveal any deviation from best practices in the injection procedure, and both the degree to which microglia were ablated and the rate at which microglia were repopulated after depletion continued to vary across subsequent trials (see Figure 34).

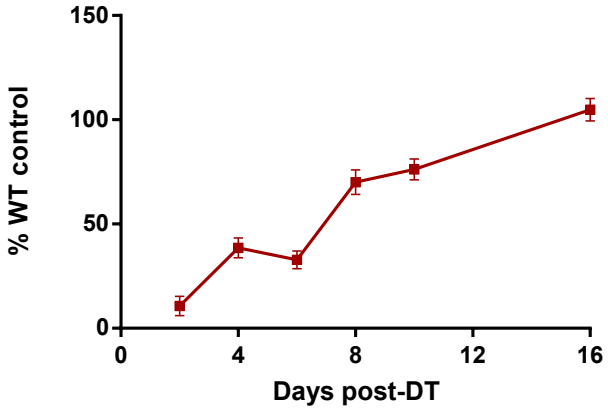


Figure 34. DT-mediated ablation of microglia, Trials 8 and 9. Microglia were substantially ablated at 2d post-DT. Microglia recovered largely by 8-10d post-DT and fully by 16d post-DT.

Trial	**TAM dose, # treatments**	**DT dose, *cumulative DT***	**# DT injections # days post-TAM**	**Results**
7 2 DT doses	75 mg/kg, ip 5 daily	10 µg/kg *30 µg/kg* 50 µg/kg *150 µg/kg*	3 every other day 6d post-TAM	No gross evidence of ablation. Did not quantify.
8 and 9	75 mg/kg, ip 5 daily	50 µg/kg *150 µg/kg*	3 every other day 6d post-TAM	Ablation observed. Full recovery by 16d. See Figure 34.
10	75 mg/kg, ip 5 daily	50 µg/kg *150 µg/kg*	3 every other day 6d post-TAM	No gross evidence of ablation. Did not quantify.
11	75 mg/kg, ip 5 daily	50 µg/kg *150 µg/kg*	3 every other day 6d post-TAM	No gross evidence of ablation. Did not quantify.

Table 6. DT-mediated microglial ablation trials with tamoxifen prepared in corn oil. Tamoxifen was administered intraperitoneally.

B.3 Discussion

I experienced, across a moderately large number of trials, significant variability in both the extent of microglial ablation and recovery of microglia, despite varying different parameters in an attempt to generate consistent outcomes.

Although ablation of microglia using DT in $CX_3CR1^{CreER/+}$:$R26^{iDTR/+}$ mice has been used extensively, the various published manuscripts that have used this method have striking differences in the protocols followed; the doses (individual and cumulative) of tamoxifen and DT varied across publications, as did the number of treatments and the interval between the last tamoxifen injection and the first DT injection. For example, Parkhurst et al. (2013) treated animals with two doses of 10 mg tamoxifen dissolved in corn oil by gavage with 48 hours between doses, and approximately 30 days later administered 1 μg DT (ip) for three consecutive days. Bruttger et al. (2015) administered two doses of 2 mg tamoxifen (sc) suspended in olive oil with 48 hours between doses, and then waited six weeks before three doses of 500 ng DT with 1 day between each DT injection. Parkhurst and colleagues (2013) reported only 15% recovery of microglia at six days post-DT, while Bruttger et al. (2015) reported $\sim$75% recovery at seven days post-DT. Not only did these two groups report differences in microglial depletion and recovery, but they found differences in astrocyte response to depletion and behavioral consequences (or lack thereof) in the mice.

Although this general approach has long been used (Saito et al., 2001), it appears that the method may be much less reliable and/or sensitive than generally realized. Some reasons for these disparities could include the genetic background of the animals, the exact parameters and age of the tamoxifen administration, the interval between recombination and subsequent DT treatment, and age and dose of DT-mediated ablation. I addressed, to some degree, several of these parameters without satisfactorily resolving the issues.

Because of the striking variability in the effectiveness of DT-mediated central ablation of

cells, I felt that further efforts using this approach would not be useful in addressing key issues in the sequelae of PFC microglial ablation (see Chapter 8). Moreover, systemic administration of DT should presumably target all microglia, not just those in the PFC, rendering interpretation of microglial manipulations difficult (direct central DT injection, while possible, likely induces changes in synaptic (dendritic spine) due to the injection procedure). Based on these considerations, I therefore did not pursue further studies involving DT-mediated ablation of microglia.

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