Dietary Choline, Inflammation, and Neuroprotection Across the Lifespan

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

The cholinergic system is intricately linked with hippocampal memory. As well, choline is anti-inflammatory in the brain and periphery (Terrando et al., 2011; Rivera et al., 1998). However, few have analyzed the anti-inflammatory properties of choline as an alternate means by which cholinergic manipulations affect hippocampal memory throughout the lifespan. The first aim of this dissertation work sought to determine if dietary choline supplementation protects against the deleterious effects of air pollution in the developing brain. Pregnant mice were given a high-choline diet (approximately 4.5x the choline chloride in the control diet) or a synthetic control diet. As well, dams were exposed to a series of diesel particulate (DEP) or saline vehicle sessions throughout pregnancy. Mice were sacrificed and tissues were collected on embryonic day 18. The activation state of microglia, identified by quantifying morphology using Iba1+ immunohistochemical staining, was examined in the dentate gyrus of the hippocampus (DG), the paraventricular nucleus (PVN) of the hypothalamus, the basolateral amygdala (AMY), and the parietal cortex (PCX). As expected, we found that DEP led to increased microglial activation in the fetal DG in males. Choline supplementation partially prevented this increase in activation. Interestingly, these effects were region-specific: the opposite pattern is seen in the PVN, and no significant diesel effect was seen in the AMY and PCX. These findings suggest that prenatal choline supplementation throughout

pregnancy may protect the fetal hippocampus against the neuroinflammation associated with air pollution. To analyze whether the acute effects of dietary choline seen prenatally also occur in adulthood, adult dietary choline supplementation alongside the tibial fracture model of post-operative cognitive dysfunction (POCD) was used. POCD occurs when increased neuroinflammation due to peripheral surgery leads to impairments in cognition. Differences were found in almost hippocampal-dependent behavior, astrocytic activation, and cell proliferation. Differences were time point-specific. In the hippocampus, astrocytic activation, cell proliferation, and hilar granule cells all increased 1 day after surgery, and these increases were blunted by dietary choline. An increase in hippocampal young neurons was found 2 weeks after surgery. However, both were blunted by choline supplementation. At both time points assessed, tibial fracture impaired novel object recognition performance, and dietary choline rescued these impairments. As well, dietary choline supplementation did not mitigate the increase in anxiety-related behavior – specifically implicating hippocampal actions of the nutrient. Because the hippocampal-dependent memory impairment and rescue is not time point-specific, but the neural effects of tibial fracture are each specific to a certain timepoint, the mechanisms of behavior are likely different at each time point. Building upon aim 2, aim 3 explores if perinatal choline supplementation can act via "programming" of the neuroimmune system in development to prevent POCD in adulthood. Perinatal choline supplementation prevented POCD and neuroinflammation

due to peripheral surgery, but did not protect against increases in young neurons or hilar neurons. Perinatal choline nutrition, in addition to its already-known neuroprotection, is additionally protective against POCD and its associated neuroinflammation in adulthood. Taken together, this body of work concludes that dietary choline supplementation at various administration dates is protective in neuroinflammatory models in behavior and brain.

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1. Introduction

The critical role of the cholinergic system in cognitive functioning has been widely examined. Disruption of cholinergic circuitry is likely to be responsible for the cognitive impairments seen in neurodegenerative disorders (Bartus et al., 1982; Drachman & Leavitt, 1974). Recent studies have also revealed deficits in cholinergic signaling in disorders of attention and cognitive control (Ballinger et al., 2016). Though the mechanism by which cholinergic signaling influences cognitive processes has been assumed to be direct cholinergic stimulation of neurons, a neglected area of investigation is the role of cholinergic anti-inflammatory effects on cognition. Not only do neurons respond directly to cholinergic agonists, but so do microglia, astrocytes, and peripheral macrophages.

The focus of this work was to determine whether dietary choline supplementation either perinatally or in adulthood provides neural protection via its anti-inflammatory actions in the hippocampus following an immune assault. More specifically, I have analyzed 1.) the effects of maternal dietary choline supplementation and a maternal immune assault on neuroimmune cells in the fetal brain; 2.) the effects of adult dietary choline supplementation and an adult immune assault on behavior, neuroimmune cells, cell proliferation and survival, and neurons; and 3.) perinatal choline supplementation and an adult immune assault on behavior, neuroimmune cells, cell survival, and neurons. In this introduction, I will first summarize the current

literature on the cholinergic system and memory (based on Maurer & Williams, 2017), then discuss the potential role of neuroimmune cells in these phenomena, and lastly outline a plan to examine the effects of dietary choline supplementation to mitigate cognitive, neuroimmune, and neuronal effects of real-world immune assaults.

1.1 The cholinergic system and memory

1.1.1 The cholinergic system

1.1.1.1 Synthesis and synaptic actions

Otto Loewi first discovered the chemical ability of acetylcholine (ACh) to decrease heart rate and act as a peripheral chemical messenger in 1921. He initially named it "Vagusstoff" because it is released from the vagus nerve. Since then, the intricate workings of ACh synthesis and synaptic communication have been identified.

First, ACh is synthesized from choline and AcetylCoA via the Choline AcetylTransferase (ChAT) enzyme. The uptake of ACh into storage vesicles occurs through an energy-dependent pump. ACh is then released into the synaptic cleft. It can bind to the muscarinic and/or nicotinic ACh receptors on the post-synaptic cell. Within the synapse, ACh is broken back down into choline and acetic acid by the AcetylCholinEsterase (AChE) enzyme. Choline reuptake occurs via a high-affinity choline transporter, and then choline is recycled in the synthesis of new ACh.

1.1.1.2 Receptors

There are two kinds of ACh receptors: nicotinic (nAChR) and muscarinic (mAChR). NAChRs are ligand-gated receptors which have five subunits. Muscle-type nAChRs are found in the neuromuscular junction, and neuronal-type nAChRs are found throughout the central nervous system (CNS). NAChRs are also functionally different from mAChRs, in addition to the former being ionotropic and the latter being metabotropic, in that nAChRs are all excitatory, but mAChRs can be either excitatory or inhibitory.

Using selective agonists such as nicotine and α-bungarotoxin, nAChRs have been mapped in the rodent brain (Decker et al., 2000), and, to a lesser extent, the human brain (Gotti et al., 1997). Critically, choline itself also activates α7 nAChRs (Alkondon et al., 1997). The hippocampus has almost every subtype (Dineley-Miller & Patrick, 1992), most strongly expresses α7 nAChRs, and has a high density of cholinergic receptors both pre- and post-synaptically (Fabian-Fine et al., 2001). This high receptor density may be a mechanism behind the cholinergic impact on hippocampal-dependent memory. This distribution of receptors is similar in humans (Gotti et al., 1997). Microglia (Egea et al., 2015; Suzuki et al., 2006), astrocytes (Shen & Yakel, 2012), and macrophages (Wang et al., 2003) also express α7 nAChRs (Liu et al., 2012; Suzuki et al., 2006). A7 nAChRs are likely the main cholinergic receptor that is involved with suppressing inflammation, as

previous work showed that the $\alpha 7$, but not $\alpha 1$ or $\alpha 1$, subtypes suppressed inflammation from macrophages (Wang et al., 2003).

1.1.1.3 Cholinergic circuits

In the CNS, cholinergic neurons reside in three major areas: the brain stem, striatum, and basal forebrain. The most critical of these for hippocampal-dependent memory is the basal forebrain. The cholinergic neurons in the basal forebrain reside mainly in the medial septum, vertical limb of the diagonal band (MS/VDB), horizontal limbs of the diagonal band, and nucleus basalis. These cells project to the olfactory bulb, neocortex, hippocampus, and amygdala (Mesulam et al., 1983; Perez-Lloret & Barrantes, 2016; Woolf, 1991). Cholinergic neurons in the MS/VDB project to all subregions of the hippocampus (Khakpai et al., 2013; Teles-Grilo Ruivo & Mellor, 2013). There are cholinergic interneurons in the cortex itself, but they are scarce (von Engelhardt et al., 2007). Basal forebrain neurons, specifically those in the nucleus basalis, selectively degenerate in Alzheimer's disease (Whitehouse et al., 1982). Concurrently, cholinergic degeneration in the diagonal band of the basal forebrain has been mechanistically linked to spatial memory impairments in an Alzheimer's disease mouse model (Zhu et al., 2017). There are also projections from the basal forebrain to the frontal cortex, which are involved in attentional processes (Himmelheber et al., 2000).

1.1.1.4 Dietary choline supplementation

Choline, a α7 nAChR agonist (Alkondon et al., 1997), is a vital nutrient for the human brain and body (reviewed in Zeisel, 1991). Choline has various roles: from synthesizing ACh, to contributing to cell membranes, to epigenetic modifications. Though choline can be synthesized in the liver *de novo*, the quantity produced is not sufficient to prevent liver damage (Zeisel et al., 1991). Hence, dietary consumption of choline is needed. High dietary choline intake at the time of assessment has been shown to enhance cognitive performance in human adults (Poly et al., 2011). In the same study, mild cognitive protective effects of high dietary choline intake were observed 3-10 years later. High choline intake (> 454 mg/day) four years prior to behavioral testing protects against cognitive aging in adult men (Ylilauri et al., 2019). In a rodent model, adult dietary choline supplementation (500 mg/kg/day) has also been shown to be protective against memory 3 months later (Teather & Wurtman, 2005). Dietary choline is also preventative against memory impairments associated with status epilepticus (Holmes et al., 2002), possibly through the prevention of hippocampal cell loss (Holmes et al., 2002). Though there has been work in humans demonstrating that dietary choline intake is inversely correlated with levels of inflammatory cytokines in blood samples (Detopoulou et al., 2008), few have analyzed the interaction of dietary choline supplementation and inflammation as it pertains to cognition.

Much research has been devoted to dietary choline supplementation in the context of brain development, particularly in prevention of neural tube defects and the development of memory capabilities (reviewed in Zeisel & da Costa, 2009). The need for choline is great during fetal development. In fact, serum choline concentrations are usually six to seven times higher in the fetus and newborn than they are in adults (Zeisel & Wurtman, 1981). During pregnancy, the placenta stores choline as ACh and preferentially delivers large amounts of choline to the fetus (Leventer & Rowell, 1984). After birth, the neonate has a particularly high capacity for choline transport across the blood-brain barrier to the brain (Cornford & Cornford, 1986). A novel form of phosphatidylethanolamine N-methyltransferase (PEMT), an enzyme involved in synthesizing ACh *de novo*, is present in the neonatal brain, but not the adult brain (Blusztajn et al., 1985). This enzyme is induced by estrogen, leading to enhanced *de novo* synthesis of choline during the high-estrogen environment of pregnancy (Resseguie et al., 2007). Because of the importance of choline during development, many advantages are conferred during pregnancy and early life to ensure adequate delivery.

Even though there are multiple mechanisms to preferentially supply the fetus with choline, maternal dietary choline supplementation is still critical. This is likely because, due to the increased fetal demand, maternal choline stores are depleted during pregnancy and lactation (Zeisel et al., 1995). In humans, even Adequate Intake (AI) of choline recommended by the Food and Drug Administration (FDA) was not sufficient to

prevent maternal depletion of choline-derived methyl donors (Yan et al., 2012). Hence, replenishing those stores with dietary choline supplementation is critical to maintain the high levels of choline and its derivatives required for fetal development. Cholinergic manipulations early in life are neuroprotective, but few have analyzed this phenomenon with a neuroimmune lens.

1.1.2 Memory and plasticity

Reviews of basal forebrain cholinergic systems in memory and cognition (Ballinger et al., 2016; Hasselmo & Sarter, 2011; Ferreira-Vieira et al., 2016; Blake & Boccia, 2017; Knox, 2016) largely focus on the septohippocampal pathway, which is widely known to be implicated in memory processes. There are multiple lines of evidence to support the view that hippocampal ACh is important for memory. First, during spatial memory tasks, cholinergic markers such as ChAT are upregulated (Park et al., 1992). Second, ACh levels in the hippocampus are correlated with memory function. For example, there is a positive correlation between age-related cognitive decline and decreases in hippocampal ACh (Baxter et al., 1999). There is a positive correlation as well between spatial memory and ACh release both in the hippocampus (Stancampiano et al., 1999) and basal forebrain (Tian et al., 2004). Also, damage to the septum leads to decreases in both spatial memory performance and hippocampal levels of ACh (Herzog et al., 2000). Third, both the direct infusion of ACh into the hippocampus and direct pharmacological activation of nAChRs in the hippocampus

reverse the cognitive deficits caused by damage to the septum (Decker et al., 1992; Hodges et al., 1991; Levin et al., 1993; Maho et al., 1988). In addition to neurons, hippocampal astrocytes (Gahring et al., 2004) and microglia (De Simone et al., 2005) also express nAChRs. These findings leave open the possibility that some of the actions of ACh on the hippocampus may be via nicotinic activation of cells of the neuroimmune system.

1.1.2.1 ACh prevents interference

The classically-held view is that ACh is the "decider" between encoding and retrieval in memory processing (reviewed in Easton et al., 2012, and Hasselmo, 2012). ACh suppresses old associations and inhibits interference. Rats with cholinergic basal forebrain lesions perform comparably to controls in a hippocampal-dependent Morris water maze (MWM) spatial memory task unless the location of the platform changed daily (Baxter et al., 1995). An explanation for this finding is that the lack of ACh in the hippocampus leads to more expression of a previously encoded association (which would be the previous location of the platform). However, rats with intact cholinergic systems are able to inhibit the previous association and form a new one. Hasselmo (2012) in addition to Easton et al. (2012) outlined the likely neural underpinnings of this phenomenon. Briefly, the hippocampal region Cornu Ammonis 1 (CA1) receives input from two brain regions: entorhinal cortex 3 (EC3, associated with sensory perception – "extrinsic input") and CA3 (associated with previously-formed associations – "intrinsic

input"). ACh reduces the relative input from CA3, allowing sensory inputs to be encoded, free from retroactive inhibition. Hence, hippocampal ACh "prioritizes encoding" in novel contexts.

1.1.2.2 Markers of plasticity

1.1.2.2.1 LTP

ACh "biases the system" toward increased long-term potentiation (LTP), a molecular correlate for memory, by decreasing the induction threshold required (Pyapali et al., 1998; Seol et al., 2007). In addition, in an *in vitro* high ACh environment, stimulation that normally produces long term depression (LTD) produces LTP (Sugisaki et al., 2011). The specific neuronal mechanisms underlying this effect have largely been identified. ACh, when it binds to a muscarinic receptor, leads to a signaling cascade activating phospholipase-C, which contributes to LTP (Cohen et al., 1998). Additionally, impaired LTP has been linked to malfunctioning α7 nAChRs (Chen, Yamada et al., 2006). A blockade of α7 nAChRs blunted LTP, showing that it is necessary for this form of plasticity to occur. Additionally, α7 nAChR knockout mice can show decreased LTP (Freund et al., 2016). The cholinergic systems' impact on LTP has been interpreted as a direct synaptic action, but it is also possible that ACh is acting on non-neuronal α7 nAChRs. Impairments in hippocampal LTP have also been linked to microglial overactivation, and minocycline normalizes these impairments (Hoshino et al., 2017), further supporting the view that perhaps one mechanism of action is by stimulating microglial α7 nAChRs.

1.1.2.2.2 BDNF

ACh modulates plasticity via brain-derived neurotrophic factor (BDNF), which aids cognitive function. In cortical culture, choline resulted in dose-dependent increases in BDNF (Johansson et al., 2009). Additionally, after loss of basal forebrain cholinergic neurons, hippocampal BDNF decreases (Turnbull & Coulson, 2017). Finally, following chronic nicotine exposure (which activates nAChRs), BDNF in the hippocampus is upregulated (Czubak et al., 2009; Kenny et al., 2000). BDNF is one result of cholinergic stimulation that affects plasticity, and may be one mechanism behind the cholinergic link to memory.

Critically, neurons are not the only cells in the brain that release BDNF. Microglia (Trang et al., 2011) and astrocytes (de Pins et al., 2019) also release BDNF. De Pins and colleagues (2019) performed an elegant series of experiments that genetically increased BDNF from astrocytes. In addition to the rescue of neural correlates of memory such as dendritic spines, they also found that BDNF specifically from astrocytes rescues the hippocampal-dependent memory impairments in an Alzheimer's disease model. Non-neuronal cells in the brain, and the BDNF they produce, may yet be a concurrent mechanism by which the cholinergic system aids hippocampal memory.

1.1.2.2.3 Neurogenesis

The dentate gyrus (DG) of the hippocampus is well-known for its neurogenic properties throughout adulthood, and basal forebrain projections to the DG are extremely beneficial for neurogenesis (Cooper-Kuhn et al., 2004; Kotani et al., 2008;

Mohapel et al., 2005). Moreover, enhanced adult hippocampal neurogenesis appears to improve pattern separation ability, temporal separation of events in memory, beneficial forgetting to reduce proactive interference, and cognitive flexibility (reviewed in Hvoslef-Eide & Oomen, 2016).

Neural stem cells in the hippocampus express ACh receptors, including $\alpha 7$ nAChRs (Mohapel et al., 2005), providing a possible mechanism by which the cholinergic system influences neurogenesis. In general, *in vivo* and *in vitro* studies show that cholinergic receptor stimulation increases neural stem cell proliferation (Itou et al., 2011). Activation of the $\alpha 7$ nAChR via increased ACh levels enhances new neuron survival, but not differentiation or proliferation (reviewed in Kita et al., 2014, and Narla, et al., 2013). These manipulations may be influencing neuronal progenitors through ACh receptors, but they also may be impacting neuronal survival by acting on non-neuronal cells.

Increasing ACh levels through electrode or optogenetic stimulation of cholinergic neurons promotes adult hippocampal neurogenesis, and decreased ACh levels impair it (Jeong et al., 2014; Paez-Gonzalez et al., 2014). There is some evidence that a choline-supplemented diet in adulthood, leading to increased ACh synthesis (Cohen & Wurtman, 1976), increases proliferation of hippocampal neurons (Wong-Goodrich et al., 2008). AChE inhibitors also upregulate proliferation (Itou et al., 2011; Kotani et al., 2008; Mohapel et al., 2005). Exercise-induced proliferation of neural stem

cells is prevented by lesions of the septal cholinergic system (Ho et al., 2009; Itou et al., 2011). Taken together, the cholinergic system increases hippocampal neurogenesis, which aid hippocampal-dependent memory (Clelland et al., 2009).

1.2 Cholinergic actions on the neuroimmune system: an alternate mechanism for hippocampal memory protection

1.2.1 The neuroimmune system

The neuroimmune system is the crosstalk between the nervous and immune system. There are some cells that can cross the blood-brain barrier during an immune challenge, such as macrophages and T cells (Fiala et al., 1998; Engelhardt, 2006). The immune cells that are largely present in the brain under non-pathological circumstances are microglia, astrocytes, and mast cells.

Microglia are the resident macrophages of the brain. During non-pathological states, microglia actively monitor surrounding cells and synapses (Nimmerjahn et al., 2005; Wake et al., 2009). During states of immune activation, these cells dramatically change morphology. They quickly rush to a site of injury (Nimmerjahn et al., 2005), and undergo a host of diverse changes in response to an immune assault, such as the release of chemical signals called cytokines (reviewed in Hanisch & Kettenmann, 2007). Though this immune response is necessary to protect the brain from assaults, a hyper-inflammatory immune milieu is detrimental to brain health (reviewed in Chen, Zhang et al., 2016).

Cytokines, which can be released from microglia (Hanisch, 2002), astrocytes (Zhang et al., 2018), mast cells (Bradding et al., 1993; 1994), and neurons (Lim et al., 2016), are chemical messengers that have largely diverse roles depending on the specific cytokine and context. They are typically classified along a spectrum of "pro-inflammatory" and "anti-inflammatory," although some, like interleukin-6 (IL-6), can exhibit varying roles (reviewed in Scheller et al., 2011). Two classic pro-inflammatory cytokines are tumor necrosis factor (TNF, aka TNFα; see Clark, 2007 for the difference in nomenclature) and interleukin-1β (IL-1β).

Astrocytes have varied support roles in the brain. One of the many roles they play is immune: astrocytes release cytokines that, like microglia, can both help and harm the brain (Choi et al., 2014). Specifically, astrocytes largely make up the "glial scar" after neural injury (reviewed in Fawcett & Asher, 1999). This scar prevents the remyelination of axons, preventing full recovery from injury. Additionally, astrocytes are able to present antigens to T cells (Cornet et al., 2000). Combined, astrocytes have the capability to lead to an exacerbated neuroinflammatory milieu through varied actions, such as secretion of cytokines (reviewed in Dong & Benveniste, 2001).

Mast cells and their effects on many behaviors still require more exploration. These cells are largely involved in the allergy response, releasing histamines by degranulation. Neonatal alcohol exposure leads to an increase in hippocampal degranulation of mast cells two days after alcohol induction (Dause, 2017).

Degranulation of histamines leads to increased inflammation (reviewed in Dong et al., 2014). The role of mast cells in adult hippocampal-dependent behaviors has been linked to mast cell production of serotonin (Nautiyal et al., 2012). For this reason, this cell type will not be explored further in the context of cholinergic influences on hippocampal-dependent memory.

1.2.2 The neuroimmune system and memory

In a non-pathological state, the neuroimmune system is needed for learning and memory (reviewed in Yirmiya & Goshen, 2011). In fact, the process of learning itself induces microglial activation (Williamson et al., 2011). Additionally, microglia are critical for the proper progression of adult hippocampal neurogenesis (reviewed in Gemma & Bachstetter, 2013). Astrocytes are required for hippocampal-dependent memory, in part through IL-1 (Menachem-Zidon et al., 2011). In terms of other immune cells, both B cells and T cells are required for memory functioning: severe combined immunodeficiency (SCID) mice, which are deficient in both cell types, show large hippocampal-dependent memory impairments even without an immune assault (Brynskikh et al., 2008). These SCID mice also have impaired hippocampal neurogenesis (Ziv et al., 2006), which has been inversely correlated with hippocampal-dependent memory (Clelland et al., 2009). These deficits in neurogenesis are rescued by the addition of T cells (Ziv et al., 2006). The neuroimmune system is essential for hippocampal-dependent memory.

In a pathological state, heightened neuroinflammation leads to sickness behavior (Henry et al., 2008), and explicitly causes hippocampal-dependent memory deficits (Zhao et al., 2019). Lipopolysaccharide (LPS), a component of gram-negative bacteria, activates an immune cascade by binding to toll-like receptor 4 (TLR4). The immune cascade includes production of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which leads to increased transcription of genes that lead to the production of cytokines (reviewed in Lu et al., 2008). Because it activates an immune response but does not cause an actual infection that is difficult to empirically control, LPS is used to study the effects of immune activation. Critically, in a study that artificially attenuated the functioning of TLR4, the hippocampal-dependent memory impairments due to LPS were rescued (Zhao et al., 2019). In a similarly targeted study, administration of an anti-IL-1β antibody shortly before infection protected against hippocampal-dependent memory impairments (Gibertini et al., 1995). Hippocampal microglia and the TNF they produce are responsible for short-term memory deficits immediately after acute stress (Ohgidani et al., 2016). With the addition of a TNF inhibitor, memory deficits were completely recovered. Astrocytes in turn react to this increase in TNF to alter synapses in the hippocampus, which impairs hippocampal-dependent memory (Habbas et al., 2015). Astrocytic activation was increased after LPS administration, and this upregulation was correlated with impairments in an object recognition task (Da Ré et al., 2020). Clearly, astrocytes play a role in memory impairment due to an immune

challenge. Another study analyzed both microglial and astrocytic activation in the context of sleep deprivation-dependent spatial memory deficits (Wadhwa et al., 2017), and found that only morphological differences in microglia lead to memory deficits. Another group found that astrocytic, not microglial, production of IL-1 impaired spatial memory (Garber et al., 2018). The neuroimmune system is required for hippocampal-dependent memory, but activation due to an immune challenge is detrimental to it.

The specific role of each cell type in hippocampal-dependent memory impairment, and how they interact, is not currently clear. Together, these findings reaffirm that these immune cells are needed for typical brain functioning, but aberrations in their inflammatory production and reactions lead to impairments.

1.2.3 The cholinergic system is anti-inflammatory

The connection between peripheral ACh and inflammation has been researched extensively – even earning the name "the cholinergic anti-inflammatory pathway" (Steinberg et al., 2016). Stimulation of the vagus nerve, either endogenously or through electrical stimulation, leads to increased ACh production from splenic T cells. This ACh then acts on α7 nAChRs on splenic macrophages. This receptor activation leads to a decreased production of inflammatory cytokines by these macrophages, such as TNF, IL-1β, and IL-6 (reviewed in Gallowitsch-Puerta & Pavlov, 2007). ACh also acts on lymphocyte α7 nAChRs to suppress inflammation (De Rosa et al., 2005; Kawashima & Fujii, 2003; Nizri et al., 2006; Sopori & Kozak, 1998). TNF production is upstream of IL-

1β (Terrando et al., 2010) and IL-6 production (Shalaby et al., 1989), suggesting that blunting TNF will subsequently block the other pro-inflammatory cytokines. Vagal stimulation also produces a dose-dependent inhibition of TNF, IL-6, and IL-1β production in human macrophages (reviewed in Pavlov & Tracey, 2005; Borovikova et al., 2000) and in the whole-blood of patients who had an autoimmune disorder (Koopman et al., 2016). Vagotomy leads to increases in IL-6 and, to a lesser extent, TNF (Borovikova et al., 2000), indicating that the vagus nerve is critical in preventing the production of these cytokines.

Critically, the vagus nerve's anti-inflammatory action is also bi-directional: the afferent vagus nerve detects peripheral cytokines, then communicates through the medulla to the hypothalamus, which subsequently communicates via the efferent vagus nerve to inhibit inflammation in the periphery (Watkins et al., 1995). In other words, the bidirectional anti-inflammatory communication between the brain and periphery relies on the vagus nerve and ACh signaling. Termed the "inflammatory reflex," the afferent vagus immediately regulates production of pro-inflammatory cytokines to avoid overproduction (Tracey, 2002). The relationship between the inflammatory reflex and the cholinergic anti-inflammatory pathway can be seen in Figure 1.

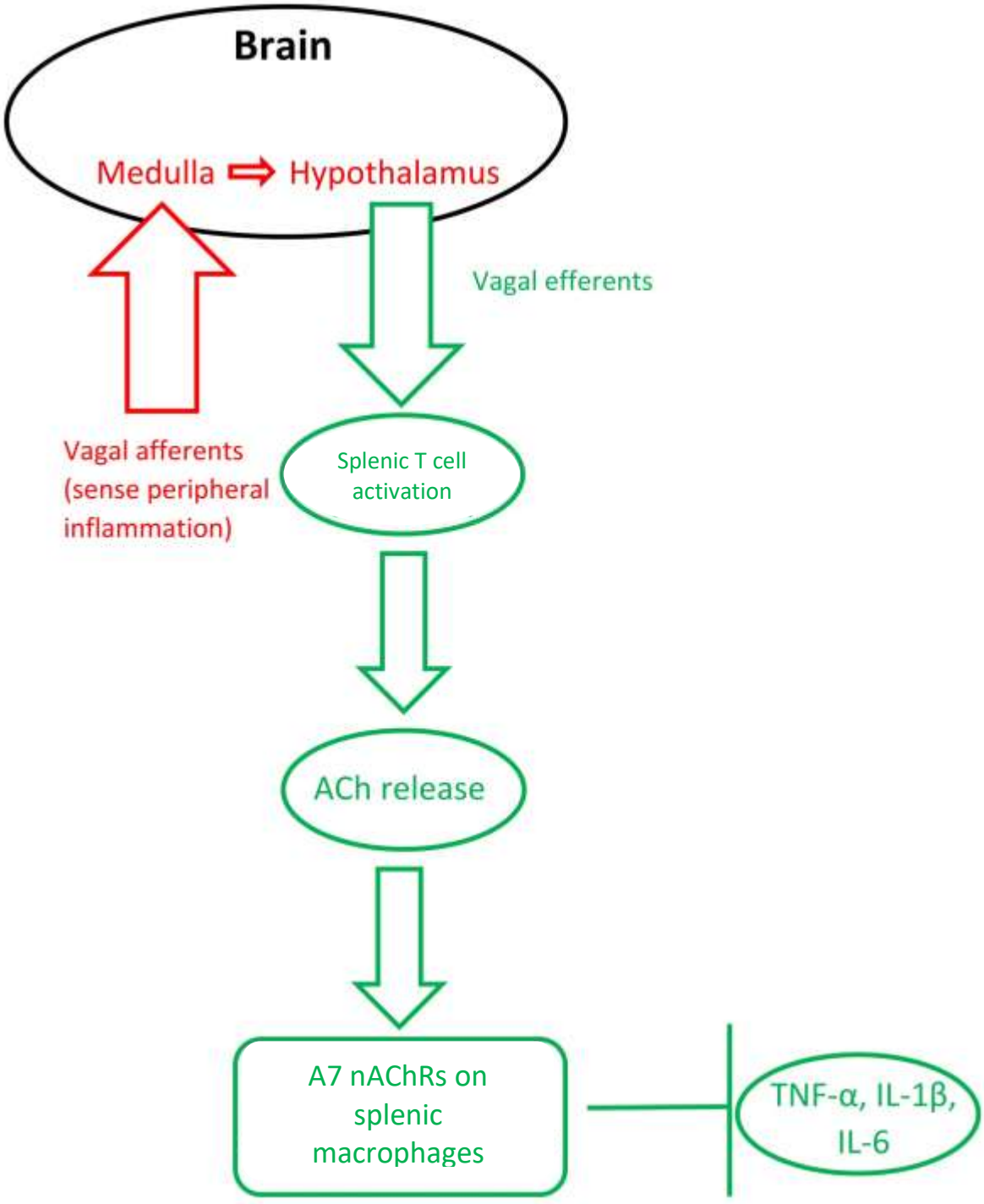

Brain
Medulla ⇨ Hypothalamus
Vagal efferents
Vagal afferents
(sense peripheral
inflammation)
Splenic T cell
activation
ACh release
A7 nAChRs on
splenic
macrophages
TNF-α, IL-1β,
IL-6

Figure 1: A schematic of the relationship between the inflammatory reflex and the cholinergic anti-inflammatory pathway. From Maurer & Williams, 2017.

There is also evidence that there is a *central* cholinergic anti-inflammatory pathway by which ACh released from neurons inhibit microglial secretion of inflammatory cytokines. One study implicating the baseline necessity of ACh for neuroimmune control showed that the loss of basal forebrain cholinergic input to the hippocampus causes microglial activation (Roßner et al., 1995). Like splenic macrophages, microglia express α7 nAChRs, which, when activated, suppress pro-inflammatory cytokine release (De Simone et al., 2005; Shytle et al., 2004). In mouse and human cell culture studies, α7 nAChRs on microglia are necessary for blunting TNF and downstream IL-1β production in the brain (Shytle et al., 2004; Yamada-Nomoto et al., 2016). AChE inhibitors suppress TNF secretion from microglia, and addition of α-bungarotoxin blunted these effects (Giunta et al., 2004) – indicating that the increased activation of nAChRs blunts cytokine release from microglia.

Because high choline consumption leads to high activation of nAChRs – partly through increased ACh synthesis (Cohen & Wurtman, 1976), and partly through direct binding of choline to these receptors (Alkondon et al., 1997) – microglial activation is suppressed, possibly by acting directly on microglial nAChRs. The influence of dietary choline supplementation on neuroimmune cells may be one way in which dietary choline is neuroprotective.

1.2.4 Cholinergic impacts on the neuroimmune system in memory

Recent work has, in fact, demonstrated that ACh acts directly on hippocampal astrocytes, which then leads to alterations in firing of hippocampal neurons (Pabst et al., 2016). Consistent with Hasselmo's view (2006) that high levels of ACh aid encoding by suppressing inappropriate activations, specific optogenetic stimulation of septal cholinergic neurons led to decreased firing of dentate granule cells. However, disrupting astrocytic function prevented this inhibition. Astrocytes are critical for the prevention of interference due to ACh. Similarly, microglial depletion has also been shown to impair spatial memory (Torres et al., 2016), in addition to fear and novel-object memory.

Astrocytes and microglia are integrated in the process by which ACh improves memory capability – however, as mentioned previously, overactivation of these cells can lead to memory deficits. The role of the cholinergic system in pathological states has been explored in several studies of preclinical post-operative cognitive dysfunction (POCD).

POCD is the phenomenon in which cognitive function (especially memory and executive functions) is impaired post-peripheral surgery. POCD is a part of the broad umbrella of perioperative neurocognitive disorders, which also includes short-term delirium (Eckenhoff et al., 2020). This phenomenon in particular can persist for several months (Monk et al., 2008). Peripheral surgery in a tibial fracture model not only increases pro-inflammatory cytokines in the periphery, but also upregulates

neuroinflammation (Terrando et al., 2010). Critically, these effects are not due to anesthesia, as mice given anesthesia without surgery do not show this inflammatory profile (Terrando et al., 2010). Macrophage-produced TNF promotes POCD by altering the permeability of the blood-brain barrier to increase macrophage infiltration to the hippocampus after tibial fracture surgery (Terrando et al., 2011). Prevention of macrophage-produced TNF prevented the increased permeability of the blood-brain barrier, and also prevented the increased macrophage migration into the brain after surgery. The hippocampal-dependent memory deficits following tibial fracture can be rescued by dampening neuroinflammation.

One way to dampen this neuroinflammation is by using cholinergic agonists. Stimulation of the $\alpha 7$ nAChR just prior to surgery prevents the macrophage migration into the brain and hippocampal-dependent memory deficits 3 days after surgery, and administration of an $\alpha 7$ nAChR antagonist increased microglial activation (Terrando et al., 2011; 2014). Of note, cholinergic antagonists administered intraperitoneally (i.p.) 30 minutes prior to surgery further impair freezing in a trace fear conditioning task after tibial fracture (Terrando et al., 2011), suggesting that stimulation of nAChRs dose-dependently rescues memory after tibial fracture.

In the tibial fracture model of POCD, minocycline (which decreases microglial activation, but may also have effects on neurons) given i.p. 12 hours before surgery not only decreases hippocampal levels of inflammatory cytokines TNF, IL-1β, and

interferon-γ, but also rescues the spatial memory deficits seen following surgery (Wang et al., 2016). Additionally, blocking IL-1β release in the CNS (presumably of microglial origin, Williamson et al., 2011) prevents the memory impairment caused by overactivation of microglia (Bilbo, Biedenkapp et al., 2005). Decreasing microglial activation in adulthood can rescue hippocampal-dependent memory deficits in a highly inflammatory environment – cholinergic suppression of immune activation is likely similarly protective.

1.3 Development of the cholinergic and neuroimmune system

The development of the cholinergic system has been well-described (reviewed in Abreu-Villaça et al., 2011). Cholinergic neurons in the basal forebrain appear in a rostral to caudal gradient between embryonic day (E) 12 and E18 in rats (Brady et al., 1989; Semba & Fibiger, 1988) and between E14 and E17 in mice (Schambra et al., 1989). The earliest development of ChAT activity occurs first in regions containing cholinergic cell bodies (e.g., medial septum and diagonal band) and later by their target areas (e.g., hippocampus and cortex). ACh receptors develop at about the same time as the cell bodies, with *α7* nAChRs appearing on E13 in rats (Adams et al., 2002; Tribollet et al., 2004).

Currently, we do not know when cholinergic receptors on microglia or astrocytes first appear during development. However, we do know that microglia derived from the yolk sac migrate into the CNS between E8.5 and E9.5 (Ginhoux et al., 2010). Because the

neuroimmune system develops slightly before cholinergic neurons differentiate in the basal forebrain, and we know that nAChRs are expressed as early as E12, it is possible that ACh modulation of microglial activation may contribute to brain and behavioral development.

1.3.1 "Priming"

Thus far, this literature review has focused on the joint influences of ACh and inflammation in hippocampal neural plasticity and memory in the adult. However, inflammation itself may not be enough to induce cognitive impairment. Instead, early-life inflammatory events "prime" microglia toward a more exaggerated phenotype (Bilbo, Biedenkapp, et al., 2005; Bilbo, Levkoff, et al., 2005; Williamson et al., 2011) such that overactivated microglia and cognitive impairments are only "unmasked" when another immune challenge is encountered in adulthood (Williamson et al., 2011). Adult microglial overactivation, like that caused by early life "priming," has been previously implicated in decreased hippocampal BDNF (Barrientos et al., 2011) and may increase neurodegeneration in adulthood (reviewed in Cárdenas-Tueme et al., 2020).

Prenatal choline supplementation has many beneficial long-term effects on hippocampal plasticity, neuroprotection, and hippocampal-dependent memory function (reviewed in McCann et al., 2006, and Meck et al., 2008), which may also stem from neuroimmune "priming." For example, adult offspring that had prenatal dietary choline supplementation show higher levels of hippocampal BDNF, enhanced neurogenesis,

lowered threshold for LTP, increased hippocampal spine density, increased size of basal forebrain cholinergic neuron cell bodies, and more ACh storage in presynaptic vesicles (reviewed in Meck et al., 2008; Li et al., 2004; Pyapali et al., 1998; Blusztajn & Mellott 2013; Williams et al., 1998). Choline supplementation early in life also improves adult working memory (Meck & Williams, 1999) and improves scores on the cognitive domain of the Bailey Scales of Infant Development-III in human infants, but not any other domain (receptive language, expressive language, fine motor, and gross motor, Wu et al., 2012). Large effects of early-life choline supplementation are seen in more difficult memory tasks (Meck & Williams, 1997), and in preventing age-related memory decline (Meck et al., 2008).

Evidence that the immune and cholinergic systems influence each other in development is sparse. For example, prenatal immune activation on E12.5 with the viral mimic poly(I:C) led to increased numbers of ChAT+ cells and increased ChAT levels in the basal forebrain (Pratt et al., 2013). Hence, early in development, the immune system influences development of the cholinergic system. In the same model of early-life inflammation, addition of a high-choline maternal diet completely prevents heightened IL-6 in the fetal brain (Wu et al., 2015), which was dependent on the $\alpha 7$ nAChR. Manipulating the levels of choline in the embryonic environment is anti-inflammatory and neuroprotective against immune assaults in development. These data support

another explanation for how prenatal choline supplementation might alter hippocampal function and lead to long-term protection of brain and behavior.

1.4 Experimental outline

The hypothesis tested in this dissertation work is: Does dietary choline supplementation mitigate neuroinflammation and hippocampal-dependent cognitive deficits within inflammatory models – and if so, does choline act via neuroimmune priming or only acutely? While some studies have analyzed the impact of pharmaceutical cholinergic stimulation on neuroimmune activation and hippocampal-dependent memory in models of heightened inflammation (Terrando et al., 2011), few have analyzed these two variables using dietary cholinergic manipulations. Through the lens of the neuroimmune system, the current work aims to explore the acute and lifelong protective effects of dietary choline. In addition to assessing fetal and adult acute exposure to dietary choline and an immune assault, the "programming" effect of perinatal dietary choline supplementation in the context of an adult immune assault is assessed. Translationally, humans are most likely to encounter cholinergic manipulations via diet, not pharmacologically. Analyzing the effects of dietary choline supplementation is critical to understanding how a high-choline diet can impact the neuroimmune system, and potentially protect against memory decline following neuroinflammatory events.

The mouse models of inflammation used in the current work were chosen because of their prevalence in human life: prenatal diesel exposure and adult tibial fracture. These models were also chosen because they profoundly impact the immune system and cognitive outcomes (Bolton et al., 2013; Terrando et al., 2011).

We first determined whether prenatal dietary choline supplementation altered microglial activation caused by prenatal diesel exposure in the dentate gyrus, paraventricular nucleus of the hypothalamus, amygdala, and parietal cortex of fetal mouse brains. Microglial activation was quantified based on morphology after immunohistochemical staining for the macrophage marker Iba1. The largest changes in microglial activation were found in the dentate gyrus and paraventricular nucleus, with the former exhibiting an increase in activation and the latter exhibiting a decrease. Both changes due to diesel exposure were mitigated by maternal dietary choline supplementation. Prior to birth, microglia are in a morphologically activated state (Schwarz et al., 2012), and this activation is necessary for critical synaptic pruning (Paolicelli et al., 2011). As discussed previously, a delicate balance between activity and overactivity is needed for optimal brain development. These data provide key evidence that maternal choline supplementation blunts microglial alterations due to maternal diesel exposure in the fetal brain, and may therefore "prime" microglia against overreacting to an immune challenge in adulthood.

The second and third aims of this dissertation work tested whether dietary choline supplementation protects hippocampal-dependent memory, neuroinflammation, and neuronal changes due to an adult immune activator, tibial fracture surgery. The second aim tested whether acute adult dietary choline supplementation is protective against the cognitive, neuroimmune, and neuronal alterations due to tibial fracture. The third tested whether perinatal dietary choline supplementation could "program" brain and behavior to be similarly protective against tibial fracture surgery. Though previous work has analyzed cholinergic agonists and hippocampal-dependent memory in the tibial fracture model (Terrando et al., 2011), the current work is novel in a number of ways. First, this is the first analysis that focuses on a long-term (two-week) effect of tibial fracture on brain and behavior; previous work in the tibial fracture model focused on POCD just three days after surgery (Terrando et al., 2011; 2014). Second, though others have analyzed pharmaceutical cholinergic agonists (Terrando et al., 2011), this is the first to analyze dietary choline supplementation in the context of POCD. Third, novel object recognition was utilized to asses choline's protective effects on hippocampal-dependent memory. Previous work analyzed memory via a trace fear conditioning task (Terrando et al., 2011), which may in itself cause hippocampal neuroinflammation (Brevet et al., 2010). Fear conditioning tasks that include a foot shock induce an increase in corticosterone, indicative of a physiological stress response (Chester et al., 2014). Because stressful experiences contribute to

widespread microglial alterations (reviewed in Rohan Walker et al., 2013), we tested short-term memory using a less stressful paradigm.

We found that surgery had widespread effects that were time point-dependent. Dietary choline supplementation for three weeks prior to tibial fracture at least partially mitigated all surgery-induced behavioral, neuronal, and neuroimmune changes. Importantly, we found an increase in young neurons that had migrated into the hilar region of the hippocampus following tibial fracture - a phenomenon that until now has not been observed in any model except for status epilepticus (Hester & Danzer, 2013). This rewiring may account for the long-term memory dysfunction seen after tibial fracture.

The first two aims of the current work demonstrate that dietary choline has acute effects. The third aim tests whether dietary choline supplementation can "prime" the developing brain to be protected against adult tibial fracture. Surprisingly, we found that, like adult dietary choline supplementation, perinatal choline supplementation in the absence of an adult dietary change at least partially prevented the long-term cognitive and neuroimmune changes due to peripheral tibial fracture in adulthood.

Taken together, this work suggests that an alternative mechanism for the beneficial effects of dietary choline supplementation, both in adulthood and perinatally, is by suppressing neuroinflammation and its associated destructive aftermath. The implications of this work are immediately translatable to human populations – though

dietary choline has already been shown to have varied behavioral and neural protective effects throughout the lifespan, this work provides yet another context in which dietary choline is protective. By consuming more high-choline foods, such as eggs and lean meats, people about to undergo surgery can prevent the cognitive and neural effects of peripheral surgery without turning to pharmaceutical manipulations. Additionally, the field of prenatal choline supplementation now has another avenue, in addition to its varied effects on neuronal number and functioning, by which it is protective across the lifespan.

2. Aim 1: Prenatal choline as an anti-inflammatory agent in the fetal brain

2.1 Introduction

2.1.1 Pollution and neuroimmune development

Pollution exposure is a public health hazard, particularly during development. Air pollution from traffic and proximity to highways leads to developmental delays in brain connectivity and working memory capability (Pujol et al., 2016; Sunyer et al., 2015). Children who live in an area with high levels of air pollution perform poorer on motor, IQ, and learning and memory tests than children in non-polluted areas (Wang et al., 2009; Calderón-Garcidueñas et al., 2011). In addition, prenatal exposure to polycyclic aromatic hydrocarbons, a common air pollutant, inversely correlates with widespread white matter alterations and behavioral problems such as ADHD symptoms, externalizing problems, and slower processing speed (Peterson et al., 2015).

It is possible to model human exposure to diesel particulate matter (a common pollutant in industrialized areas) within mice. One such model utilizes oropharyngeal aspiration to directly expose mice to precisely controlled levels of diesel exhaust particles (DEP). This model has been utilized to characterize the effects of prenatal diesel exposure in development. In this model, the adult mice whose dams were exposed to DEP show impaired hippocampal-dependent memory in male offspring only (Bolton et al., 2013). As well, deficits in anxiety-related behaviors and activity (Bolton et al., 2014; 2012) in DEP-exposed offspring compared to offspring that received maternal saline

aspirations were observed. However, these effects are only "unmasked" with the addition of a second immune assault in adulthood.

Many of the effects of early-life pollution on behavior have been traced to neuroinflammation. Pollutant exposure *in vitro* leads to microglial stimulation of pro-inflammatory cytokines – specifically, interleukin-1β (IL-1β) and tumor necrosis factor (TNF, Sama et al., 2007). Though neuroinflammation has not been studied in human development as it pertains to air pollution, children do experience increases in asthma, an inflammatory disorder, due to increased air pollution (reviewed in Patel & Miller, 2009). Consistent with this increase in inflammation, findings in the aforementioned rodent model of early-life pollution has detailed male-specific inflammatory and behavioral alterations (Bolton et al., 2012; 2013; 2014; 2017), and there is a large body of work demonstrating the impact of maternal immune stressors on offspring development (reviewed in Knuesel et al., 2014; Bilbo & Schwarz, 2009). In particular, microglial "priming" is one mechanism by which early life inflammation impacts brain and behavior in adulthood: after an adult immune challenge, microglia overreact to the stimulus, leading to increased neuroinflammation and behavioral deficits (Bilbo, Biedenkapp et al., 2005; Bilbo, Levkoff et al., 2005; Bilbo et al., 2008). The lifelong deficits due to air pollution may be due to microglial priming.

However, the neuroimmune alterations due to early pollutant exposure are not entirely pro- or anti-inflammatory, are sexually dimorphic, and differ based on brain

region and age. At embryonic day 18 (E18), whole-brain expression of three cytokines robustly increases after chronic prenatal DEP exposure; of those cytokines, two [IL-1β and interleukin-6 (IL-6)] are pro-inflammatory, and one [interleukin-10 (IL-10)] is anti-inflammatory (Bolton et al., 2012). In addition to this overall increase in cytokine production, one example of sexual dimorphism in the immune reaction to DEP is a decrease in IL-10 in E18 male brains, and an increase in IL-10 in females (Bolton et al., 2013). Finally, DEP increased male microglial antigen density after a second immune assault in adulthood, but only in the hypothalamus, amygdala, dentate gyrus, and CA1 regions of the hippocampus, not in the CA3 region (Bolton et al., 2012). In a different early-life immune activation model using the viral mimic poly(I:C), cytokine receptor expression in the cortex fluctuated widely throughout development, indicating that the neuroinflammatory profile taken at one timepoint, may not show the "whole picture" (Estes et al., 2018). Though an overall increase in cytokine production was seen at E18 (Bolton et al., 2012), more work in the maternal DEP model to characterize the inflammatory profile is required. Additionally, because one way in which pollution may be harmful is via neuroinflammation, mitigating this inflammation in a way that is easily accessible may lead to immediate therapies for those living in highly polluted areas.

2.1.2 Prenatal choline supplementation

Prenatal choline supplementation is neuroprotective throughout the lifespan. In a model of autism, prenatal choline supplementation reduced anxiety-like behaviors of adult offspring (Langley et al., 2015). Prenatal choline supplementation also mitigates hallmarks of Alzheimer's disease in aged offspring (Mellott et al., 2017) and the adult hippocampal memory deficits characteristic of Down syndrome (Velazquez et al., 2013; Moon et al., 2010). Prenatal choline supplementation also prevents age-related memory decline (Meck et al., 2008) and the decrease of hippocampal neurogenesis in advanced age (Glenn et al., 2008), indicating a lifelong effect and early-life "programming." Prenatal choline supplementation is particularly effective in preventing adult neuronal and behavioral alterations due to seizure induction, such as cell loss (Wong-Goodrich et al., 2008), loss of neurogenesis (Wong-Goodrich et al., 2010), and impaired hippocampal learning and memory (Wong-Goodrich et al., 2010; Holmes et al., 2002).

Critically, every model of cholinergic neuroprotection mentioned above has a pervasive inflammatory component. Children with autism exhibit higher plasma levels of inflammatory cytokines such as IL-1β and IL-6, and heightened levels of these cytokines are highly correlated with the severity of autistic symptoms (Ashwood et al., 2011). "The inflammatory hypothesis" of Alzheimer's disease is one with a large body of support (reviewed in Zotova et al., 2010). Peripheral blood mononuclear cells (PBMCs) of children with Down syndrome express a wide range of inflammatory genes

differently than children without Down syndrome (Zampieri et al., 2014). Aging increases systemic (Weaver et al., 2002) and central (Prechel et al., 1996; Ye & Johnson, 1999; 2001) IL-6. Seizures lead to increased glial pro-inflammatory cytokine production, which in turn exacerbates the damage caused by the seizure (Librizzi et al., 2012). Prenatal dietary choline supplementation blunts the adult upregulation of astrocytic activation due to seizure (Wong-Goodrich et al., 2008) and Alzheimer's disease (Mellott et al., 2017), providing evidence that one mechanism by which prenatal choline supplementation is likely neuroprotective in all of these models is via the immune system.

Dietary choline in adulthood is anti-inflammatory. People with high-choline diets had fewer circulating pro-inflammatory cytokines such as IL-6 (Detopoulou et al., 2008). As well, it is protective in an inflammatory disorder: dietary choline supplementation in adults with asthma reduced cytokine levels, as well as the use of asthma drugs, indicating a decrease in inflammation and in symptoms (Mehta et al., 2010). Dietary choline also prevented stimulated TNF and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) from macrophages (Rowley et al., 2010; Parrish et al., 2006). As well, *in vivo*, choline blunted serum TNF after an immune challenge (Parrish et al., 2006).

Because choline is anti-inflammatory, prenatal choline supplementation may be able to blunt the immune dysregulation due to pollutant exposure early in life. To date,

few studies have analyzed prenatal choline supplementation with a neuroimmune lens. In humans, circulating choline levels in pregnant women who experienced a recent infection was correlated with offspring protection in neurodevelopmental tests such as self-regulation and cerebral inhibition (Freedman et al., 2019), indicating that in humans, dietary choline prevents the behavioral effects of maternal immune activation. A study in a rodent model of maternal immune activation showed a protection in IL-6 expression in the fetal brain after a maternal immune assault (Wu et al., 2015). This landmark study provided evidence that the fetal immune system is impacted by maternal immune activation and maternal diet – neither of which were experienced directly by the fetus. Critically, dietary prenatal choline also normalized increases in adult autism-like behaviors due to maternal immune activation. Because of this work, we hypothesized that prenatal choline supplementation would similarly blunt the immune dysregulation in fetal brains caused by prenatal diesel air pollution exposure in mice.

2.2 Methods

2.2.1 Mice

Adult male and female C57BL/6 mice were obtained from Charles River Laboratories (Raleigh, NC, USA). Mice were time-mated using harem breeding of two females and a male. Upon confirmation of pregnancy with the visualization of vaginal plug (considered embryonic day 0, E0), females were pair-housed and given *ad libitum* access to water and the assigned diet. Specialized bedding (Alpha-Dri; Shepherd

Specialty Papers, Milford, NJ, USA) was used to minimize risk of external contaminants. The colony room within the vivarium was on a reversed 12 hour dark-light cycle (lights off at 9am). These experiments were conducted with the approval of the Duke University Animal Care and Use Committee.

2.2.2 Prenatal manipulations

2.2.2.1 Diesel Exhaust Particle (DEP) exposures

Briefly, diesel exhaust particles (DEP) were collected from a 4.8kW direct injection single-cylinder 320 mL displacement Yanmar L70V diesel generator at 3.5 rpm. Using an electrostatic precipitator, diesel exhaust particles were collected from diesel fuel. On the mornings of embryonic days 2, 5, 8, 12, and 16, dams were anesthetized with 2% isoflurane and exposed to diesel particles suspended in saline via oropharyngeal aspiration as previously described (Auten et al., 2012). Anesthetizes mice were suspended by their frontal incisors. Dams were exposed to either 50 μg of DEP dissolved in 50 μL of saline vehicle (phosphate-buffered saline [PBS] + 0.05% Tween 20), or vehicle alone. Using a 200 μL micropipette, DEP or saline solution was pipetted into the oropharynx by holding the tongue with forceps. Mice recovered from anesthesia under careful supervision. This method causes maternal lung inflammation similar to that seen after exposure to DEP via an inhalation chamber (Auten et al., 2012). This method was utilized over an inhalation chamber to administer the same amount of DEP

in each installation and in each mouse; it is difficult to control diesel inhalation of mice in a chamber.

2.2.2.2 Choline supplementation

At confirmation of pregnancy, dams were assigned to one of two diet conditions *ad libitum*: a synthetic control chow (1.1 g/kg choline chloride in formula AIN-76A with choline chloride substituted for choline bitartrate, DYET #110098, Dyets, Inc., Bethlehem, PA, USA), or the same diet with 4.95 g/kg choline chloride (DYET #110210).

2.2.3 Tissue collection

On embryonic day 18 (E18), dams were anesthetized with ketamine/xylazine (430 mg/kg ketamine; 65 mg/kg xylazine intraperitoneally, i.p.). Hysterotomy was performed to extract the fetuses, which were immediately placed on ice.

Fetuses were numbered and dissected in the order of their presence in the uterine horn from right to left. Whole heads were fixed in 4% paraformaldehyde.

2.2.4 Fetal genotyping

To confirm the sex of each fetus, tails were genotyped for the *Sry* gene. This was done by DNA extraction with phenol, chloroform, and proteinase K (Kouduka et al., 2007; Shinomiya, 1999). After DNA was obtained, the gene products of *Sry* were assessed by using polymerase chain reaction (PCR) and subsequent gel electrophoresis (Koopman et al., 1991). The primers used (Integrated DNA Technologies, Coralville, IA,

USA) were as follows: forward (5′ – 3′): TGGGCTGGACTAGGGAGGTCC; reverse (3′ – 5′): TGCTGGGCCAACTTGTGCCT.

2.2.5 Fetal immunohistochemical analysis

2.2.5.1 Iba1 immunohistochemistry

Fetal heads were cryoprotected in 30% sucrose after 48 hours in 4% paraformaldehyde, then gelatin-blocked. They were sliced using a cryostat at 14 μm and slices were thaw mounted directly onto slides in a series of 5. Briefly, slides that included the region of interest were washed with PBS before quenching with a solution of 50% methanol (VWR, Radnor, PA, USA) and 3% hydrogen peroxide (VWR) for 30 minutes. Slides were washed again in PBS and blocked in a solution of 5mL 0.01M PBS, 150μL normal goat serum (ThermoFisher Scientific, Waltham, MA, USA) and 50μL Triton-X 100 (VWR) at 20°C for one hour and rinsed again in PBS. Slides were incubated at room temperature in a 1:500 concentration of rabbit anti-Iba1 (ionized calcium binding adaptor molecule 1) primary antibody (Fujifilm Wako Chemicals, Richmond, VA, USA). The next day, sections were washed in PBS and incubated for two hours in secondary antibody of goat-anti-rabbit (Vector Laboratories, Burlingame, CA, USA) at a 1:200 concentration. Slides were washed and incubated in "Ready to use" Avidin/biotin complex (R.T.U. ABC, Vector). Sections were washed again and incubated in diaminobenzidine (DAB, SigmaFast 3,3′-Diaminobenzidine, Sigma-Adrich, St. Louis,

MO, USA) until desired color was attained. Sections were washed again in PBS before they were mounted onto slides, dehydrated, and coverslipped.

2.2.5.2 Unbiased stereology

Iba1+ cells were exhaustively counted using the optical fractionator method using StereoInvestigator software (Microbrightfield Inc., Williston, VT, USA). We set an optical dissector height of 7 μm, used a 50 μm x 50 μm counting frame, and counted cells using an 100x objective. Cells were only counted if the entire cell body was uniformly stained and had a minimum diameter of 13 μm (Kongsui et al., 2014). All brain regions analyzed were chosen because of previous research indicating an adult or fetal microglial hyperactivity in each area (Bolton et al., 2012; 2017). For each fetus, we analyzed the dentate gyrus (DG, Bolton et al., 2012; 2017), paraventricular nucleus of the hypothalamus (PVN, Bolton et al., 2012), basolateral amygdala (AMY, Bolton et al., 2012), and parietal cortex (PCX, Bolton et al., 2017). Five brain slices (including both hemispheres of the brain) per animal were quantified for DG, PVN, and PCX analyses, and 3 per animal for AMY analyses. Both sexes were quantified for all brain regions except for PCX, in which only males were quantified due to significant male microglial alterations in the other brain areas (Bolton et al., 2017). Iba1+ cells were classified into one of 4 morphological states based on cell shape, process thickness, and process number (Schwarz et al., 2012): round/amoeboid, stout processes, thick long processes, and thin long processes (Figure 4A). The first two activation states were pooled together

to form "activated" microglia. Because a vast majority of microglia are in the activated stages in the embryonic rodent brain (Schwarz et al., 2012), raw numbers of microglia were analyzed instead of the percent of activated microglia. Total number of microglia was also quantified.

2.2.6 Statistical analysis

Data were analyzed using repeated measures, one- or two-way ANOVA and Tukey HSD, when appropriate, with JMP Pro version 12.2 (SAS Institute, Cary, NC). All graphs were generated using Prism version 8.3.0 (GraphPad, San Diego, CA, USA) using XY (body weight) and grouped (all other metrics) table formats.

2.3 Results

2.3.1 Maternal weight and food consumption

There were no treatment differences in maternal weight gain through pregnancy in a repeated measures ANOVA due to diet ($F_{1,18} = 0.20$, $p > 0.05$, Figure 2) or diesel ($F_{1,18} = 0.41$, $p > 0.05$). There were no significant effects on maternal weight gain due to an interaction between diet and diesel ($F_{1,16} = 0.09$, $p > 0.05$).

There were also no group differences in food consumption analyzed by a two-way ANOVA due to diet ($F_{1,62} = 0.05$, $p > 0.05$, Figure 3) or diesel ($F_{1,62} = 0.66$, $p > 0.05$). There was no interaction of diet and diesel ($F_{1,62} = 1.72$, $p > 0.05$) on maternal food consumption.

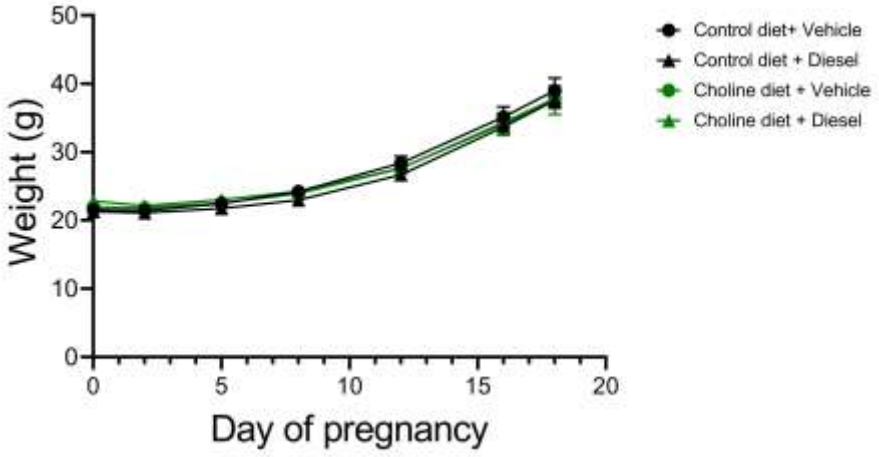


Figure 2: No differences in maternal weight gain due to choline supplementation or diesel exposure were observed. N = 4-7 pregnancies per group. Each value represents the mean ± SEM.

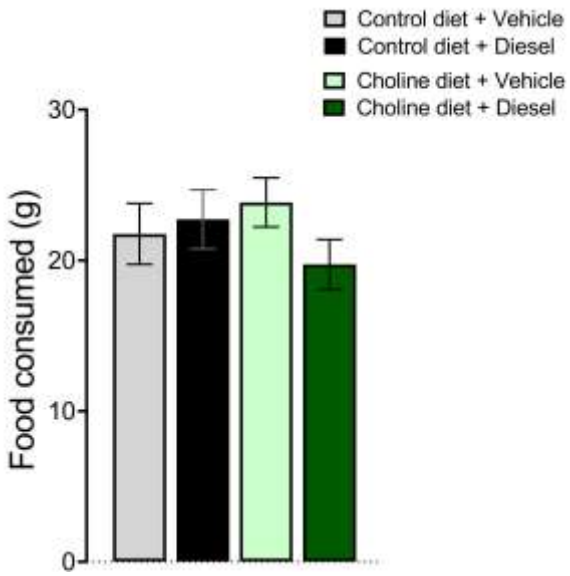


Figure 3: No differences in food consumption due to choline supplementation or diesel exposure were observed. N = 4-7 pregnancies per group. Each value represents the mean ± SEM.

2.3.2 Dentate gyrus

2.3.2.1 Activated microglia

Microglial activation was analyzed using immunohistochemical staining for Iba1 after tissue collection. To examine whether maternal choline supplementation mitigates hippocampal neuroinflammation from maternal diesel exposure, we assess the total number of "activated" ("round" +"stout") microglia by exhaustively counting Iba1+ cells in the DG. Data is expressed as the number of activated microglia in the entire DG. One-way ANOVA was utilized to assess the presence of any sex differences, and separate two-way ANOVAs were utilized to analyze males and females.

Analyses revealed a sex difference using a one-way ANOVA, indicating that males have more activated microglia overall at this age ($F_{1,35} = 4.46$, $p < 0.05$). In the male fetal DG, no significant effects of diet were observed ($F_{1,17} = 2.22$, $p > 0.05$, Figure 4). However, a diesel exposure significantly upregulated the number of activated microglia in the male DG ($F_{1,17} = 9.76$, $p < 0.01$). As well, a significant interaction between diet and diesel was observed ($F_{1,17} = 7.09$, $p < 0.05$). Tukey HSD comparisons revealed a significant increase in activated microglia in males given the control diet + diesel exposure compared to both saline-vehicle treated groups ($p < 0.05$). Mice given prenatal choline supplementation and diesel exposure did not differ in the number of activated microglia from other treatment groups, indicating a partial neuroprotection in the fetal DG. Diesel exposure significantly increased the number of activated microglia in the male DG, and this effect was partially mitigated by prenatal dietary choline supplementation. Overall,

prenatal choline supplementation protected the male fetal dentate gyrus from the increase in microglial activation due to maternal diesel exposure, as supported by several findings.

In females, no significant effects due to diet ($F_{1,18} = 0.29$, $p > 0.05$) or diesel ($F_{1,18} = 0.74$, $p > 0.05$) were observed. As well, no significant interaction of diet and diesel was observed ($F_{1,18} = 0.10$, $p > 0.05$). No effects of diet or diesel on activated microglia were observed in females, unlike the robust diesel effects in the male DG.

2.3.2.2 Total microglia

To assess the effect of choline supplementation and diesel on the total number of microglia, all three observed microglial morphologies ("round" + "stout" + "thick") were pooled. No "thin" cells were observed. No significant sex difference was found in a one-way ANOVA ($F_{1,35} = 2.99$, $p > 0.05$). In males, no main effect of diet was observed ($F_{1,17} = 1.48$, $p > 0.05$, Figure 5). However, likely because the majority of the cells counted were "activated," the number of total microglia exhibited the same pattern of significance. A main effect of diesel was observed ($F_{1,17} = 7.17$, $p < 0.05$) – diesel upregulated the number of total microglia in the male fetal DG. As well, a significant interaction was observed ($F_{1,17} = 7.48$, $p < 0.05$) between diet and diesel. Diesel upregulated the total number of microglia in mice given prenatal control diet. However, neither choline-supplemented group differed from the control diet groups, indicating that choline supplementation blunts the increase in microglial number.

In females, no significant effects due to diet ($F_{1,18} = 0.002$, $p > 0.05$) or diesel ($F_{1,18} = 2.18$, $p > 0.05$) were observed. As well, no interaction between diet and diesel affected the total number of microglia in the DG of females ($F_{1,18} = 0.0004$, $p > 0.05$). Again, in stark contrast to the diesel effects in males, females exhibit no microglial changes in the DG due to diet or diesel.

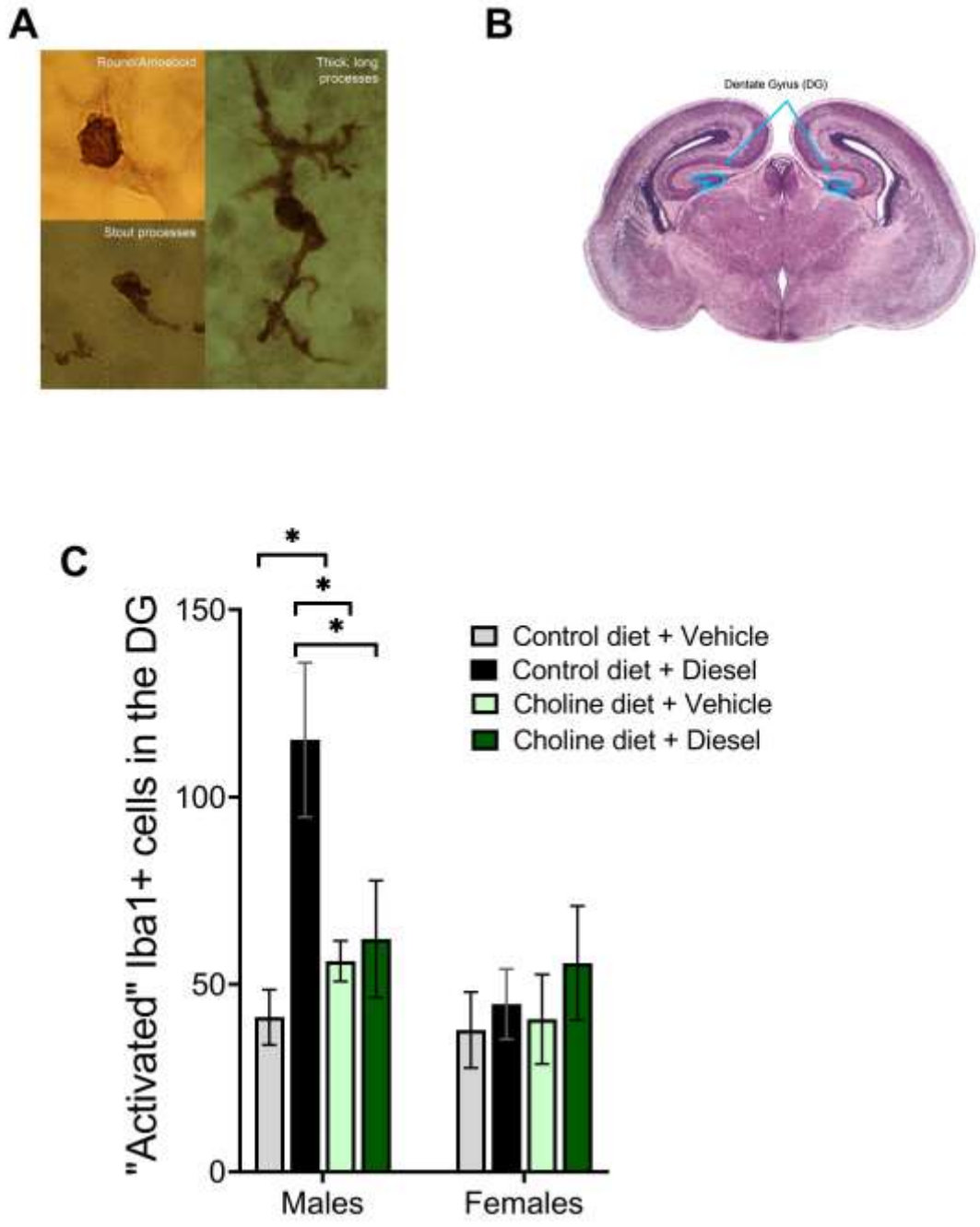


Figure 4: Choline diet and diesel inhalation effects on microglial activation in the dentate gyrus of the E18 mouse brain. (A) Representative images of microglial activation states in the fetal brain. (B) Diagram of counting area. Atlas image from Schambra, 2008. (C) Histogram showing the number of "round" + "stout" Iba1+ cells

in the dentate gyrus. N = 3-9 per group. * $p < 0.05$, Tukey HSD. Each value represents the mean ± SEM.

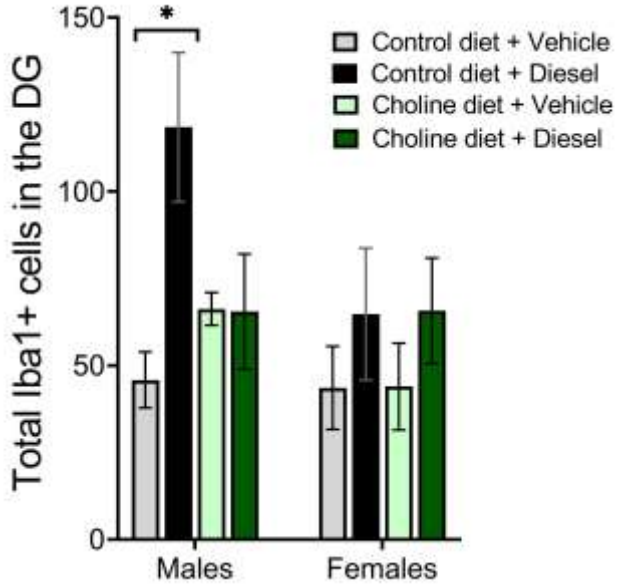


Figure 5: Choline diet and diesel inhalation effects on total microglial number in the dentate gyrus of the E18 mouse brain. Histogram showing the number of Iba1+ cells in the dentate gyrus. N = 3-9 per group. * $p < 0.05$, Tukey HSD. Each value represents the mean ± SEM.

2.3.3 Paraventricular nucleus of the hypothalamus

2.3.3.1 Activated microglia

Due to previous findings indicating that the hypothalamus is "programmed" by maternal DEP to overreact in adulthood (Bolton et al., 2012), the PVN of the hypothalamus was assessed. To answer whether prenatal dietary choline supplementation can mitigate the alterations in fetal microglia due to maternal diesel exposure, microglial morphology was exhaustively quantified. In the PVN, diesel affected microglia differently than in the DG: instead of an upregulation of activated

microglia, a decrease in activated microglia was observed in males. A significant sex difference in activated microglia was found in a one-way ANOVA, indicating that males have more activated microglia overall at this age ($F_{1,26} = 4.93$, $p < 0.05$, Figure 6B). In males, though no effects were found due to diet ($F_{1,12} = 0.02$, $p > 0.05$) or diesel ($F_{1,12} = 3.11$, $p > 0.05$), a significant interaction was observed ($F_{1,12} = 8.29$, $p < 0.05$). Diesel significantly decreased the number of activated microglia in the male fetal PVN in mice given a prenatal control diet. Neither choline-supplemented group differed from the other groups, indicating a partial neuroprotection. Choline supplementation partially prevented the decrease in activated microglia due to diesel exposure.

Activated microglia number did not vary due to any manipulation in the fetal female PVN: neither diet ($F_{1,12} = 0.41$, $p > 0.05$), nor diesel ($F_{1,12} = 0.35$, $p > 0.05$), nor an interaction between diet and diesel ($F_{1,12} = 0.03$, $p > 0.05$) yielded significant differences in microglial activation. Similarly to the DG, only males were affected by diesel. However, diesel exposure increased microglial activation in the DG, but decreased microglial activation in the PVN of the fetal male brain. Choline-supplemented diet led to no differences in microglial activation compared to either control diet group, indicating a partial neuroprotection from aberrant microglial function due to maternal DEP.

In contrast to findings of activated microglia in the dentate gyrus, in the paraventricular nucleus, diesel exposure in mice on the control diet decreased the number of activated microglia compared to saline-treated males. This indicates a

decrease in hypothalamic neuroinflammation. Again, choline supplementation mitigated the alteration in microglial activation caused by diesel exposure.

2.3.3.2 Total microglia

In total microglia of the male PVN, effects mirrored those seen in the number of activated microglia, likely because a high number of total microglia were activated in morphology. Though no differences due to diet ($F_{1,12}$ = ..07, $p > 0.05$, Figure 7) or diesel ($F_{1,12} = 2.38$, $p > 0.05$) were observed, there was an interaction of diet X diesel ($F_{1,12} = 6.91$, $p < 0.05$). A decrease in the total number of microglia due to diesel was observed, and dietary choline supplementation partially mitigated these decreases.

In females, no effects due to diet ($F_{1,12} = 0.23$, $p > 0.05$) nor due to diesel ($F_{1,12} = 0.39$, $p > 0.05$) were observed in the total number of microglia. No interaction effects were observed ($F_{1,12} = 0.10$, $p > 0.05$). Female microglial number in the fetal PVN is unaffected by diet and diesel.

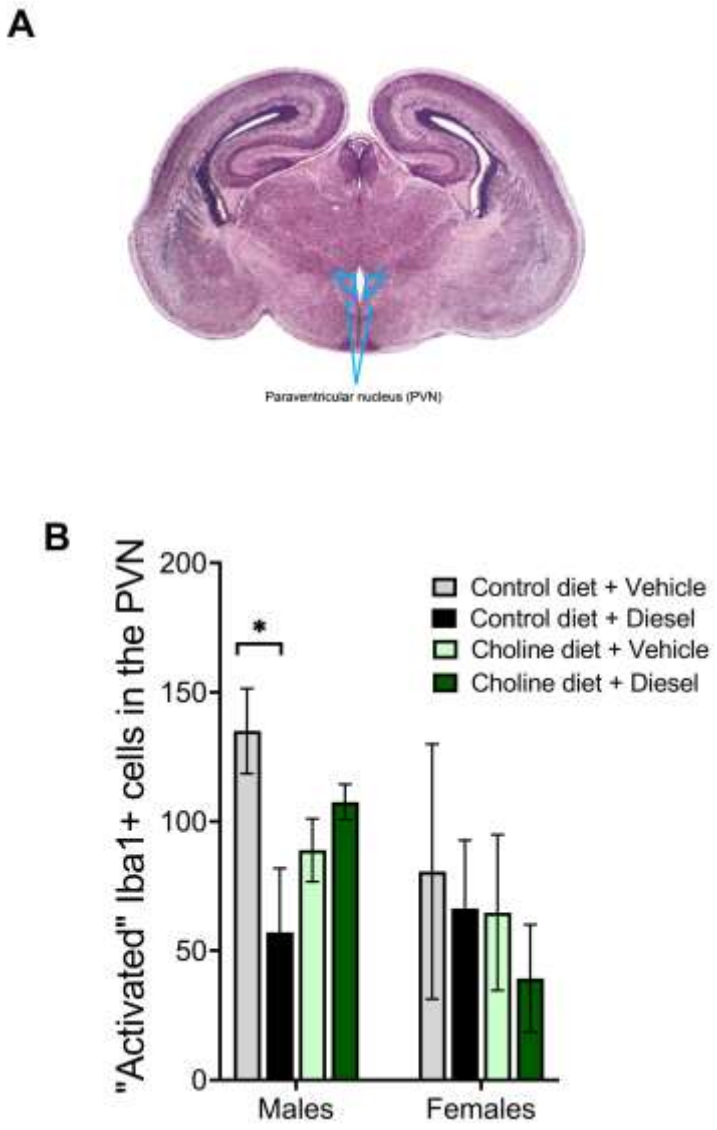


Figure 6: Choline diet and diesel inhalation effects on microglial activation in the paraventricular nucleus (PVN) of the hypothalamus in the E18 mouse brain. (A) Diagram of counting area. Atlas image from Schambra, 2008. (B) Histogram showing the number of "round" + "stout" Iba1+ cells in the PVN of the hypothalamus in E18 mouse brains. N = 3-4 per group. * $p < 0.05$, Tukey HSD. Each value represents the mean ± SEM.

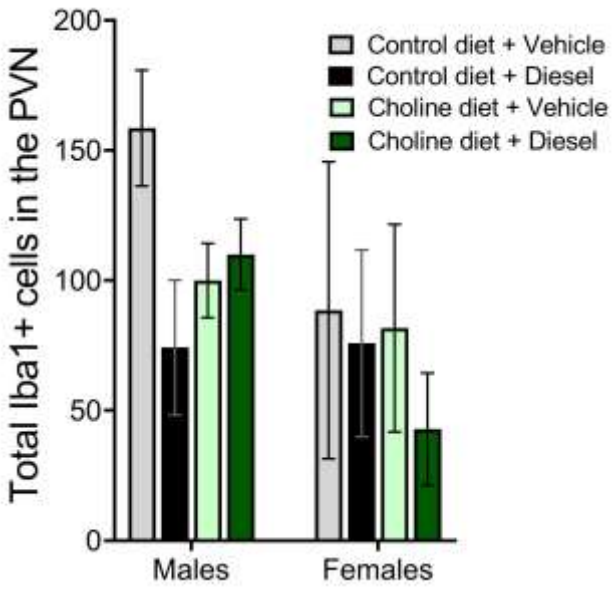


Figure 7: Choline diet and diesel inhalation effects on total microglial number in the PVN of the E18 mouse brain. Histogram showing the number of Iba1+ cells in the PVN. N = 3-4 per group. Each value represents the mean ± SEM.

2.3.4 Amygdala

2.3.4.1 Activated microglia

Unlike the DG and PVN, no significant differences were found in activated microglia. No significant sex difference in the percent of activated microglia was apparent in the amygdala (one-way ANOVA, $F_{1,26} = 1.31$, $p > 0.05$, Figure 8B). No effects due to diet ($F_{1,14} = 0.32$, $p > 0.05$) or diesel were observed in the male fetal amygdala ($F_{1,14} = 1.56$, $p > 0.05$). No interaction between diet and diesel was observed ($F_{1,14} = 2.70$, $p > 0.05$). Unlike in the DG and PVN, no effects of choline or diesel were found in the male amygdala.

In female fetuses no difference in microglial activation due to diet ($F_{1,12} = 1.84$, $p > 0.05$) was observed. No difference in female microglial activation due to diesel ($F_{1,12} = 0.25$, $p > 0.05$) was observed. No significant interaction of diet and diesel was observed in the female amygdala at this time point ($F_{1,12} = 0.19$, $p > 0.05$). Female microglial morphology was not affected by diet or diesel in the amygdala.

2.3.4.2 Total microglia

In total microglia, no significant sex difference was found in a one-way ANOVA ($F_{1,26} = 2.48$, $p > 0.05$, Figure 9). In male fetuses, two-way ANOVA analyses revealed no significant differences in microglial number due to prenatal diet ($F_{1,14} = 0.03$, $p > 0.05$), or diesel ($F_{1,14} = 1.98$, $p > 0.05$). There was a marginal interaction between diet and diesel ($F_{1,14} = 4.15$, $p = 0.07$). Tukey HSD posthoc comparisons showed no significant differences in the interaction between diet and diesel. Prenatal choline supplementation and diesel exposure combined slightly decreased the total number of microglia in the fetal male amygdala.

In females, no significant differences were found due to diet ($F_{1,12} = 0.27$, $p > 0.05$) or diesel ($F_{1,12} = 0.01$, $p > 0.05$). No significant interaction of diet and diesel was observed ($F_{1,12} = 0.36$, $p > 0.05$). This lack of difference is in contrast to the DG and PVN, in which diesel effects were mitigated by maternal choline supplementation. Though differences in microglial activation were observed in males, no differences in the total microglial number were observed in males or females.

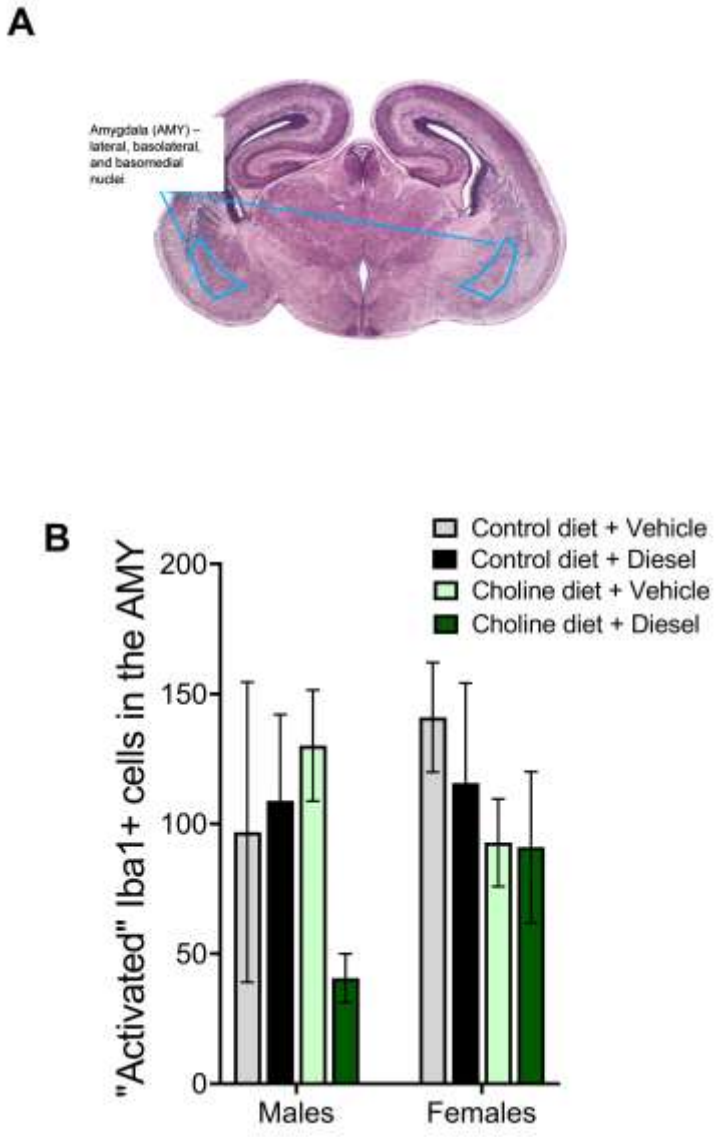


Figure 8: Choline diet and diesel inhalation effects on microglial activation in the amygdala of the E18 mouse brain. (A) Diagram of counting area. Atlas image from Schambra, 2008. (B) Histogram showing the number of "round" + "stout" Iba1+ cells in the amygdala in E18 mouse brains. N = 3-5 per group. Each value represents the

mean ± SEM.

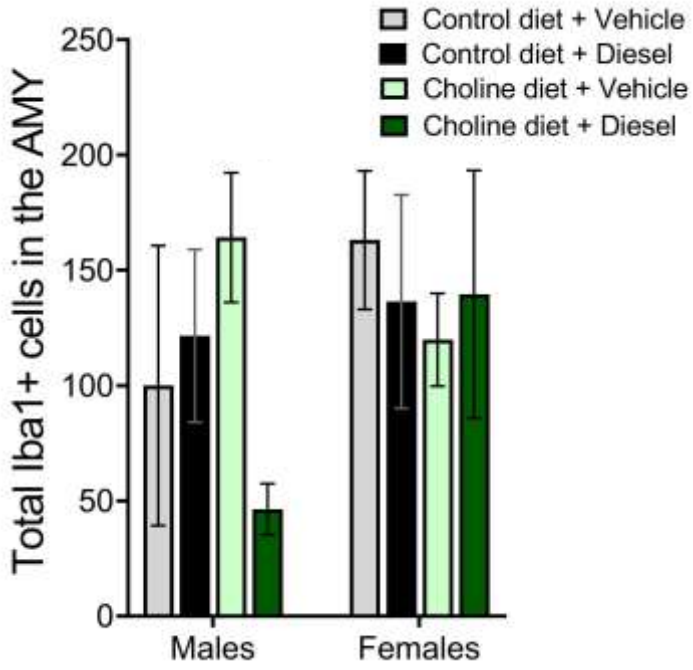


Figure 9: Choline diet and diesel inhalation effects on total microglial number in the amygdala of the E18 mouse brain. Histogram showing the number of Iba1+ cells in the amygdala. N = 3-5 per group. Each value represents the mean ± SEM.

2.3.5 Parietal cortex

2.3.5.1 Activated microglia

In quantifying the microglial activation state of this area, only males were analyzed because of previous work showing slight increases in activated microglia caused by diesel exposure in male fetuses but not female fetuses in the maternal DEP model (Bolton et al., 2017). However, in a two-way ANOVA, no significant difference due to diet was observed ($F_{1, 19} = 1.37$, $p > 0.05$, Figure 10B). No difference in activated microglia due to diesel was observed ($F_{1, 19} = 0.17$, $p > 0.05$). No significant interaction between diet and diesel was observed in the activation of microglia in the male PCX ($F_{1,}$

$_{19}$ = 0.27, $p > 0.05$). No significant differences due to diet or diesel in microglial activation were observed in the PCX, contrary to previous findings (Bolton et al., 2017). Despite the role of the PCX in episodic memory retrieval (Tayler et al., 2013), no increases in PCX microglial activation were observed due to maternal diesel exposure. However, significant upregulation in microglial activation was observed in the DG. For this reason, the "programming" effects of maternal diesel exposure on memory in adulthood are likely due to DG alterations.

2.3.5.2 Total microglia

In total microglia, no differences due to diet ($F_{1,19}$= 2.58, $p > 0.05$, Figure 11) or diesel ($F_{1,19}$= 2.26, $p > 0.05$). As well, no significant interaction between diet and diesel was observed ($F_{1,19}$= 0.15, $p > 0.05$). In both microglial measures, though no significant differences were observed, a pattern of differences emerged similar to those in the PVN. Diesel-exposed mice exhibited a decrease in microglial activation and number, but choline-supplemented mice were not affected by diesel exposure. This suggests that choline normalizes the small differences in microglia due to diesel exposure.

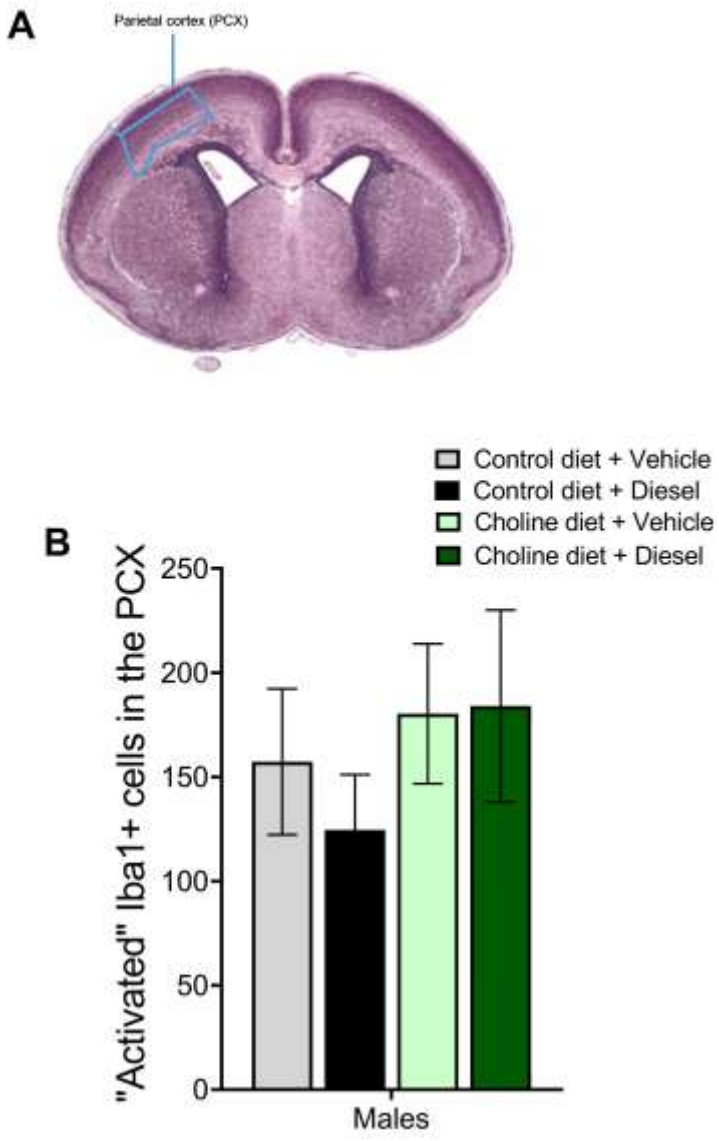


Figure 10: Choline diet and diesel inhalation effects on microglial activation in the parietal cortex of the E18 male mouse brain. (A) Diagram of counting area. Atlas image from Schambra, 2008. (B) Histogram showing the number of "round" + "stout" Iba1+ cells in the parietal cortex in E18 male mouse brains. N = 4-5 per group. Each value represents the mean ± SEM.

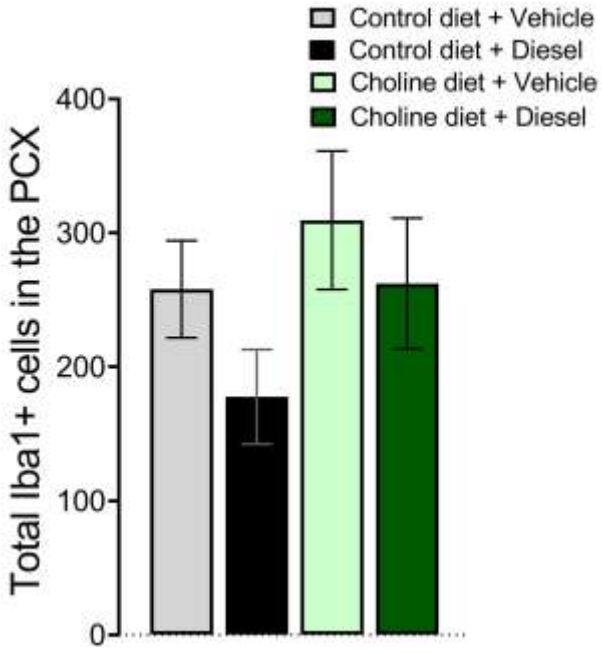


Figure 11: Choline diet and diesel inhalation effects on total microglial number in the PCX of the E18 mouse brain. Histogram showing the number of Iba1+ cells in the PCX. N = 4-5 per group. Each value represents the mean ± SEM.

2.4 Discussion

This work shows that, in the male fetal dentate gyrus, microglial activation was increased after chronic diesel exposure. This increase was partially mitigated by maternal choline supplementation. This pattern was altered in the PVN of the hypothalamus and the PCX: diesel led to a decrease in microglial activation, which was rescued by choline supplementation. Neither diesel nor choline altered microglia in the amygdala. Differences of this kind were only found in male fetuses, and not in other brain regions. Females were not affected by either diesel or choline in any brain region analyzed.

2.4.1 Sex difference

Maternal immune activation only leads to fetal immune activation in male mice; microglia of female mice do not appear to be altered by diesel or choline supplementation. Other research supports the view that male fetuses and not female fetuses are susceptible to immune activation caused by maternal DEP (Bolton et al., 2013; 2012; Ehsanifar et al., 2019). This male susceptibility to developmental delays due to maternal exposure to air pollution has also been shown in humans (Sears et al., 2019). Further supporting the hypothesis that males are impacted worse than females in models of early immune activation, early-life diesel exposure has been linked to male-specific increases in autism diagnoses in humans (Raz et al., 2018). Male and female fetal brains exhibit different inflammatory reactions to diesel exposure; specifically, male brains produce more IL-10 protein, but female brains exhibit a decrease in IL-10 protein (Bolton et al., 2013). This point is important because female brains are not necessarily resilient to DEP and maternal choline supplementation – they may be reacting differently than male brains, and these changes may not be detected by assessing microglial morphology. At baseline, neonatal male rats have more activated microglia in the DG, AMY, and PCX, which could possibly contribute to the heightened male susceptibility to inflammatory programming (Schwarz et al., 2012). Additionally, male microglia at E18 and onward have delayed maturational timelines than females (Hanamsagar et al., 2017), and immune activation leads to an acceleration in the

development of these microglia. However, female microglial maturation is not affected. These findings suggest that altered development of male microglia early in life could lead to a compensatory microglial aging in males, which could affect adult brain and behavior (Hanamsagar et al., 2017). In addition to the age- and region-dependent differences in microglial activation (Bolton et al., 2017; 2012), sex is another variable that affects the fetal brain response to maternal diesel exposure. Partly because of these male-specific differences, future experiments in the current dissertation work focused on males.

Previous research on early-life choline supplementation and hippocampal-dependent memory has largely focused on male offspring (Holmes et al., 2002; Meck & Williams, 1988; 1999; Meck et al., 1989; Wong-Goodrich et al., 2008; 2010; Yang et al., 2000). However, there is also evidence that early-life choline supplementation can enhance female spatial memory capability in adulthood (Meck & Williams, 1997). The current work found no effects of prenatal choline supplementation on microglia in females. It is possible that, though there are no neuroimmune protections in females at this age, they may be affected by cholinergic microglial impacts at a later time point.

The current study analyzed fetal microglial activation after prenatal choline supplementation. Though there is much evidence that prenatal choline supplementation leads to significant effects on hippocampal memory (Holmes et al., 2002; Meck & Williams, 1997; 1999; Meck et al., 1989; Wong-Goodrich et al., 2008; 2010; Yang et al.,

2000), there is also evidence that prenatal and postnatal choline supplementation combined is ideal (Meck & Williams, 1988). Microglia in female brains do not react as dramatically to early-life infection (Bilbo et al., 2006), but females have more microglia than males in adulthood (30-60 days of age, Schwarz et al., 2012) – suggesting that females may be more susceptible to later immune alterations.

Additionally, some groups propose that, similar to their role in male-specific susceptibility to early-life insult, microglia are implicated in the sex difference in later-life neurological disorders such as Alzheimer's disease (Brown et al., 2008; reviewed in Hanamsagar & Bilbo, 2016). Prenatal choline supplementation does prevent Alzheimer's related pathology and microglial activation in the hippocampus, but neither memory nor sex was analyzed (Velazquez, Ferreira, Winslow et al., 2019). Perhaps prenatal choline does affect female microglia, but the protective effects are only seen later in life.

To examine whether female microglial activation is suppressed due to adult administration of dietary choline, the same group administered dietary choline supplementation to female Alzheimer's disease model mice from 2.5 to 10 months of age (Velazquez, Ferreira, Knowles et al., 2019). Females were protected from both the hippocampal plaques characteristic of Alzheimer's disease, and the spatial memory impairments. In addition, in females, lifelong choline diet suppressed the microglial activation characteristic of Alzheimer's disease. Dietary choline supplementation rescued hippocampal inflammation and memory deficits in females when administered

in adulthood, suggesting that females react to the nutrient at a different developmental time point than administered in the present work.

2.4.2 Dentate gyrus

Prenatal DEP increases microglial activation in the DG in males. This finding is in contrast with previous work which found no differences from controls (Bolton et al., 2017). In a model of maternal immune activation (MIA), similarly, previous work shows no differences at this timepoint (Smolders et al., 2015). However, in the latter study, males and females were not analyzed separately, so it is possible that the lack of differences in females washed out any differences.

The difference in microglial activation due to DEP in the fetal hippocampus in the present work may explain hippocampal-dependent behavioral deficits in adulthood. In another model of prenatal DEP, pollution dose-dependently impairs spatial memory in a Morris water maze (MWM) in the absence of a second immune assault in adult male offspring (Ehsanifar et al., 2019). Further, others using MIA models have found effects in adult hippocampal-dependent memory (Schaafsma et al., 2017) specifically in male offspring (Chlodzinska et al., 2011). The differences seen in microglial activation in the fetal hippocampus could possibly have large effects on behavior.

2.4.3 Paraventricular nucleus of the hypothalamus

In the PVN, a different pattern was observed from the DG. In the male fetal PVN, DEP decreases activated microglia. This phenomenon was partially mitigated by prenatal choline supplementation. There are three possible explanations for this pattern.

First, with the exception of the hippocampus, MIA leads to a *decrease* in microglial activation in the adult brain (Schaafsma et al., 2017). Schaafsma and colleagues isolated microglia from the entire hippocampus and found that these microglia secrete more cytokines after maternal exposure to lipopolysaccharide (LPS), an immune activator, and an adult "second hit" of LPS. However, microglia isolated from the whole brain of animals with MIA show a blunted immune response after a second hit. These findings indicate that the hippocampus is an anomaly in MIA models, and that only in the hippocampus are microglia "overactive" due to MIA. The current findings in the PVN and parietal cortex support the hypothesis that maternal immune assaults lead to a nearly brain-wide decrease in microglial activation.

Second, microglia are needed for normal hypothalamic development (Rosin et al., 2018). Without microglia, more apoptosis is seen in the fetal hypothalamus, and this leads to dysregulated body weight in the adult. This finding is consistent with previous work indicating that maternal DEP dysregulates weight and initiates insulin resistance (Bolton et al., 2012; 2014). DEP-exposed offspring ate more of a high-fat diet than vehicle-exposed offspring (Bolton et al., 2012), indicating that the DEP-induced

reduction of microglia in the hypothalamus (seen here) may result in an impairment in satiety.

This hypothalamic dysregulation may also be associated with an altered stress response, which corresponds with the increases in anxiety-related behavior in DEP-exposed offspring (Bolton et al., 2014). The decrease in activated microglia in the PVN of the fetal hypothalamus due to DEP could lead to an altered hypothalamic-pituitary-adrenal (HPA) axis response. However, more work is needed to tie hypothalamic neuroinflammation in fetal life to later HPA activation. Prior research has shown that prenatal DEP leads to more cytokines in the fetal brain, such as IL-1β (Bolton et al., 2012). Critically, peripheral IL-1β activates the HPA axis to initiate a stress response (Besedovsky et al., 1986). The PVN is a critical first step in HPA axis activation (reviewed in Herman & Tasker, 2016). The PVN, specifically corticotropin-releasing hormone (CRH) neurons, has been closely tied to anxiety-related behaviors. For example, lesioning the PVN and optogenetically inhibiting CRH neurons in the PVN reduces freezing in an open field and stress-associated grooming behavior, respectively (Herman et al., 1991; Füzesi et al., 2016). It is possible that prenatal DEP activates the fetal HPA axis, starting with the PVN, via increased cytokine production prior to E18. From there, more glucocorticoids are produced, which have been shown to both be pro- and anti-inflammatory to microglia (reviewed in Tapp et al., 2019). These glucocorticoids could lead to lessened microglial activation in the fetal brain due to DEP

that we see in the current work at E18, and this hypothalamic dysfunction could contribute to altered anxiety phenotypes in DEP-exposed offspring (Bolton et al., 2013).

2.4.4 Parietal cortex

A similar pattern of microglial activation due to diesel and maternal choline supplementation was found in the PCX: a slight decrease in activated microglia due to diesel was observed, and this decrease was mitigated by prenatal choline supplementation. This is consistent with previous work in this brain region in the maternal DEP model at this age (Bolton et al., 2017). This finding is also consistent with previous work showing that prenatal DEP leads to impairments in hippocampal-dependent memory, but not in other types of cognition (Yokota et al., 2015).

2.4.5 Amygdala

In the current work, no differences in activated microglia were observed in the amygdala. In adulthood after prenatal DEP, a second immune assault unmasks an overreaction of microglia in the amygdala (Bolton et al., 2012). So, DEP has some "programming" effect on microglia in this area during fetal development that leads to alterations in adult microglia. However, this "programming" effect may or may not take place at this time point and may not manifest through microglial morphology. The current results do not support an alteration in microglial activation at this timepoint. The lack of a diesel effect in the control-diet groups does not explain the increased proclivity for anxiety-related behaviors in these offspring (Bolton et al., 2013); however, these

behaviors have been linked to other brain regions, such as the hippocampus and hypothalamus (Jimenez et al., 2018). It has been previously suggested that maternal DEP exposure may lead to a delay in microglial maturation in male brains (Bolton et al., 2017), rather than an increase in activation. Perhaps choline supplementation further alters this maturational timeline (i.e., accelerates the maturation of microglia), allowing them to be in a more vulnerable state to react to the DEP exposure, leading to a significant reduction of "activated" microglia with choline and DEP exposure together.

2.4.6 Microglia as immune mediators

Critically, the Iba1+ cells quantified herein are likely microglia and not infiltrating macrophages. Iba1 is a marker for all macrophages; though microglia are the resident macrophages of the brain, Iba1+ may be microglia or infiltrating macrophages. In adult offspring, an increase in expression of a microglia-specific gene (CX3CR1) was expressed in the hippocampus after a second immune assault (Bolton et al., 2014). Though the current body of work did not differentiate between microglia and infiltrating macrophages, the increase in CX3CR1 in adult brains suggests that microglia are primed to change their morphology in response to an immune assault. However, it is also possible that peripheral macrophages are impacted as well by prenatal DEP and choline at this time point. Future studies should aim to differentiate the effects of microglial activation and macrophage infiltration due to DEP and choline at E18.

We found that maternal dietary choline mitigated fetal neuroinflammation. Previous work in a MIA model demonstrated that maternal choline supplementation prevents the increase in IL-6 in the fetal brain caused by the viral mimic poly(I:C); however, the origin of this cytokine was not identified (Wu et al., 2015). A separate study (Pratt et al., 2013), using poly(I:C) to induce maternal immune activation, found that fetal microglia are the source of IL-6. Together, these studies and ours support the view that MIA induces microglial activation and cytokine production, and that this inflammatory response can be blunted in the fetal brain by maternal choline supplementation.

The present findings indicate that microglial activation as a result of DEP varies widely across brain regions. These data are consistent with other findings using DEP to induce maternal and offspring inflammation at E18 (Bolton et al., 2017) and in adult offspring (Bolton et al., 2012). In other models of MIA, cytokine expression has been difficult to generalize due to large age and region differences, as well as different developmental patterns of expression per cytokine (Garay et al., 2014). Hence, this variation in neuroinflammation in a sex- and region-dependent manner is consistent with other findings; how exactly these variations impact development, and how these variations interact with each other, is currently a wide area of investigation.

Maternal choline supplementation partially mitigates the increase in microglial activation due to prenatal DEP. These findings are consistent with those of Wu and

colleagues (2015) in that maternal dietary choline supplementation prevented fetal neuroinflammation due to a maternal inflammatory agent. There is currently no direct data on whether choline supplementation can protect hippocampal-dependent behavioral deficits due to prenatal DEP; however, the current data suggests that prenatal dietary choline supplementation may prime developing microglia such that they do not overreact in adulthood or old age when they confront another immune challenge. If this is the case, prenatal choline supplementation in the adult may lead to less overactivation of microglia following a second immune assault, which impairs memory (Williamson et al., 2011). Because the largest effect due to maternal diesel was found in the DG, this effect was rescued with maternal choline supplementation, and because previous work has found specific impairments in hippocampal-dependent memory (Chlodzinska et al., 2011), work continuing from this aim focuses on the hippocampus.

Taken together, the effect of maternal DEP and prenatal dietary choline supplementation is complex and appears to be both sex- and region-specific. Both diesel exposure and choline are systemic treatments, and do not work only on one tissue or cell type. These variables may cause changes in multiple biological pathways in addition to these neuroimmune changes. As well, the impact of these neuroimmune alterations on future behavior is not yet fully understood (Garay et al., 2013)

We have found that, in the dentate gyrus of the fetal hippocampus, prenatal DEP increases activated microglia. Prenatal choline supplementation partially mitigates this

activation increase. In the PVN of the hypothalamus, diesel exposure led to a decrease in activated microglia, and this decrease was rescued by maternal dietary choline supplementation. Only male fetuses were affected by diesel exposure. Clearly, both maternal inflammatory assaults and maternal diet impact the male fetal brain. Though the mechanism of maternal-fetal immune communication is not fully understood, DEP and choline supplementation have region- and sex-specific effects on microglia in the embryonic brain.

3. Aim 2: Adult choline as an anti-inflammatory and neuroprotective agent in adulthood

3.1 Introduction

3.1.1 Post-operative cognitive dysfunction

Post-operative cognitive dysfunction (POCD), one perioperative neurocognitive disorder (Eckenhoff et al., 2020), is the phenomenon in which increased neuroinflammation due to peripheral surgery leads to impaired cognitive function. POCD affects patients of all ages, but older populations are particularly susceptible (Monk et al., 2008). Though POCD resolves for most of the population, 12.7% of patients age 60 and older still display cognitive impairments 3 months after surgery (Monk et al., 2008). Indeed, other human studies have found cognitive impairments that last for years post-surgery (Abildstrom et al., 2000; Selnes et al., 2008). POCD is a pervasive health problem that is explicitly linked to neuroinflammation.

In a tibial fracture mouse model of POCD, increased neuroinflammation is observed. Specifically, increased infiltrating macrophage number in the hippocampus, increased systemic pro-inflammatory cytokines, and decreased systemic anti-inflammatory cytokines are all observed in the tibial fracture model (Terrando et al., 2011). These neuroinflammatory changes are tied to impairments in hippocampal-dependent memory in a contextual fear response task (Terrando et al., 2011).

3.1.2 Neuroinflammation and hippocampal-dependent memory

Neuroinflammation alone is sufficient to induce hippocampal-dependent memory impairments, indicating that neuroinflammation is likely the mechanism behind short-term POCD (less than a week post-surgery). Intracerebroventricular administration of inflammatory cytokines - specifically, IL-1β genetic overexpression in the hippocampus or intracerebroventricular administration causes deficits in spatial memory in adult mice and rats (respectively: Moore et al., 2009; Oitzl et al., 1993; Pugh et al., 2001). As well, direct hippocampal injection of IL-1β leads to deficits in hippocampal-dependent fear conditioning in rats (Barrientos et al., 2002). Preventing the neuroinflammation associated with POCD also prevents contextual memory impairments that occur 3 days after surgery (Terrando et al., 2011). Neuroinflammation is integral to POCD, and preventing neuroinflammation can mitigate the cognitive impairments that occur following surgery.

3.1.2.1 Cell types involved in POCD

The cell-type specificity of neuroinflammation after peripheral surgery has been disputed. There have been findings implicating astrocytes (Xu et al., 2017), microglia (Feng et al., 2017), infiltrating macrophages (Terrando et al., 2011), and mast cells (Zhang, Dong et al., 2016) as the initiating cellular mechanism behind the inflammatory and behavioral deficits in POCD models. Surely none of these cell types is isolated in its actions; however, the specific cell type primarily involved in POCD is still unclear.

Perhaps because neuroimmune changes in a variation of cell types have been previously shown to initiate POCD, neuronal changes have not been as explicitly researched. Though neuroimmune cells are known to interact with neurons and to lead to neuronal death in POCD (Ge et al., 2015), and though heightened neuroinflammation blunts neurogenesis (Ekdahl et al., 2003), few rodent studies have directly linked POCD with markers of *young* neurons. One such study observed a decrease in a marker for young neurons 24 hours after surgery (Xiong, Zhang et al., 2018). However, further quantification of young neurons at this time point and at later time points is required. **The first question this aim sought to answer was**: What are the effects of peripheral surgery on glial activation and neuronal measures?

3.1.2.2 Post-inflammation POCD: modeling long-term POCD

The inflammatory responses to tibial fracture are transient. One study characterized the time course of the systemic and hippocampal inflammation due to tibial fracture (Cibelli et al., 2010). Plasma levels of interleukin-1β (IL-1β) and interleukin-6 (IL-6) were elevated 6 hours and 24 hours after surgery; however, this increase resolved 72 hours after surgery. This same time course was observed in the hippocampal transcription of these two cytokines. Cytokine expression due to tibial fracture resolves between 1 and 3 days post-surgery. Further, quantification of the percent area stained by an anti-CD11b (cluster of differentiation molecule 11b) antibody to show the activation state of microglia revealed that microglial activation persists both

1 day and 3 days after surgery. However, this activation resolves by 7 days after surgery. Critically, no differences in microglial activation or hippocampal IL-1β were observed if mice were treated with IL-1 receptor antagonist (IL-1Ra) before surgery. For this reason, many studies on the neuroinflammatory mechanisms of POCD have focused no later than 3 days post-surgery (Ni et al., 2018; Terrando et al., 2011; Zhang et al., 2020; Zhu et al., 2016) – if the mechanism of behavioral impairment is solely neuroimmune, no effects would likely be seen after the neuroinflammation disappears.

The second question examined in this aim is: Do the effects of peripheral tibial fracture surgery and dietary choline supplementation extend to 2 weeks post-surgery? Two weeks after tibial fracture, long after the neuroinflammation resolves, can the tibial fracture mouse model reflect the persistent and long-term cognitive impairments seen in humans (Monk et al., 2008)? And if so, what could the possible mechanisms be – could they be due to differences in young neurons? Other work in non-tibial fracture models of POCD has analyzed behavioral impairments long-term as late as one week after surgery in mice (Liang et al., 2018), and 2 weeks after surgery in rats (Hovens et al., 2015). However, to our knowledge, no studies have analyzed POCD in the mouse tibial fracture model 2 weeks after surgery. We sought to quantify any behavioral and neural alterations due to surgery at a relatively late time point compared to previous studies in the mouse tibial fracture model.

3.1.2.3 Dietary choline supplementation as a possible mitigator of POCD

Choline and its derivative acetylcholine (ACh) are critical for learning and memory, specifically in the hippocampus. An acetylcholinesterase inhibitor increases resilience against memory loss (Wang et al., 2019) and increases spatial memory ability in normal rats (Ahsan et al., 2019). Increased cholinergic signaling helps hippocampal-dependent memory by preventing interference from old memories (reviewed in Hasselmo, 2012) by acting on hippocampal neurons. However, one of the goals of the present work is to characterize the contribution of non-neuronal cells in the effects of dietary choline supplementation on hippocampal-dependent memory.

Dietary choline in adulthood has previously been shown to be beneficial for hippocampal neurogenesis and memory. Previous findings have shown that rats given only adult, not prenatal, dietary choline supplementation show more hippocampal neurogenesis than controls (Wong-Goodrich, Glenn et al., 2008). Increased nutritional choline intake and higher free plasma levels have also been associated with enhanced episodic memory, verbal memory, and visual memory in human adults (Nurk et al., 2013; Poly et al., 2011). Dietary choline supplementation is beneficial for the hippocampus, though the precise cellular mechanisms are unknown.

One of the many lesser-known roles of choline (in that it is a specific nAChR agonist) is that it is an anti-inflammatory agent (reviewed in Pavlov et al., 2003; Wang et al., 2003; Terrando et al., 2011). A7 nicotinic ACh receptor (α7 nAChR) stimulation with

selective agonists such as nicotine decreases the lipopolysaccharide (LPS)-induced microglial release of inflammatory cytokines (De Simone et al., 2005), but not in α7 nAChR knockout mice (Pavlov et al., 2009; Wang et al., 2003). Administration of α7 nAChR agonists, including a pharmacological bolus dose of choline, prevents both the inflammatory phenotype, specifically the infiltration of peripheral macrophages, and POCD seen shortly after surgery, indicating that the cholinergic system intricately ties together these two phenomena in adulthood (Terrando et al., 2011; 2014).

The third goal of this aim is: Does dietary choline supplementation mitigate the neuroinflammation and memory deficits due to peripheral surgery? Even though POCD 3 days after surgery can be ameliorated with a pre-surgical bolus dose of choline in a mouse model, no previous research has examined dietary choline supplementation in the tibial fracture mouse model of POCD. Humans are much more likely to encounter choline in their diets than via pharmacological doses; hence, one motivation behind this work is to examine the effect of the equivalent of a "high choline diet" in POCD. Can the anti-inflammatory actions of dietary choline supplementation imitate the effects of a bolus dose (Terrando et al., 2011) and mitigate the short-term inflammation at 1 day post-surgery, at which time neuroimmune activation in the tibial fracture model is at its highest (Cibelli et al., 2010)? And, should any differences in behavior or brain be observed long-term (2 weeks post-surgery), does dietary choline rescue those effects as well?

This work sought to answer these questions: 1. What are the effects of peripheral surgery on glial activation and neuronal measures? 2. Do the effects of peripheral tibial fracture surgery and dietary choline supplementation extend to 2 weeks post-surgery? 3. Does dietary choline supplementation mitigate the neuroinflammation and memory deficits due to peripheral surgery? To answer these questions, dietary choline supplementation (4.95 g/kg choline chloride) was given to adult male mice for three weeks before tibial fracture surgery. Either 1 day or 2 weeks after surgery, novel object recognition testing or sacrifice for histology was carried out. Using immunohistochemistry, the following cell types were quantified in the hippocampus: microglia, infiltrating macrophages, astrocytes, dividing and surviving cells, and young neurons.

3.1 Methods

This study was conducted in two cohorts of mice: the first cohort underwent behavioral testing, and the second cohort was used for histological analysis (Figure 12). For both cohorts, the experimental design was 3 (treatment condition: control diet, control diet + surgery, choline supplemented diet + surgery) x 2 (time point: 1 day after surgery, two weeks after surgery). These time points were chosen because of previous findings that neuroinflammation is high 24 hours after surgery (Cibelli et al., 2010), and that tibial fracture impairs hippocampal-dependent memory at this time point (Xiong, Liu et al., 2018). Additionally, very few studies have examined the longer-term

behavioral, glial, and neuronal changes due to tibial fracture surgery (Hovens et al., 2015), and no work has analyzed this time point in mice. These experiments were conducted with the approval of the Duke University Institutional Animal Care and Use Committee.

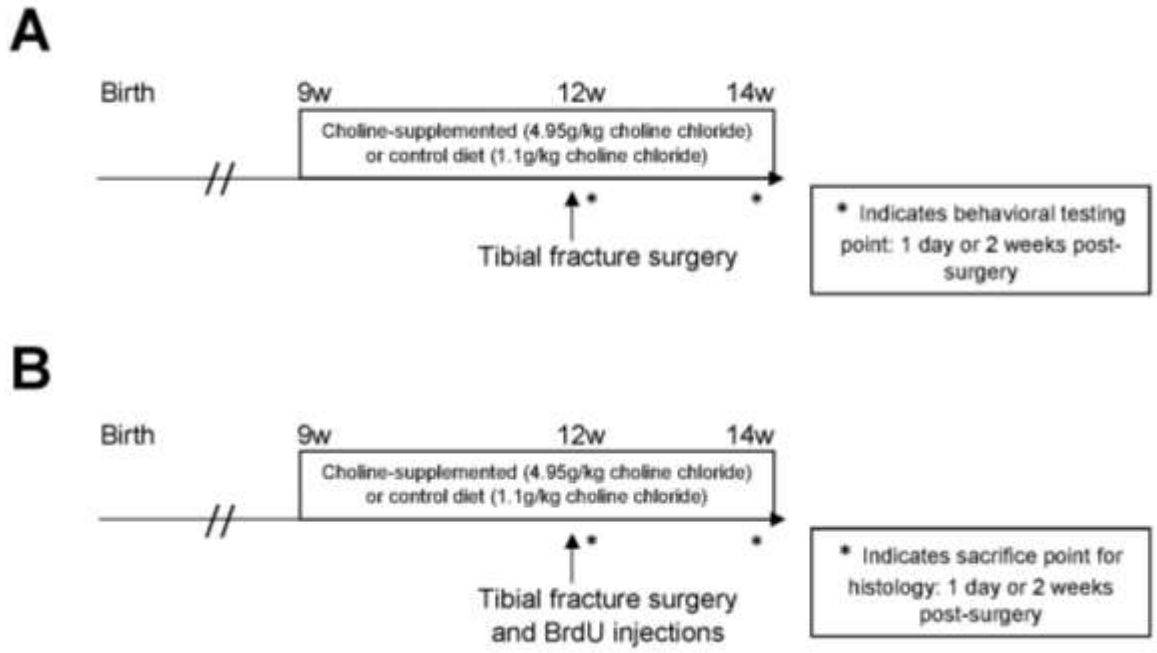


Figure 12: Experimental timelines. (A) The effect of choline and tibial fracture surgery on hippocampal-dependent behavior. Nine-week-old C57/Bl6J male mice were treated with a choline-supplemented diet or a synthetic control diet for 3 weeks before a tibial fracture surgery. All mice underwent behavioral testing (NOR) one day before surgery. Mice received another two weeks of choline-supplemented or control diet after surgery. After these two weeks, they underwent the same behavioral testing. (B) The effect of choline and tibial fracture on histological measures. Nine-week-old C57/BL6J male mice were treated with a choline-supplemented diet or a synthetic control diet for 3 weeks before a tibial fracture surgery and subsequent BrdU injections. The first group of mice was euthanized one day after surgery to analyze microglial activation, macrophage infiltration, astrocyte activation, cell proliferation, and young neurons. The second group received another two weeks of choline-

supplemented or control diet after surgery. After these two weeks, they were euthanized to measure microglial activation, macrophage infiltration, astrocyte activation, cell survival, and young neurons.

3.1.1 Mice

Nine-week old male C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, USA) were bred, raised, and housed in the Duke University vivarium. They were housed in groups of 2-5. All mice were housed in individually ventilated polypropylene cages with 6.35mm corn-cob bedding in a room with a 12 hour dark-light cycle, with lights off at 12pm. Filtered water was available *ad libitum*.

3.1.2 Choline supplementation

Beginning at 9 weeks of age, mice were given their assigned diet until sacrifice at either 1 day or 2 weeks post-surgery. Mice in both experimental cohorts were assigned to one of two *ad libitum* diet conditions: a synthetic control chow (1.1 g/kg choline chloride in formula AIN-76A with choline chloride substituted for choline bitartrate, DYET #110098, Dyets, Inc., Bethlehem, PA, USA), or the same diet with 4.95 g/kg choline chloride (DYET #110210) based on previous work using these diets (Meck & Williams, 1999). Mice were raised eating standard rodent chow (2.2 g/kg choline chloride; PicoLab Mouse Diet 5058, Lab-Diet, Philadelphia, PA, USA) until 9 weeks of age.

3.1.3 Tibial fracture surgery

At twelve weeks of age, mice were assigned to either a naïve group (no anesthesia or surgery) or a tibial fracture group. Mice in the tibial fracture group were

given 2.1% isoflurane (Isothesia; Butler Animal Health Supply, Dublin, OH, USA) in 0.30 FiO_2. Mice received a tibial fracture of the left hind paw with an intramedullary fixation as previously described (Cibelli et al., 2010; Harry et al., 2008; Xiong, Zhang et al., 2018). Briefly, the left hind paw of surgical mice was disinfected with povidone iodine (Dynarex, Orangeburg, NY, USA) while on a heating pad. Mice were given buprenorphine-SR (0.1mg/kg, ZooPharm, Laramie, WY, USA) as analgesia. A median incision on the left hind paw was performed. A 0.38mm pin was inserted in the intramedullary canal. Then, the periosteum was stripped, and osteotomy was performed. Post-fracture, bupivacaine (0.25%; McKesson, Irving, TX, USA) was administered in the wound, and the skin was sutured. Mice were allowed to recover from anaesthesia in an empty clean cage, were returned to their home cage after the recovery of ambulatory behaviour, and were carefully monitored day for recovery.

3.1.4 Behavioral testing

Novel object recognition (NOR) testing was administered using a modified version of a previously published protocol (Leger et al., 2013). This task is dependent on the hippocampus when the inter-trial interval is more than 10 minutes (reviewed in Cohen & Stackman, 2015). This task was chosen because it does not elicit a stress response, and can be refined to assess subtle differences in hippocampal-dependent pattern recognition ability (Tognoni, 2014). Mice in all diet and surgery groups were allowed to habituate for 5 minutes to an empty plexiglass arena covered on the outside

with white paper one day before testing. Mice were placed in individual cages in a dark, quiet anteroom for a half hour before habituation and testing. Each cage had adequate corncob bedding, the assigned diet, and water in bottles. The testing room itself was kept in dim light, in which the ceiling light bulbs were distributed to ensure even light distribution. Light bulbs were a warm white light with 3000 kelvin color temperature. The testing room was kept entirely quiet during testing. A plastic tube was used to transport mice from room to room to minimize stress (Hurst & West, 2010). Mice then underwent 4 trials, each consisting of 5 minutes of automated motion tracking (HVS Image, Buckingham, UK) and video recording in a 406.4mm L x 203.2mm W x 254mm H Plexiglass arena on a white table 762mm above the ground. A tubular spirit level was utilized at the beginning of each testing day to ensure the table and arena were flat. The arena was covered with paper to hide possible distractions and navigational cues. The bottom of the arena was divided into 63.5mm squares in a 6 x 3 grid. Adhesive was placed on the bottom of the arena to secure objects in place. Between each trial, the arena and objects were disinfected with 70% ethanol. The trials were approximately 30 minutes apart for each mouse. The four trials were habituation [in an empty arena, during which open field (OF) data was collected], training 1 (with two identical objects, Figure 17C), training 2 (with the same identical objects), and test (with one of the objects replaced with a novel object). Objects were no taller than 50mm, and were counterbalanced with each group. Objects were wooden and painted high-contrast

colors, such as napkin rings and miniature candlestick (Figure 17C). Data for open field was quantified as the distance traveled (activity), and percent of time spent in the inner 4 squares of the arena grid (anxiety-related behavior, reviewed in Prut & Belzung, 2003). Data for the NOR was quantified as percent of time spent in each third of the arena and was verified for accuracy using video recordings. Mice were tested on all measures the day after surgery and were retested 2 weeks after surgery.

3.1.5 BrdU injection

All mice were given two bromodeoxyuridine (BrdU; ThermoFisher Scientific, Waltham, MA, USA) injections: one 1 hour after surgery, and one 10 hours after surgery. Each injection contained 200 mg/kg of BrdU in 0.9% saline (Balu et al., 2009).

3.1.6 Immunohistochemistry

Mice were sacrificed at either 1 day or 2 weeks after surgery. Under anesthesia, the thoracic cavity was opened, and the right atrium of the heart was cut. A solution of ice-cold 0.1M phosphate-buffered saline (PBS) was perfused into the left ventricle of the heart. The brains were harvested and fixed in 4% paraformaldehyde for 3 days, then cryoprotected in 30% sucrose for 3 more days. Then, brains were freeze-mounted in OCT (VWR International, Radnor, PA, USA), and sliced on a cryostat at -20°C in 40μm sections in a 1:4 series. Free-floating sections were suspended in PBS with .1% sodium azide preservative.

Briefly, sections that included the dorsal dentate gyrus (DG) of the hippocampus (bregma -1.28 through -2.12; Rosen et al., 2000) were washed with PBS before quenching with a solution of 50% methanol (VWR) and 3% hydrogen peroxide (VWR) for 30 minutes. Sections were washed again in PBS and blocked in a solution of 5mL 0.01M PBS, 150μL normal donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) and 50μL TX100 (Sigma-Aldrich, St. Louis, MO, USA) at 20°C for one hour and rinsed again in PBS. Sections were incubated at room temperature in one of three combinations of primary antibodies: to analyze microglia and macrophages, a 1:500 concentration of goat anti-Iba1 primary antibody (Novus Biologicals, Littleton, CO, USA) and a 1:2000 concentration of rabbit anti-P2Y12 (Anaspec Inc., Fremont, CA, USA); to analyze astrocytes, a 1:500 concentration of rabbit anti-GFAP (glial fibrillary acidic protein; Santa Cruz Biotechnology, Inc., Dallas, TX, USA); to analyze neurons, cell division and survival, a 1:200 concentration of rabbit anti-doublecortin (DCX) antibody (Cell Signaling Technologies, Danvers, MA, USA) and a 1:500 concentration of goat anti-BrdU primary antibody (Abcam, Cambridge, UK). The next day, sections were washed in PBS and incubated for two hours in secondary antibody of donkey-anti-rabbit AlexaFluor 647 (ThermoFisher) and/or donkey-anti-goat AlexaFluor 488 (ThermoFisher) at a 1:200 concentration. Sections were washed in PBS before they were mounted onto slides, coverslipped using a fluorescent medium (Vectashield; Vector Labs, Burlingame, CA, USA), and sealed with nail polish. Z-stack images were collected on a Zeiss SP8

microscope (Zeiss, Oberkochen, Germany) in the Duke University Light Microscopy Core Facility with 40x objectives. Images were taken as 150 μm x 150 μm "tiles," which were then merged together to view the entire hippocampus or cortex. Cells were quantified using manual cell counting (Iba1, Iba1+/P2Y12-, BrdU, BrdU+/DCX+, and hilar DCX), unbiased stereology (DCX in SGZ) or densitometry (GFAP) using ImageJ software (NIH, Bethesda, MD, USA).

3.1.7 Unbiased stereology

Unbiased stereology for DCX+ cells in the subgranular zone of the dentate gyrus was conducted based on a previous protocol using ImageJ (NIH; Ip et al., 2017). A 150 μm x 150 μm counting frame was utilized in 5 sections that were sliced in a series of four. Optical fractionator estimates were multiplied by 2 to account for both hemispheres. Contours were traced around the granule cell layer, excluding the hilus.

3.1.8 Densitometry

Astrocytic density was examined using densitometry as previously described for microglial density (Bilbo & Tsang, 2010). Using ImageJ software, signal pixels were defined as pixels within the dentate gyrus with a gray value 3 standard deviations higher than the mean gray value of a cell-poor area within the dentate gyrus. The number of signal pixels and their average gray values above background were multiplied to give an integrated density measurement for each section.

3.1.9 Statistical analysis

Data were analyzed using one- or two-way ANOVA and Tukey HSD post-hoc test, when appropriate, with JMP Pro version 12.2 (SAS Institute, Cary, NC). A two-way ANOVA between treatment (control diet + naïve, control diet + tibial fracture, or choline-supplemented diet + tibial fracture) and time point (1 day or 2 weeks post-surgery) was utilized in all measures except for body weight and food consumption, which used repeated measures and one-way ANOVAs. All graphs were generated using Prism version 8.3.0 (GraphPad, San Diego, CA, USA) using XY (body weight) and grouped (all other metrics) table formats.

3.2 Results

3.2.1 Body weights and food consumption

There were no observed differences in body weight change due to dietary choline supplementation in a repeated measures ANOVA ($F_{1,55} = 0.22$, $p > 0.05$, Figure 13). There were also no differences due to diet in food consumption in a one-way ANOVA ($F_{1,175} = 1.58$, $p > 0.05$, Figure 14). Variation in following measures are not due to the quantity of diet consumed.

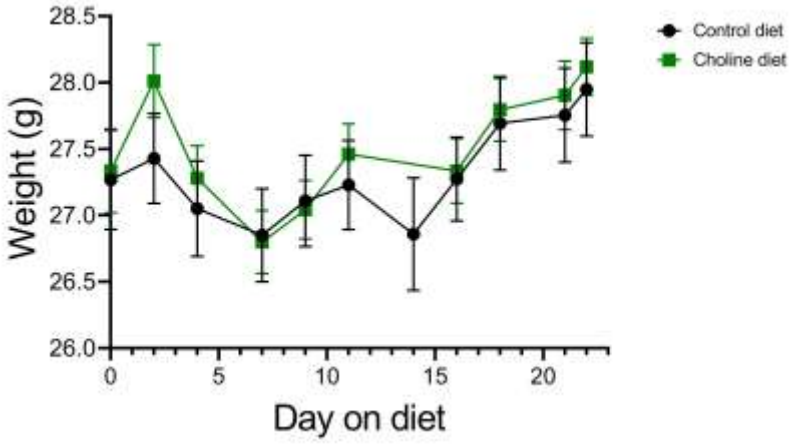


Figure 13: No differences in weight due to diet condition were observed. N = 13-31 per group. Each value represents the mean ± SEM.

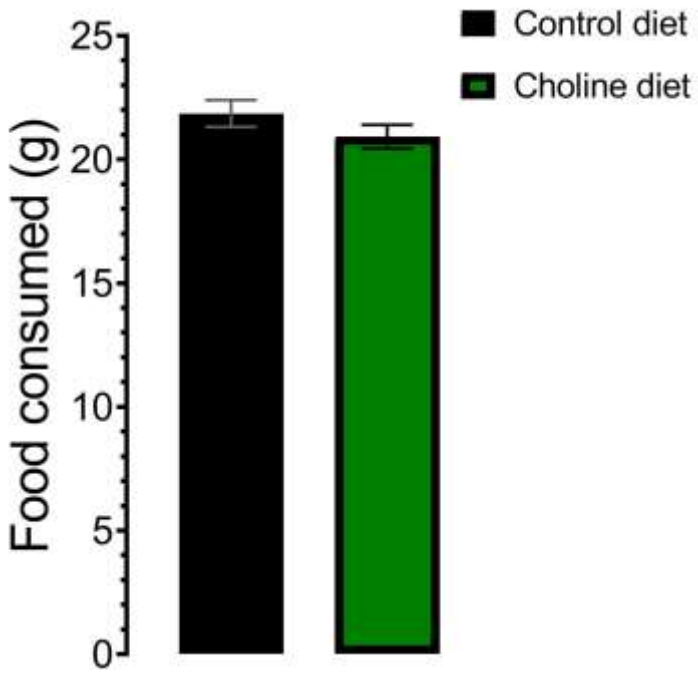


Figure 14: No differences in food consumption due to dietary condition were observed. N = 13-31 per group. Each value represents the mean ± SEM.

3.2.2 Activity and anxiety-related behavior in an open field

Activity was measured by the total distance traveled in meters during the 5 minute trial in an empty arena. A two-way ANOVA between treatment (control diet + naïve, control diet + tibial fracture, or choline-supplemented diet + tibial fracture) and time point (1 day or 2 weeks post-surgery) was utilized in all subsequent measures. A marginal difference in activity was found due to treatment in a two-way ANOVA ($F_{2,41}$ = 2.48, p = 0.10, Figure 15). Across both time points, tibial fracture led to an increase in activity, which is partially mitigated by dietary choline supplementation. At the 2 week time point, tibial fracture marginally increased activity. At the 2 week time point, this increase was not rescued by dietary choline supplementation.

There were no significant differences due to time point ($F_{1,41}$ = 0.78, p > 0.05), and no treatment x time point interaction effect was found ($F_{2,41}$ = 0.39, p > 0.05). Tibial fracture and dietary choline supplementation did not affect activity at either time point analyzed. Importantly, differences in the novel object recognition task were not due to impairments in activity due to impeded movement from tibial fracture surgery.

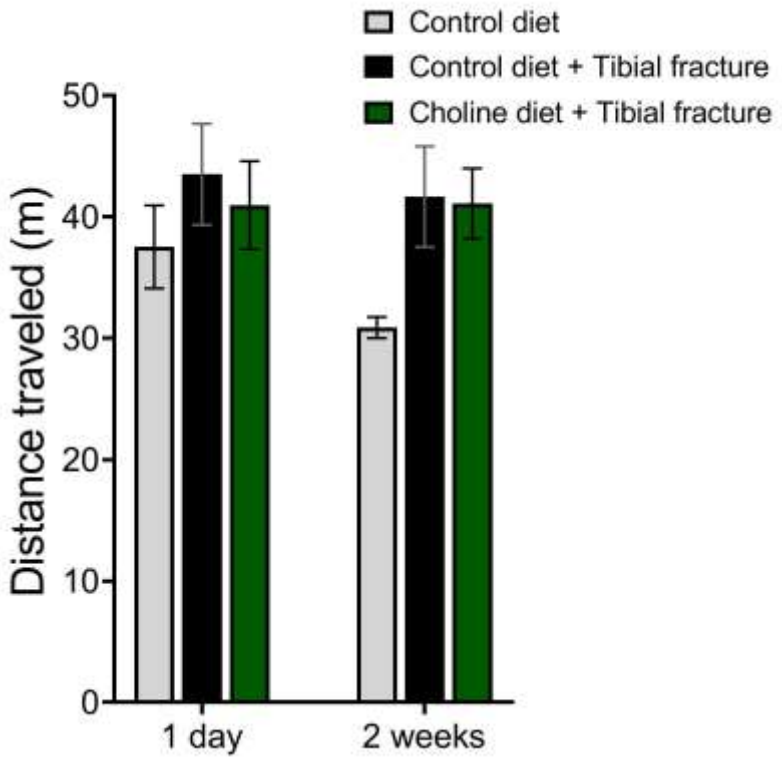


Figure 15: Tibial fracture surgery and choline diet exhibited no significant effects on activity in an open field. Histogram showing the distance traveled in meters over the 5 minute trial. N = 5-10 per group. Each value represents the mean ± SEM.

Anxiety-related behavior was assessed by the percent of time spent in the center of the arena during the open field trial. Using a two-way ANOVA, significant effect due to treatment was found ($F_{2,41} = 10.95$, $p < 0.001$, Figure 16). Tibial fracture induced less time spent in the center of the arena, indicating an increase in anxiety (reviewed in Prut & Belzung, 2003). Dietary choline supplementation did not mitigate this decrease in time spent in the center of the open field. A significant effect of time point was observed ($F_{1,41} = 5.04$, $p < 0.05$). Two weeks after surgery, mice spent a higher percentage of total time in the center of the open field. No interaction between treatment and time point was

observed ($F_{2,41} = 0.14$, $p > 0.05$). Anxiety-related behaviors increased due to tibial fracture, and unlike hippocampal-dependent memory deficits, this behavioral alteration was not affected by dietary choline supplementation.

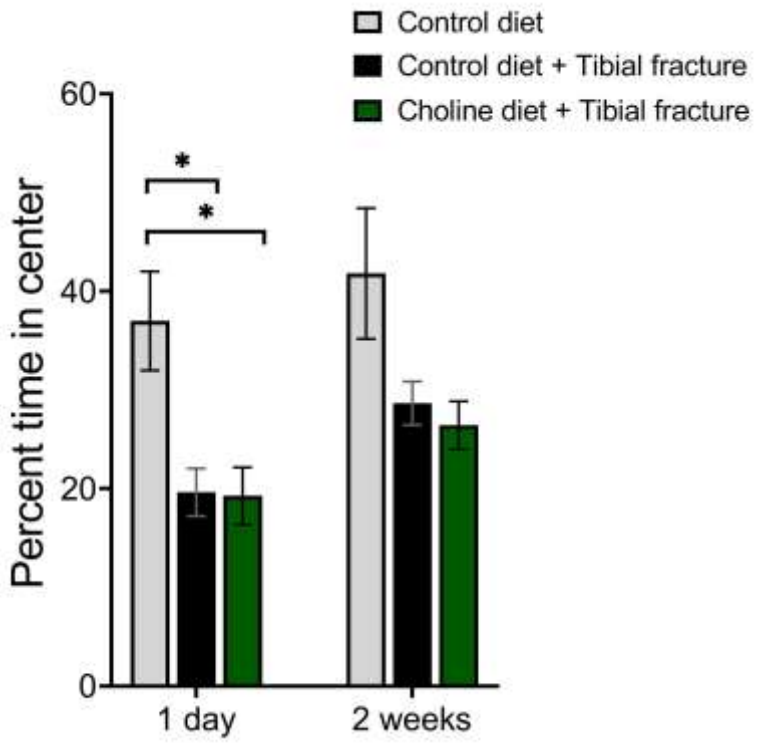
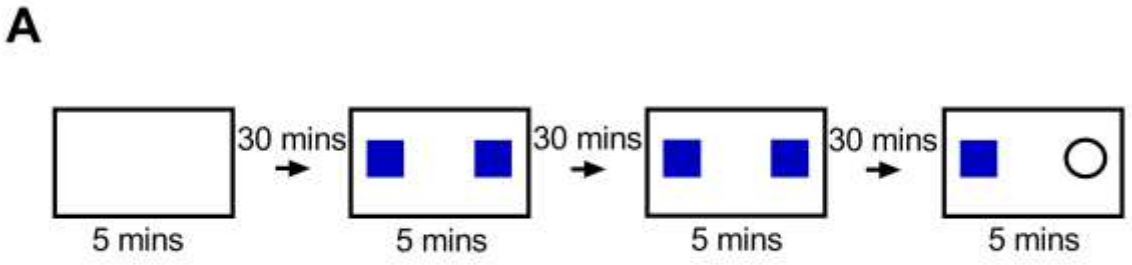


Figure 16: Tibial fracture surgery and choline diet effects on percent of time spent in the center of the arena (anxiety-related behavior) in an open field. Histogram showing the percent of time spent in the center of the arena over the 5 minute trial. N = 5-10 per group. * $p < 0.05$, Tukey HSD. Each value represents the mean ± SEM.

3.2.3 Novel object recognition

To assess hippocampal-dependent memory, a novel object recognition paradigm was administered 1 day and 2 weeks after surgery (Figure 17). The discrimination index (DI: (Novel object time – Old object time)/(Novel object time + Old object time)) was used to assess whether mice spent more time with the novel object or old object

(Ennaceur & Delacour, 1988). A significant effect of treatment was observed ($F_{2,37} = 5.57$, $p < 0.01$, Figure 17B). Tibial fracture surgery decreased the time spent with the novel object as measured by DI. Astonishingly, dietary choline supplementation completely prevented this impairment. No differences due to time point ($F_{1,37} = 0.001$, $p > 0.05$) and no treatment x time point interaction ($F_{2,37} = 0.01$, $p > 0.05$) were observed. Our finding that tibial fracture surgery leads to hippocampal-dependent cognitive deficits 24 hours after surgery is consistent with previous results using a contextual fear conditioning task (Ni et al., 2018; Terrando et al., 2011). Dietary choline supplementation for three weeks prior to surgery completely prevented the hippocampal-dependent memory impairment due to tibial fracture surgery at both time points.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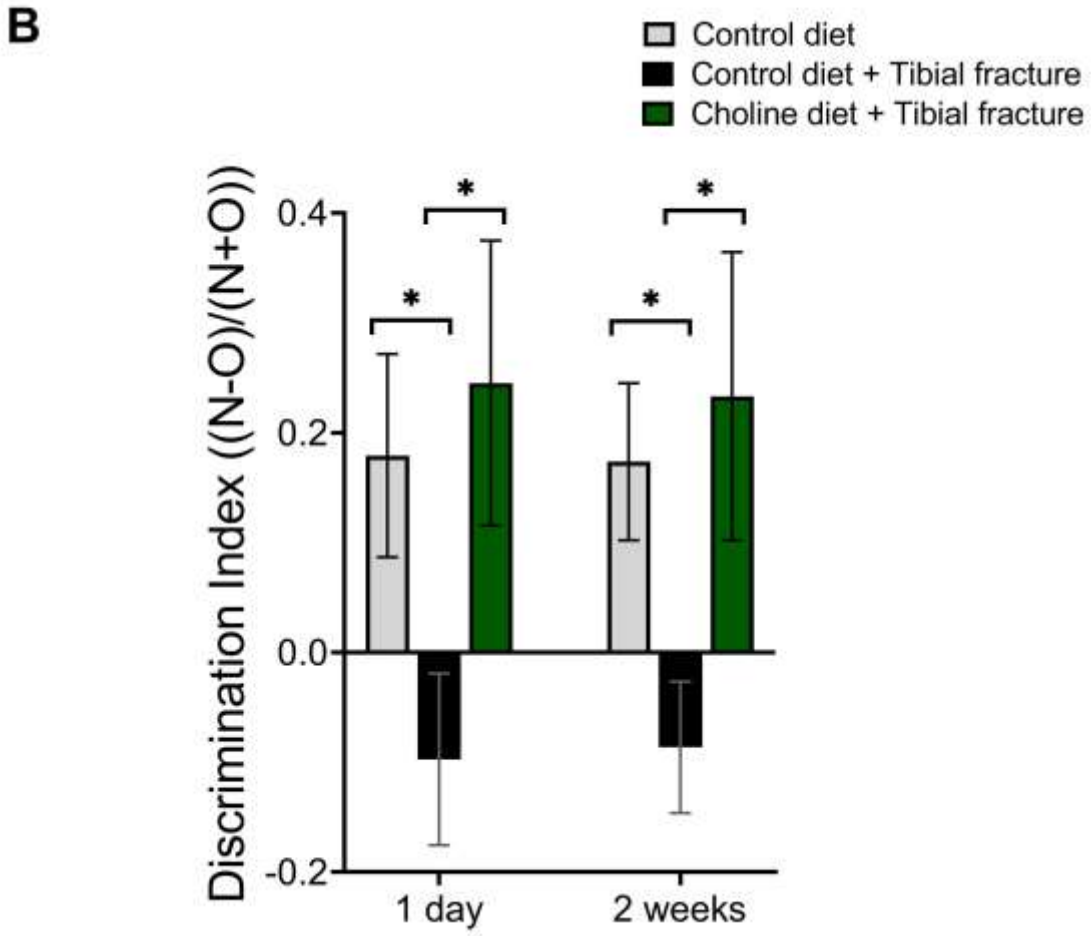

B
Control diet
Control diet + Tibial fracture
Choline diet + Tibial fracture
Discrimination Index ((N-O)/(N+O))
0.4
0.2
0.0
-0.2
*
*
*
*
1 day
2 weeks

C

Figure 17: Tibial fracture and choline diet effects on discrimination index in a novel object recognition task. (A) Schematic indicating NOR protocol. (B) Histogram showing discrimination index ((Novel object time – Old object time)/(Novel object time + Old object time)) 1 day and 2 weeks after surgery. N = 7-10 per group. * $p < 0.01$, Tukey HSD. Each value represents the mean ± SEM. (C) Example objects used in the NOR task. The order in which mice were exposed to objects was counterbalanced per mouse.

3.2.4 Microglial activation and number

3.2.4.1 Dentate gyrus

To assess the activation state of neuroimmune cells, microglial activation in the DG was analyzed using immunohistochemical staining for Iba1. Activation was quantified as the percent of microglia in "activated" stages ("amoeboid" and "stout") as previously described (Schwarz et al., 2012, Figure 18). There was a marginal effect of treatment ($F_{2,29} = 3.08$, $p = 0.06$, Figure 19). Tibial fracture led to a non-significant increase in activated microglia, which is mitigated by dietary choline supplementation. There was a significant effect of time point on microglial activation in the dentate gyrus ($F_{1,29} = 9.46$, $p < 0.01$). One day after surgery, a higher percentage of microglia were in an "activated" state compared to 2 weeks after surgery. This is consistent with previous findings indicating microglial activation resolves one week after tibial fracture (Cibelli et al., 2010). No significant interaction of treatment and time point was observed ($F_{2,29} =$

0.93, $p > 0.05$). The non-significant increase in microglial activation 1 day after surgery is mitigated by dietary choline supplementation.

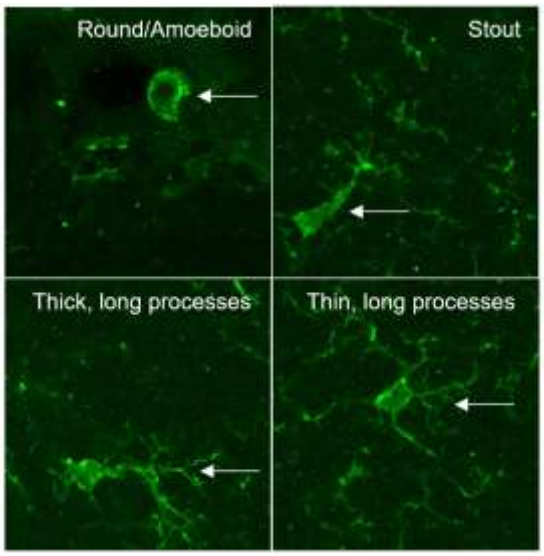


Figure 18: Representative images of each microglial morphology. Sections were immunostained with Iba1. Each image is 37.5 μm x 37.5 μm. White arrows indicate each morphology type: round/amoeboid (upper left), stout (upper right), thick long processes (bottom left), and thin long processes (bottom right). Quantification states based on Schwarz et al., 2012.

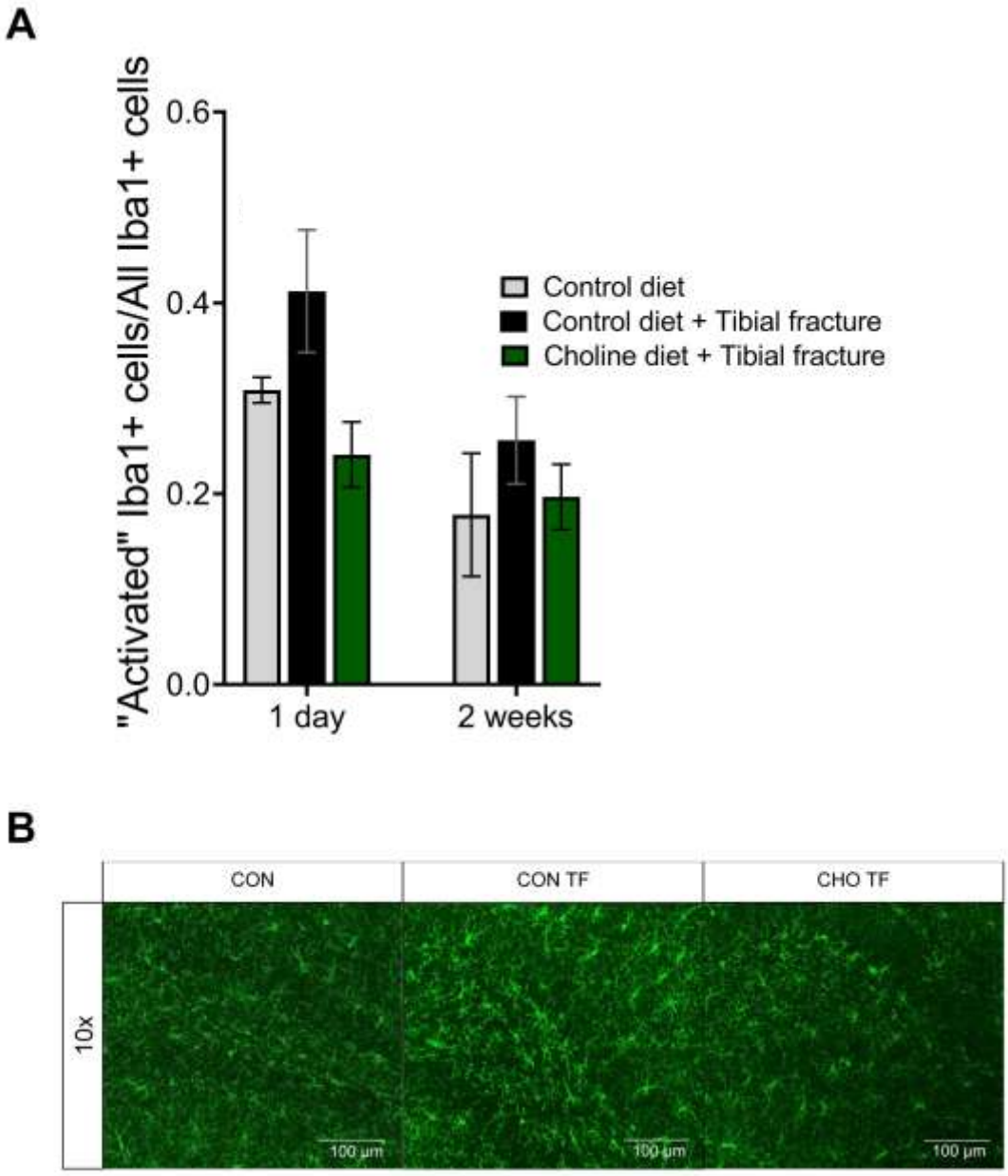


Figure 19: Tibial fracture and choline diet effects on microglial activation in the dentate gyrus of the hippocampus. (A) Histogram showing the percentage of "round" + "stout" Iba1+ cells in the dentate gyrus of the hippocampus 1 day and 2 weeks after surgery. N = 5-7 per group. (B) Representative fluorescent micrographs of the dentate gyrus taken one day after surgery, immunostained with Iba1 in mice given control diet and left naïve (left), given control diet and administered a tibial fracture (middle), and given choline-supplemented diet and administered a tibial

fracture (right). Scale bar represents 100 μm. CON: control diet + naïve. CON TF: control diet + tibial fracture surgery. CHO TF: choline diet + tibial fracture surgery. Each value represents the mean ± SEM.

Critically, differences were only found in the percent of microglia in "activated" morphologies, not total microglial number, in the dentate gyrus. No differences due to treatment ($F_{2,29} = 2.20$, $p > 0.05$, Figure 20), time point ($F_{1,29} = 1.35$, $p > 0.05$), or interaction between treatment and time point ($F_{2,29} = 0.30$, $p > 0.05$) were observed. Microglial activation was upregulated in the DG due to tibial fracture surgery, and this increase was mitigated by dietary choline supplementation 1 day after surgery.

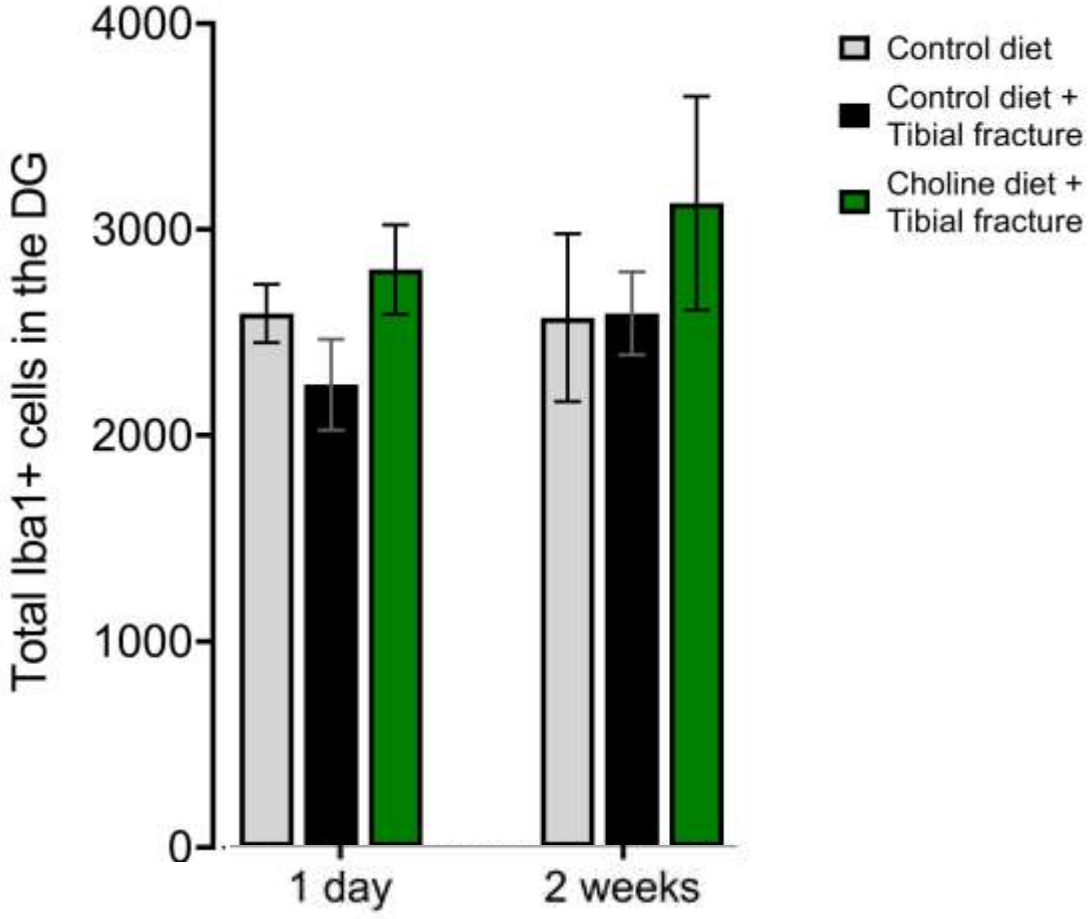


Figure 20: Tibial fracture and choline diet exhibited no effects on total number of microglia. Histogram showing the total number of Iba1+ cells in the dentate gyrus 1 day and 2 weeks after surgery. N = 5-7 per group. Each value represents the mean ± SEM.

3.2.4.2 Cortex

To assess the hippocampal specificity of these microglial differences, we quantified microglial activation and total microglial numbers in the retrosplenial cortex. This region is involved in spatial navigation and episodic memory (reviewed in Vann et al., 2009), and also receives cholinergic input (Gage et al., 1994). To further isolate the

actions of tibial fracture and dietary choline supplementation to the hippocampus, we assessed whether these variables impacted a distinct region with many hippocampal connections (reviewed in Wyass & Van Groen, 1992).

In the retrosplenial cortex, no effects of treatment ($F_{2,16} = 1.13$, $p > 0.05$, Figure 21, time point ($F_{1,16} = 0.88$, $p > 0.05$), or interaction between treatment and time point ($F_{2,16} = 1.22$, $p > 0.05$) were found in microglial activation using two-way ANOVA. As well, no differences in treatment ($F_{2,16} = 0.90$, $p > 0.05$, Figure 22), time point ($F_{1,16} = 0.18$, $p > 0.05$), or an interaction between treatment and time point ($F_{2,16} = 0.22$, $p > 0.05$) were seen. Though effects are marginal, the effects of tibial fracture and dietary choline supplementation on microglial activation are larger in the DG than in the retrosplenial cortex, suggesting a hippocampal-specific effect.

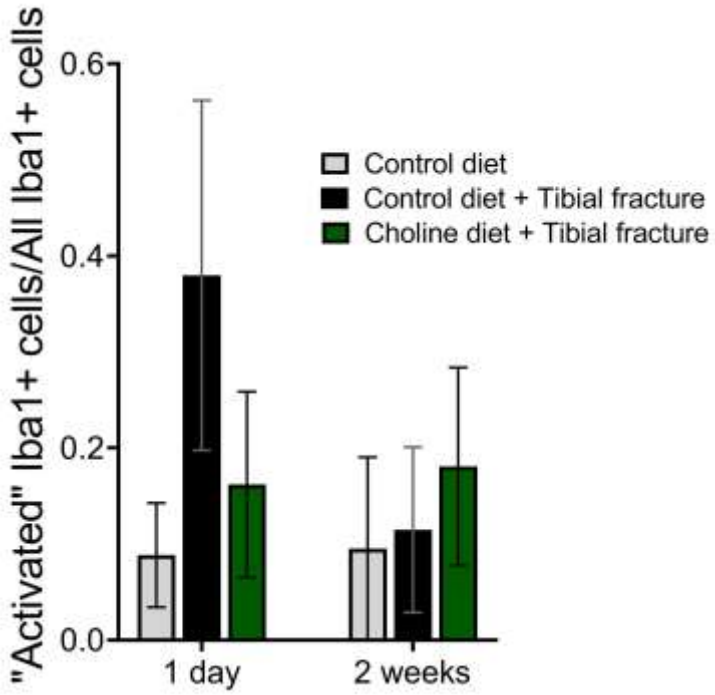


Figure 21: In the cortex, no significant differences in microglial activation were observed. Histogram indicating the percentage of "round" + "stout" Iba1+ cells in the retrosplenial cortex 1 day and 2 weeks after surgery. N = 3-5 per group. Each value represents the mean ± SEM.

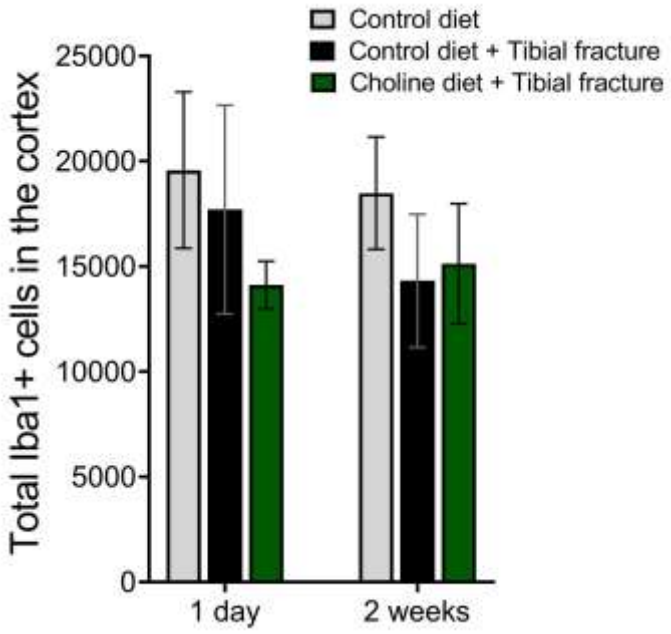
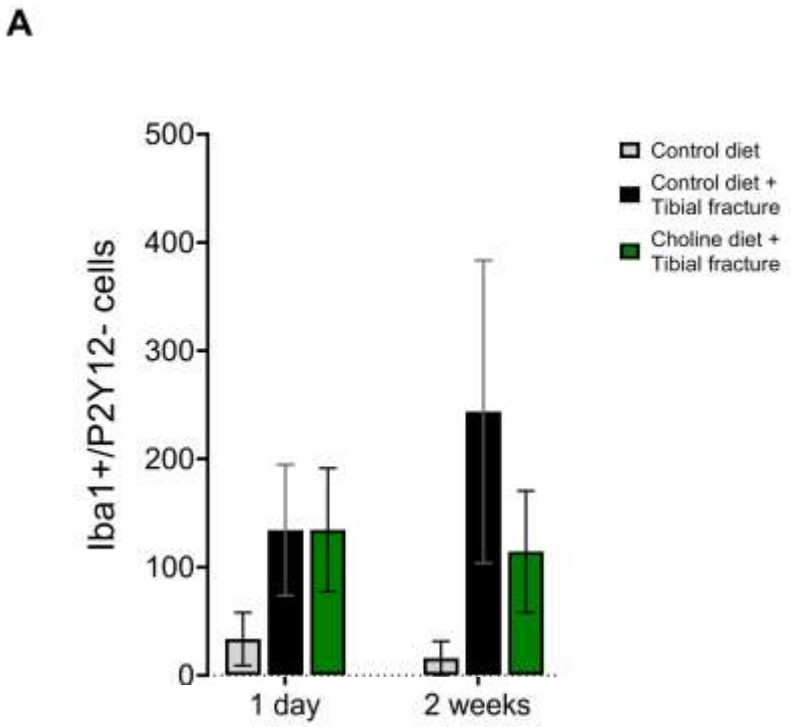


Figure 22: In the cortex, no differences in microglial number were observed. Histogram indicating the total number of Iba1+ cells in the retrosplenial cortex 1 day and 2 weeks after surgery. N = 3-5 per group. Each value represents the mean ± SEM.

3.2.5 Macrophage infiltration

To differentiate between infiltrating macrophages and resident microglia, we used a double-stain for Iba1 (a general macrophage marker) and P2Y12 (a specific microglial marker). Hence, any cell that is only labeled with Iba1 and not P2Y12 is not a microglial cell and is a non-resident macrophage. This experiment was conducted because previous work in the tibial fracture mouse model has shown that increases in macrophage number in the hippocampus are due to infiltrating macrophages and not microglia (Terrando et al., 2011). However, in the dentate gyrus, no significant differences were found in macrophage infiltration due to treatment condition ($F_{2,25}$ =

2.46, $p > 0.05$, Figure 23), time point ($F_{1,16} = 0.16$, $p > 0.05$), or an interaction between treatment and time point ($F_{2,25} = 0.54$, $p > 0.05$) were found.



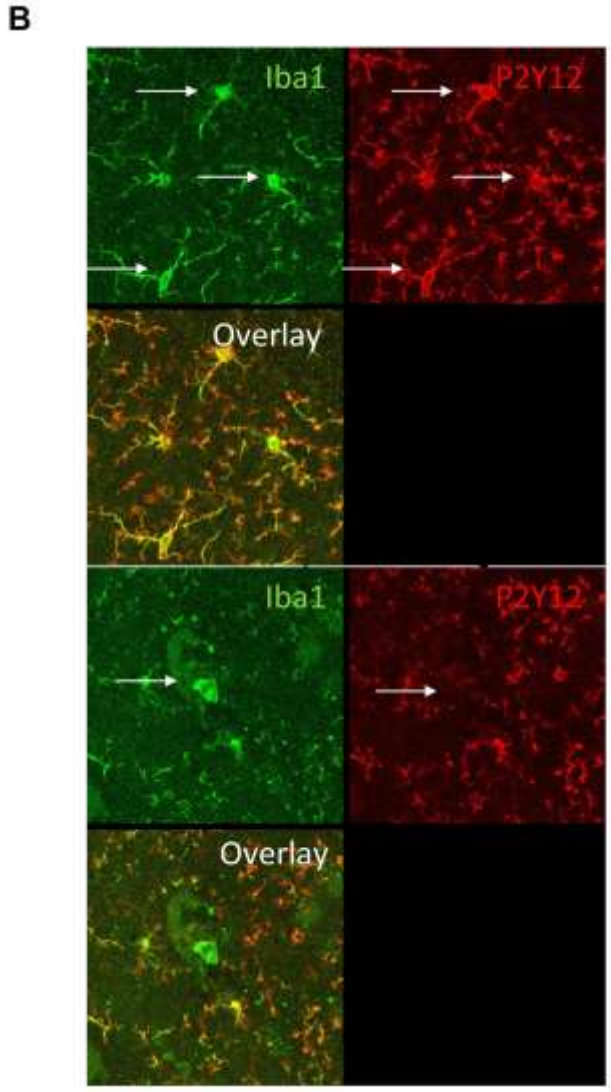


Figure 23: Tibial fracture and choline supplementation effects on hippocampal macrophage infiltration.(A) Histogram showing the number of Iba1+/P2Y12- cells in the dentate gyrus of the hippocampus 1 day (left) and 2 weeks (right) after surgery. N = 4-6 per group. (B) Fluorescent micrographs of the dentate gyrus, immunostained with Iba1 (green) and P2Y12 (red). The top three panels show double-labeled cells, indicated with white arrows. These are microglia. The bottom three panels show a single-labeled Iba1+/P2Y12- cell, indicated with a white arrow. This is a non-microglial macrophage. Each image is 150 μm x 150 μm. Each value represents the mean ± SEM.

Similarly, no effects of treatment ($F_{2,18} = 0.02$, $p > 0.05$, Figure 24), time point ($F_{1,18} = 0.005$, $p > 0.05$), or an interaction between treatment and time point ($F_{2,18} = 0.40$, $p > 0.05$) on macrophages in the cortex were seen in a two-way ANOVA. Contrary to previous findings implicating infiltrating macrophages in POCD (Terrando et al., 2011), we did not see any differences in either brain region analyzed.

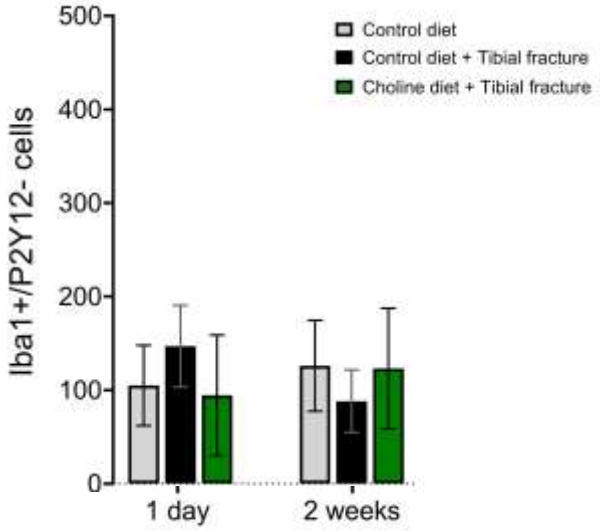


Figure 24: In the cortex, no differences in non-microglial macrophages were observed. Histogram showing the number of Iba1+/P2Y12- cells in the retrosplenial cortex 1 day (left) and 2 weeks (right) after surgery. N = 3-5 per group. Each value represents the mean ± SEM.

3.2.6 Astrocytic density

To further assess neuroinflammation, astrocytic antigen density was analyzed using immunohistochemical staining and densitometry for GFAP after sacrifice either 1 day or 2 weeks after surgery. A significant effect of treatment was observed in GFAP antigen density ($F_{2,34} = 5.27$, $p < 0.05$, Figure 25). Tibial fracture led to a significant increase in GFAP density, which was rescued with the addition of dietary choline

supplementation. Marginal effects due to time point ($F_{1,34} = 3.23$, $p = 0.08$) and an interaction between treatment and time point ($F_{2,34} = 3.09$, $p = 0.06$) were observed. The increase in GFAP density 1 day after tibial fracture is likely carrying these marginal effects. One day after surgery, dietary choline supplementation led to the complete amelioration of astrocytic activation. As well, 2 weeks after surgery, no effects were seen. Previous research implicating astrocytes in tibial fracture-induced POCD focused 1 day after surgery (Xu et al., 2017). However, the findings that microglial activation resolves one week after tibial fracture (Cibelli et al., 2010) corroborates with the current work: neuroinflammation resolves before the 2 week time point. Because tibial fracture surgery led to behavioral impairments 2 weeks after surgery, and because no increases in inflammation were found due to surgery at that time point, we next explored cell proliferation and survival in an attempt to find the mechanism behind long-term impairments and rescue with dietary choline supplementation.

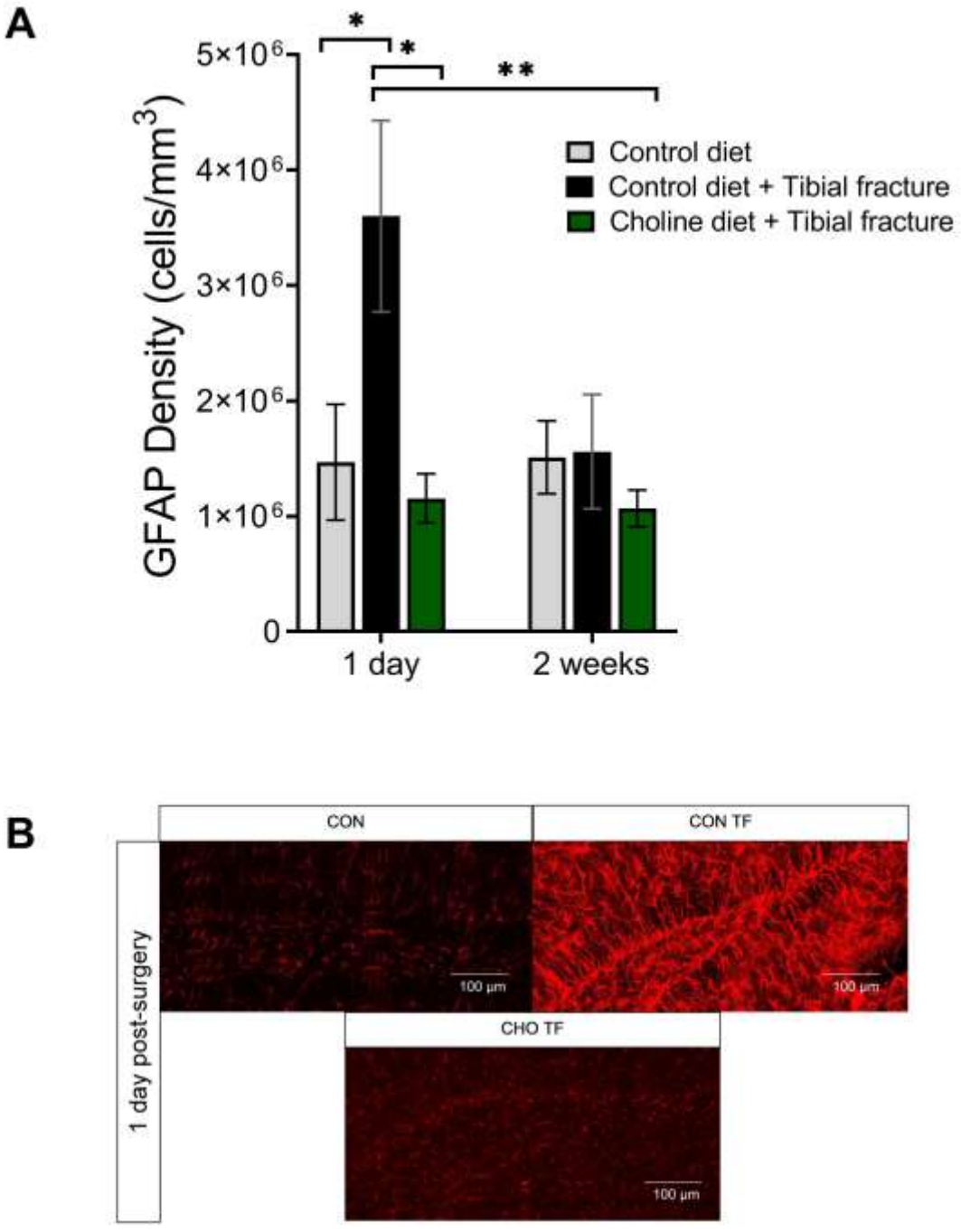


Figure 25: Tibial fracture and choline diet effects on astrocytic activation. (A) Histogram showing the antigen density of GFAP in the dentate gyrus of the hippocampus. N = 6-8 per group. (B) Representative fluorescent micrographs of the

dentate gyrus taken one day after surgery, immunostained with GFAP in mice given control diet and left naive (upper left), given control diet and administered a tibial fracture (upper right), and given choline-supplemented diet and administered a tibial fracture (bottom). CON: control diet + naïve. CON TF: control diet + tibial fracture surgery. CHO TF: choline diet + tibial fracture surgery. * $p < 0.05$, ** $p < 0.01$, Tukey HSD. Each value represents the mean ± SEM.

3.2.7 Cell proliferation and survival

BrdU was administered via intraperitoneal (i.p.) injections at both 1 hour and 10 hours post-surgery to capture the cells proliferating in response to tibial fracture. Using anti-BrdU antibody, immunohistochemistry was performed to quantify BrdU+ cells 1 day and 2 weeks after tibial fracture. Across both time points, tibial fracture non-significantly increased BrdU+ cells, and the addition of dietary choline supplementation significantly decreased the number of BrdU+ cells ($F_{2,36} = 4.54$, $p < 0.05$, Figure 26). As well, a significant effect of BrdU+ cells was observed due to time point ($F_{1,36} = 62.54$, $p < 0.0001$). BrdU+ cells labeled shortly after surgery did not survive to 2 weeks post-surgery, as indicated by there being significantly fewer BrdU+ cells at the later time point. A marginal interaction between treatment and time point was observed ($F_{2,36} = 3.21$, $p = 0.05$). Tibial fracture increases the number of BrdU+ cells in the dentate gyrus, and this increase is more pronounced 1 day after surgery, and dietary choline supplementation blunted this increase.

Because the control diet + tibial fracture group had larger numbers of BrdU+ cells 1 day after surgery, but this effect resolved by 2 weeks post-surgery, we inferred that these proliferating cells did not survive to 2 weeks post-surgery. Though tibial fracture

surgery led to an increase in proliferation, it also led to a higher rate of cell death because the group difference resolved by 2 weeks post-surgery. The number of BrdU+ cells was significantly lower in all of the 2 weeks post-surgery groups than the 1 day post-surgery groups, indicating a low rate of survival of the proliferating cells shortly after surgery.

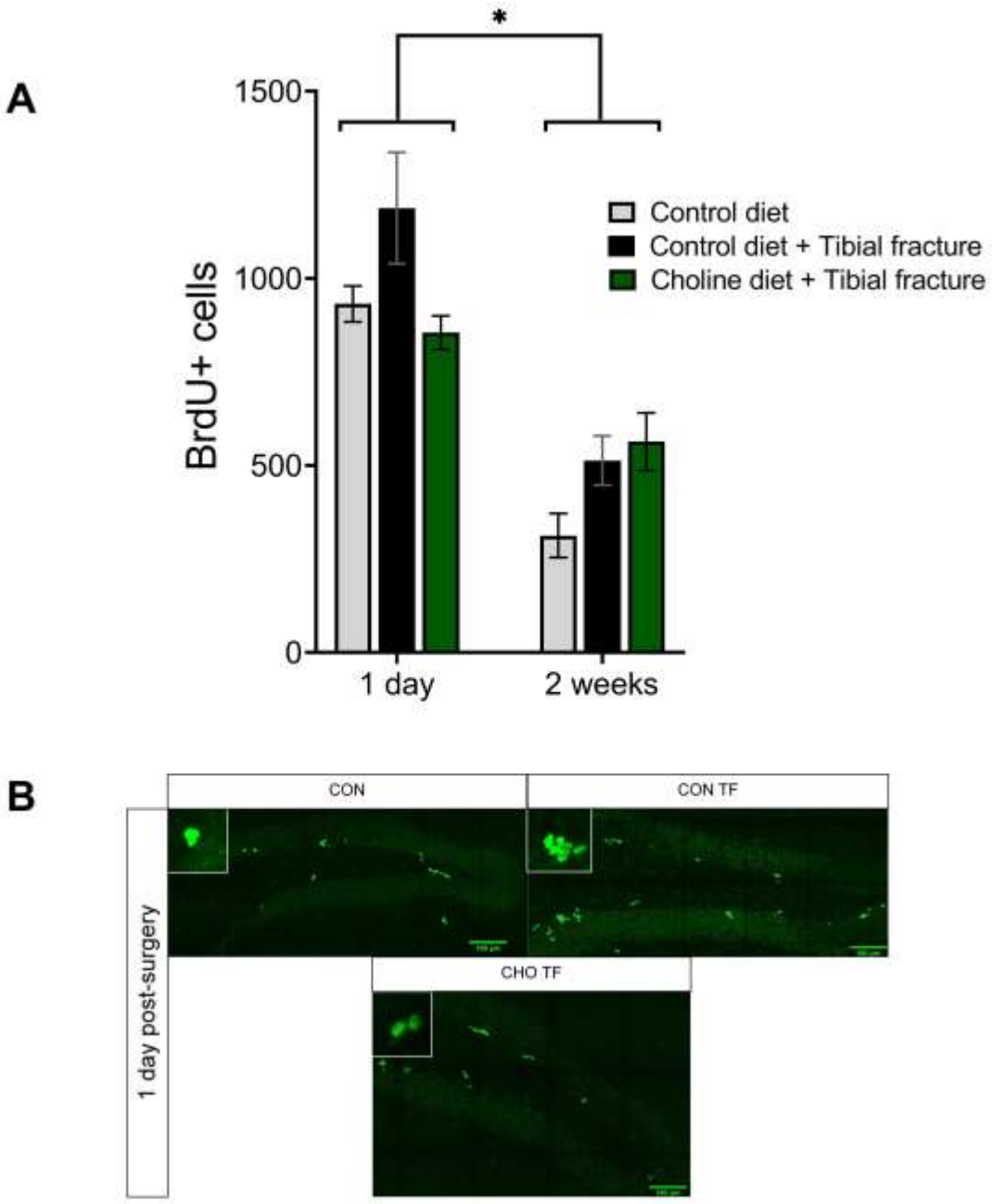


Figure 26: Tibial fracture surgery and choline diet effects on cell proliferation and survival. (A) Histogram showing the number of BrdU+ cells in the subgranular zone (SGZ) of the dentate gyrus. N = 7 per group. (B) Representative fluorescent micrographs of dentate gyri taken one day post-surgery, immunostained with BrdU in mice given control diet and left naive (upper left), given control diet and administered

a tibial fracture (upper right), and given choline-supplemented diet and administered a tibial fracture (bottom). Inset: Higher magnification micrograph of BrdU+ cells. CON: control diet + naïve. CON TF: control diet + tibial fracture surgery. CHO TF: choline diet + tibial fracture surgery. * $p < 0.05$, Tukey HSD. Each value represents the mean ± SEM.

To determine if the increase in neuroinflammation was due to an increase in glial proliferation, double labeling of BrdU with Iba1 or GFAP was quantified, respectively. No treatment, time point, or treatment x time point differences were found in dividing microglia (treatment: $F_{2,32} = 0.02$, $p > 0.05$; time point: $F_{1,32} = 0.02$, $p > 0.05$; interaction: $F_{2,32} = 0.68$, $p > 0.05$) or astrocyte numbers (treatment: $F_{2,33} = 2.92$, $p > 0.05$; time point: $F_{1,33} = 0.08$, $p > 0.05$; interaction: $F_{2,33} = 0.84$, $p > 0.05$). The lack of differences is possibly due to an extreme paucity of these dividing cells.

To further investigate the fate of the dividing cells, a double-label of BrdU and DCX was exhaustively quantified. A significant difference due to treatment was found in dividing neurons ($F_{2,34} = 6.41$, $p < 0.01$, Figure 27). Tibial fracture led to a non-significant increase in BrdU+/DCX+ cells, which was significantly blunted with the addition of dietary choline supplementation. No differences due to time point ($F_{1,34} = 0.76$, $p > 0.05$) or an interaction between treatment and time point ($F_{2,34} = 0.18$, $p > 0.05$) were observed. At both time points, choline diet led to fewer BrdU+/DCX+ cells than the control diet + tibial fracture treated mice, but not the control diet + naïve mice. This indicates that choline blunts cell division, but the cells that were dividing and differentiated into young neurons still survived 2 weeks later.

Because increases in cell proliferation immediately after surgery were identified, and because neuronal proliferation and were altered due to choline but not surgery, we next sought to analyze currently existing neurons (DCX+ cells 1 day after surgery) and neurons born in the time between surgery and sacrifice (DCX+ cells 2 weeks after surgery).

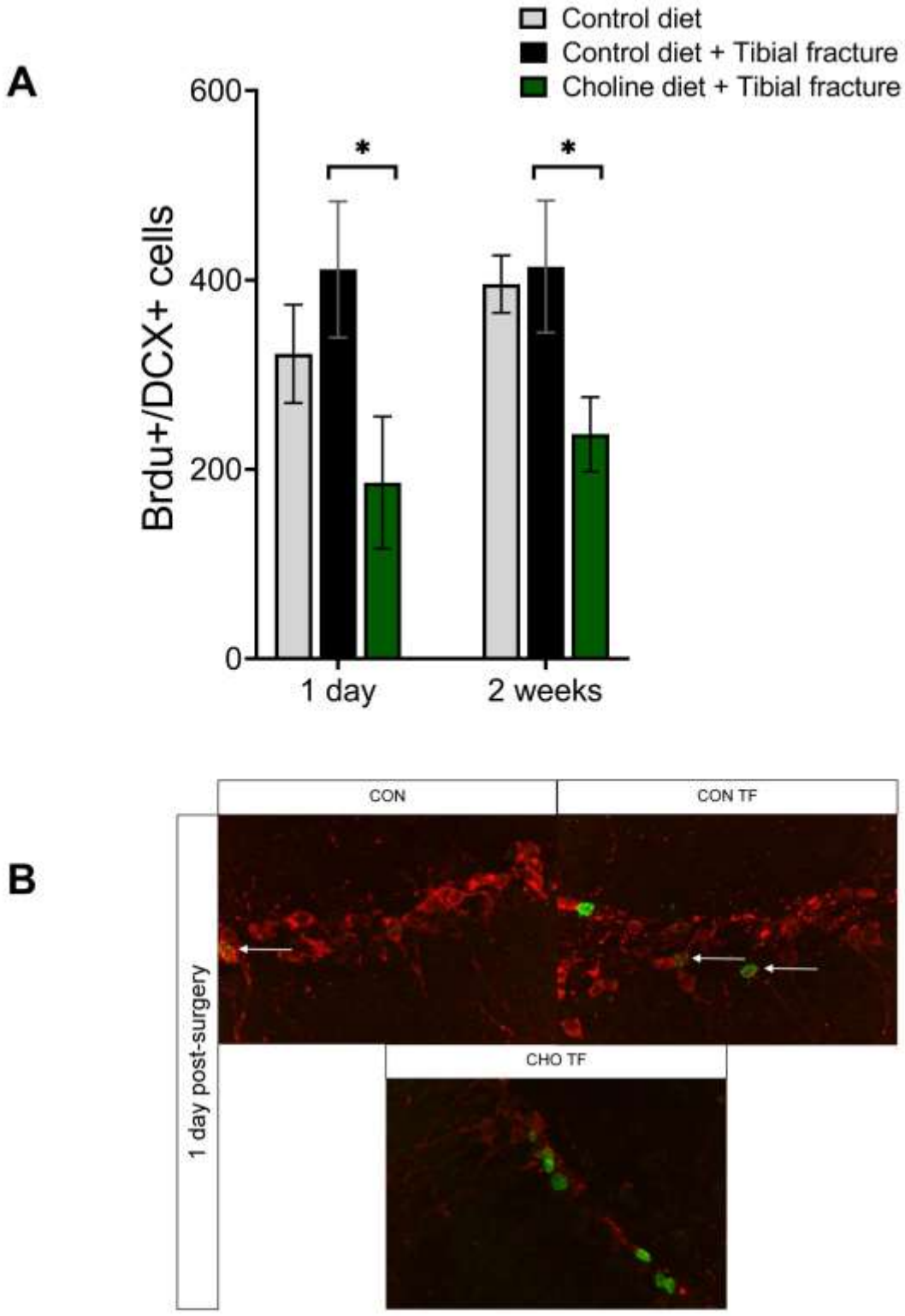

A
Control diet
Control diet + Tibial fracture
Choline diet + Tibial fracture
Brdu+/DCX+ cells
600
400
200
0
*
*
1 day
2 weeks
B
CON
CON TF
1 day post-surgery
CHO TF

Figure 27: Tibial fracture surgery and choline diet effects on dividing and surviving neurons. (A) Histogram showing the number of BrdU+/DCX+ cells in the dentate gyrus. N = 7 per group. (B) Representative fluorescent micrographs of dentate gyri taken one day post-surgery, immunostained with BrdU (green) and DCX (red) in mice given control diet and left naïve (upper left), given control diet and administered a tibial fracture (upper right), and given choline-supplemented diet and administered a tibial fracture (bottom). White arrows indicate double-labeled cells. Each image is 150 μm in width. CON: control diet + naïve. CON TF: control diet + tibial fracture surgery. CHO TF: choline diet + tibial fracture surgery. * $p < 0.05$, Tukey HSD. Each value represents the mean ± SEM.

3.2.8 Young neurons

Young neurons in the subgranular zone (SGZ) of the dentate gyrus (DG) were analyzed using immunohistochemical staining for DCX after sacrifice either 1 day or 2 weeks after surgery. Though neither treatment ($F_{2,36} = 2.14$, $p > 0.05$, Figure 28) nor time point ($F_{1,36} = 2.45$, $p > 0.05$) led to differences alone in DCX+ neurons, there was a significant treatment x time point interaction ($F_{2,36} = 3.34$, $p < 0.05$). Tibial fracture leads to an upregulation in DCX+ cells 2 weeks after surgery. This increase is partially mitigated by dietary choline supplementation. In contrast to the findings found 1 day after surgery in GFAP and BrdU, tibial fracture leads to an increase in young neurons 2 weeks after surgery. This increase is partially blunted by dietary choline supplementation.

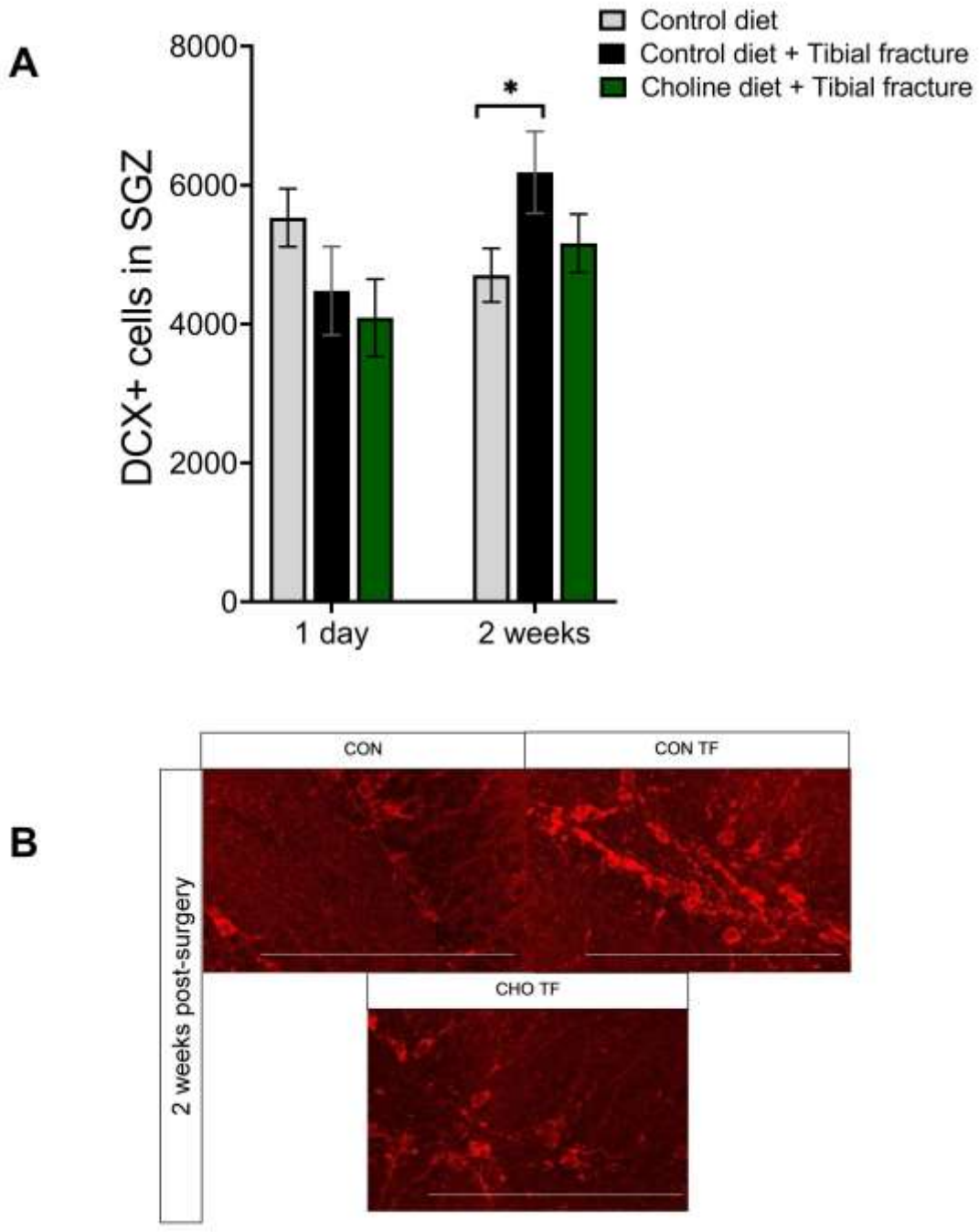


Figure 28: Tibial fracture surgery and choline diet effects on young neurons. (A) Histogram showing the number of DCX+ cells in the subgranular zone (SGZ) of the dentate gyrus. N = 7 per group. (B) Representative fluorescent micrographs of the SGZ taken two weeks after surgery, immunostained with DCX in mice given control

diet and left naïve (upper left), given control diet and administered a tibial fracture (upper right), and given choline-supplemented diet and administered a tibial fracture (bottom). Scale bar: 100 μm. CON: control diet + naïve. CON TF: control diet + tibial fracture surgery. CHO TF: choline diet + tibial fracture surgery. * $p < 0.05$, Tukey HSD. Each value represents the mean ± SEM.

3.2.9 Aberrant neuronal migration

Aberrant neuronal migration was measured by counting DCX+ cells in the hilus of the hippocampus (ectopic granule cells). Compared to mice that received control diet but otherwise were left naïve, tibial fracture led to a significant increase in hilar granule cells ($F_{2,36} = 7.09$, $p < 0.01$, Figure 29). Dietary choline supplementation partially mitigated this increase. No differences in hilar granule cells were seen due to time point ($F_{1,36} = 1.69$, $p > 0.05$) or an interaction between treatment and time point ($F_{2,36} = 1.50$, $p > 0.05$) were observed. Across both time points, tibial fracture increased the number of ectopic granule cells, which is partially mitigated by choline, which could be a mechanism behind impairments in the NOR task due to tibial fracture surgery.

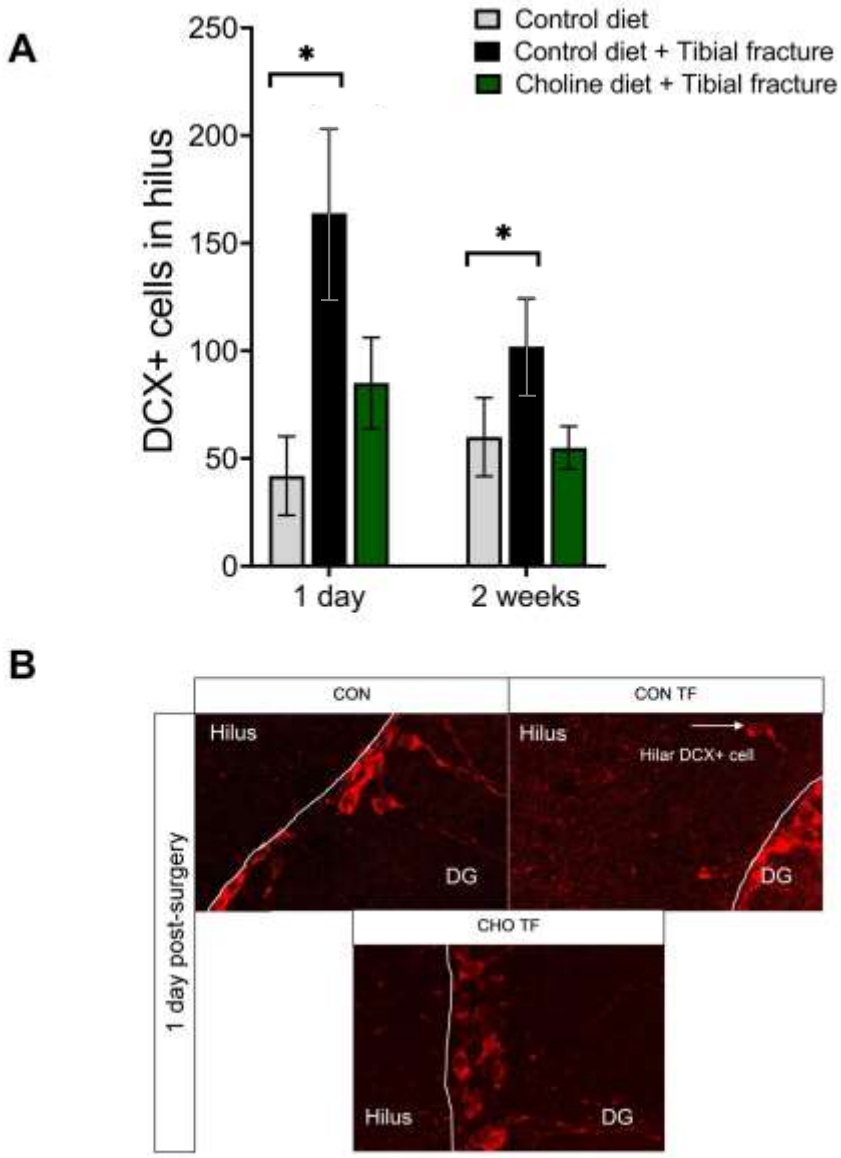


Figure 29: Tibial fracture surgery and choline diet effects on hilar neurons. (A) Histogram showing the number of DCX+ cells in the hilus of the dentate gyrus. N = 7 per group. (B) Representative fluorescent micrographs of the SGZ taken two weeks after surgery, immunostained with DCX in mice given control diet and left naïve (upper left), given control diet and administered a tibial fracture (upper right, with a hilar neuron noted), and given choline-supplemented diet and administered a tibial fracture (bottom). Each image is 150 μm in width. CON: control diet + naïve. CON TF:

control diet + tibial fracture surgery. CHO TF: choline diet + tibial fracture surgery. * $p < 0.05$, Tukey HSD. Each value represents the mean ± SEM.

3.3 Discussion

Astonishingly, 2 weeks after tibial fracture, hippocampal-dependent memory was still impaired. Adult dietary choline supplementation prior to and following surgery prevented the memory impairments that occurred both 1 day and 2 weeks following surgery. This data on the cognitive protection of dietary choline supplementation exemplifies that this mouse model is capable of mirroring the long-term POCD seen in humans (Monk et al., 2008). Additionally, the largest neural effects seen as a result of tibial fracture and dietary choline supplementation are in astrocytic density, DCX+ cells in the SGZ, and DCX+ cells in the hilus. How each of these factors may be a likely candidate mechanism for POCD will be explored, in addition to other neural correlates of hippocampal-dependent memory.

The interaction between microglia, astrocytes, and neurons has been analyzed previously in the context of POCD (reviewed in Lin et al., 2019). Our findings of both slight microglial and robust astrocytic activation support previous work showing the synergistic activation between microglia and astrocytes. Due to conflicting evidence, whether microglia activate astrocytes (Abudara et al., 2015) or astrocytes activate microglia (Xu et al., 2017) is not clear. However, the current research shows significant upregulation of astrocytic density due to tibial fracture and no significant differences in microglia, supporting the astrocytic hypothesis of POCD. Additionally, much research

on the effects of these two neuroimmune cell types on neurons in POCD (reviewed in Lin et al., 2019) focuses on neuronal death. Our findings show the opposite: tibial fracture leads to an increase in cell proliferation and the number of young neurons.

Though hippocampal-dependent memory impairment was prevalent at both time points examined, many of the differences in neural cellular measures differed based on the time point assessed. Specifically, increases in astrocytic density and cellular proliferation were seen 1 day after surgery, but increases in DCX+ cells in the SGZ were seen 2 weeks after surgery. Some alterations were persistent through both time points, such as hilar DCX+ cells and BrdU+/DCX+ cells. Critically, the largest neuroimmune effect of astrocytic activation was found 1 day after surgery, supporting the view that POCD-associated neuroinflammation is transient. This research supports a dichotomous mechanism, in which immediate neuroinflammation is responsible for short-term impairments and some other mechanism - which may be reactive neurogenesis, delayed neuronal maturation, decreased cell survival, or hilar granule cells - may be responsible for the longer-term cognitive impairment seen in these studies.

3.3.1 Hippocampal-dependent memory

Not only is POCD present one day after surgery, impairments in the NOR are also observed two weeks after surgery. The tibial fracture model is hence an applicable model for the many people who have persistent POCD (Monk et al., 2008). With these findings showing a long-term hippocampal impairment, further studies can build off of

the current findings to explore the mechanism of this behavioral impairment. Additionally, dietary choline supplementation mitigates the tibial fracture-induced memory impairments both short- and long-term, further solidifying the nutrient as a possible preventative remedy for substantive POCD.

A critical behavioral finding in the current work points to specific hippocampal actions of dietary choline: tibial fracture led to decrease in percent time spent in the center of an open field, and dietary choline supplementation did not prevent this decrease. Therefore, while choline supplementation mitigated the deficits in hippocampal-dependent memory caused by the tibial fracture, this treatment did not prevent the tibial fracture-induced anxiety-related behavior. Because anxiety begins with increased excitation in the central amygdala (Ahrens et al., 20118), these findings suggest that choline is acting via a hippocampal mechanism. However, analysis of other regions, such as the amygdala, would be a direction to confirm this hypothesis.

3.3.2 Astrocytes

Consistent with previous results, peripheral surgery led to an increase in immune activation in the hippocampus. Previous work has implicated astrocytes in POCD (Xu et al., 2017). Specifically, the chemokine CCL2, expressed by astrocytes, causes microglial activation and cognitive dysfunction following tibial fracture surgery (Xu et al., 2017). This specific chemokine has been shown to be downregulated by cholinergic agonists in peripheral tissues (Wang et al., 2011; Li et al., 2018). The current

findings support this astrocyte-dependent hypothesis: upstream of microglial activation, tibial fracture increases astrocytic density, which may indicate higher activation and/or numbers of astrocytes.

Additionally, adult dietary choline supplementation significantly prevented the increase in GFAP antigen density. This is consistent with previous work indicating that the central "cholinergic anti-inflammatory pathway" also includes astrocytes. For example, stimulation of $\alpha 7$ nicotinic acetylcholine receptors specifically on astrocytes decreases pro-inflammatory cytokines (Patel et al., 2017). This blunting was reversed with a $\alpha 7$ nicotinic acetylcholine receptor antagonist, indicating the critical importance of choline's actions on astrocytes in blunting neuroinflammation and its detrimental consequences.

3.3.3 Hilar granule cells

The current research showed significant effects of a non-seizure stimulus on hilar granule cells. Status epilepticus leads to a dramatic increase in neurogenesis, and some of these new neurons migrate not along the granule cell layer but instead enter the hilus (Parent et al., 1997; reviewed in Scharfman et al., 2007). These hilar granule cells are electrophysiologically different from non-ectopic granule cells, and lead to more excitable circuits, further perpetuating epilepsy and likely memory impairments seen with this disorder (Cameron et al., 2011). Though tibial fracture did not induce seizures, it did induce a persistent neuronal change that might make these mice more prone to

seizures later in life. Though the exact mechanism of how these cells migrate aberrantly has yet to be pinpointed, some suspected mechanisms are doublecortin itself (which is needed for neuronal migration; Jessberger et al., 2005), pro-inflammatory cytokines (Monje et al., 2003), and astrocyte activation, which has been shown to inhibit neuronal migration (Kaneko et al., 2018). Previous work has shown that status epilepticus leads to persistent impairments in hippocampal-dependent memory (Wong-Goodrich et al., 2010). Aberrant neuronal migration could possibly be the cause of these deficits (Scharfman et al., 2000), lending support for the hypothesis that these ectopic granule cells and their aberrant neural circuitry underlie the cognitive deficits seen following tibial fracture.

Though no work has been done on the potential effects of a cholinergic agonist on reactive neurogenesis and subsequent migration of granule cells in the hippocampal hilus, one study has examined the effect of a prenatal choline-supplemented diet on status epilepticus and the associated neuronal and behavioral deficits in rats (Wong-Goodrich et al., 2010). The prenatal diet rescued the memory impairments, but did not rescue the increase in hilar granule cells. No other work has been done pertaining to this nutrient and hilar granule cells, and it is likely that the mechanisms of behavior are different based on the time of choline administration. Choline supplementation prior to and following surgery blunted the increase in hilar granule cells that occurs after tibial fracture surgery. DCX+ cells are not stem cells, but are young neurons. For this reason,

these hilar granule cells are likely already-born neurons that are exhibiting a "hijacked" migration pattern.

3.3.4 DCX+ cells

Though previous work has analyzed hippocampal DCX in the tibial fracture model of POCD (Zhang, Barde et al., 2016), the current work assesses it at a longer time point, and the first to find increases in DCX+ cells due to peripheral surgery. Though the current work found no differences at the one day time point in this measure, previous work has shown a decrease in DCX+ cells in the SGZ one day after tibial fracture (Xiong, Zhang et al., 2018). Such a swift decline implies a death of these neurons – in the current work, we did not detect significant hippocampal neuronal death immediately after surgery. Instead, our results suggest that tibial fracture causes reactive neurogenesis, which is consistent with models of neural injury. For example, traumatic brain injury results in significant increases in DCX+ cells in the rat dentate gyrus (Bregy et al., 2012), and neuroinflammation (reviewed in Mayer et al., 2013). Therefore, one possible reason why we see more young neurons after tibial fracture is that the heightened neuroinflammation that occurs after peripheral surgery leads to a decrease in apoptosis of young neurons or a rapid maturation of newly-born neurons, leading to a long-term increase. The anti-inflammatory actions of supplemental choline may prevent this decrease or delay apoptosis due to surgery. Alternately, dietary choline supplementation may promote cell survival via p53 and NF-κb (Holmes-McNary et al.,

2001). Dietary choline deficiency leads to an increase in cell death via two separate molecular pathways. Inhibition of phosphatidylcholine, a metabolite of choline, has also been directly linked to cell death (reviewed in Cui & Houweling, 2002). Additionally, choline supplementation has been shown to prevent cell death due to ischemia in rats (Borges et al., 2014). These findings support those presented in the current study, indicating that choline may prevent apoptosis.

An alternative explanation for the increase in DCX+ cells is not proliferation, but instead a blunting of maturation. DCX is a marker for *young* neurons. These neurons, under non-pathological conditions, mature into "fully-grown" neurons that express other markers, like NeuN (Shepherd et al., 2016). An increase in DCX+ may mean there is a developmental block; the same rate of neurogenesis is occurring, but because fewer of these cells are maturing, there is a "buildup" of these young cells. Further study analyzing the lifespan of these neurons, using markers for neuronal stem cells and mature neurons, is needed to confirm whether this is a neurogenesis effect or a blunting of maturation.

Another critical aspect of this finding is the timing of it. Unlike the other significant effects reported here, an increase in DCX+ cells was observed only 2 weeks after surgery. For this reason, it is a possible mechanism for long-term POCD: an increase in DCX+ cells, either from reactive neurogenesis or delay of neuronal

maturation, promote too much forgetting (Akers et al., 2014) and impede hippocampal-dependent memory.

3.3.5 Cell proliferation and survival

Though chronic inflammation leads to a decrease in adult hippocampal cell proliferation, acute inflammation increases cell proliferation (reviewed in Whitney et al., 2009). While brain traumas such as seizure (Cho et al., 2015) and traumatic brain injury (Bregy et al., 2012) result in reactive hippocampal neurogenesis, these findings may be the first to document reactive cell proliferation following tibial fracture. Consistent with this upregulation, previous work has shown an increase in BDNF due to tibial fracture surgery (Zhang, Barde et al., 2016). Though an modest increase in hippocampal neurogenesis has widely been associated with increased memory capacity (reviewed in Frankland et al., 2013), our findings show that this type of reactive cell proliferation may be more akin to the very large increases in neurogenesis seen after seizure (Cho et al., 2015), which lead to memory impairment. Additionally, though adult dietary choline has been shown to increase cell proliferation (Wong-Goodrich, Glenn et al., 2008), it has also been shown to prenatal choline in a seizure model has been shown to blunt the upregulation of cell division (Wong-Goodrich, Mellott et al., 2008), similar to the findings presented here. However, in the current study, these conflicting results do correlate with memory impairment and rescue, and hence the increase in cell proliferation may be one mechanism for short-term POCD.

While cell proliferation in the dentate gyrus was increased shortly after tibial fracture surgery, many of these cells died in the next two weeks. But, this apoptosis was blunted in mice consuming the high-choline diet. This finding supports previously established work indicating that pro-inflammatory cytokines from activated microglia decrease cell survival (Monje et al., 2003).

3.3.6 Microglia

Our findings showed a slight increase in activated microglia, quantified as the percent of total in "round" and "stout" microglial phenotypes one day after surgery. These differences resolved by 14 days post-surgery. As well, a slight increase in infiltration of peripheral macrophages was found after surgery, but this was not revealed until the two-week timepoint. In these effects we find possible candidate mechanisms for the short- and long-term effects of tibial fracture, respectively. Perhaps the POCD seen at 1 day post-surgery is due to microglial activation, and the POCD at 2 weeks post-surgery is due to infiltrating macrophages. Previous work has associated postoperative memory deficits to infiltrating macrophages from the periphery due to the breakdown of the blood-brain barrier (Terrando et al., 2011). Critically, this phenomenon is ameliorated after microglial ablation, indicating that the process of macrophage infiltration due to injury relies on microglia, the resident macrophages of the brain (Feng et al., 2017). An important conclusion of this body of work is that the activation of microglia precedes the infiltration of macrophages. Though both contribute to the

neuroinflammation after surgery, they occur at different timepoints. Dietary choline supplementation partially mitigates both effects, indicating a possible means by which choline might protect the hippocampus and hippocampal-dependent memory from the deleterious, short- and longer-term, neuroimmune effects of peripheral tibial fracture.

Our findings also support our hypothesis that a choline-supplemented diet blunts the neuroinflammation one day after surgery. A bolus dose of a cholinergic agonist, or of choline itself, completely prevented the short-term increase in CD11b immunoreactivity, a marker for macrophage activation, after tibial fracture surgery (Terrando et al., 2011; 2014). Activation of the α7 nicotinic acetylcholine receptor on macrophages and microglia leads to decreased inflammatory cytokine production (Steinberg et al., 2016; reviewed in Maurer & Williams, 2017). Specifically, cholinergic agonists act on astrocytic α7 nicotinic acetylcholine receptors to blunt pro-inflammatory cytokine expression (Revathikumar et al., 2016). Cholinergic blunting of neuroinflammation in microglia and astrocytes has been found previously (Terrando et al., 2011; Revathikumar et al., 2016; reviewed in Maurer & Williams, 2017). However, previous work used pharmaceutical agents - this study is the first to use dietary choline supplementation to as an anti-inflammatory agent in the tibial fracture mouse model.

3.3.7 Infiltrating macrophages

An increase in infiltrating macrophages was observed 2 weeks after peripheral surgery. Consistent with previous findings implicating microglia as the necessary

catalyst for peripheral immune cell infiltration (Prinz & Piller, 2017), microglial activation was observed 1 day after surgery. Because the pattern of infiltrating macrophages was similar to the pattern of memory impairments, and because dietary choline supplementation reduced macrophage infiltration and improved hippocampal-dependent memory, macrophage infiltration may be one step in the mechanism by which dietary choline rescues behavior. However, further mechanistic studies are required to support this hypothesis.

One limitation of the current study is that we only studied male mice. Therefore, these findings may or may not be generalizable to females. However, a recent study (Schenning et al., 2019) reported that there are minimal sex difference in the occurrence of POCD in humans. A second limitation of this work is that we examined young adult mice, not aged mice. POCD is particularly exacerbated with age (Hovens et al., 2015). Nevertheless, it was impressive that we saw substantial hippocampal-dependent memory loss following tibial fracture in young adult mice. With an increase in POCD in older individuals, we would expect to see an even greater loss of function. Whether or not choline supplementation might rescue this memory loss in a more susceptible population is an important area for future research.

In summary, these findings provide interesting new evidence that there may be multiple neural mechanisms underlying the short- and longer-term cognitive impairment seen after tibial fracture surgery. These data also provide encouraging

evidence that consuming a choline-rich diet both before and after peripheral surgery may be a sufficient treatment to combat POCD.

4. Aim 3: Perinatal choline "programming" and adult POCD

Choline is an essential nutrient for neural development. It is a required precursor to the neurotransmitter acetylcholine (Blusztajn & Wurtman, 1983), is an essential component of all cellular membranes (Blusztajn et al., 1987), and donates methyl groups for epigenetic modifications (Niculescu & Zeisel, 2002), making it indispensable for proper brain development. As a possible indicator of choline's critical importance in development, choline is preferentially transported to the fetus via the placenta during pregnancy, depleting maternal stores (Sweiry et al., 1986). Choline sufficient for fetal development is not adequately produced by *in vivo* synthesis in the liver and possibly fetal brain (Zeisel et al., 1995); hence, dietary choline is needed for optimal fetal development.

In addition to the basic requirement of this nutrient for fetal development, additional choline supplementation above the current guidelines appears to be neuroprotective. In fact, there is considerable evidence that nutritional supplementation with choline during prenatal and early postnatal life provides substantial lifelong protection against various brain assaults. For example, numerous studies have shown that prenatal choline supplementation alone can completely protect rodents from age-related cognitive decline (Meck & Williams, 2003). Perinatal choline supplementation also dampens hippocampal amyloid-β in an Alzheimer's disease model (Mellott et al.,

2017), rescues hippocampal-dependent memory impairments in a Down syndrome model (Velazquez et al., 2013) and status epilepticus model (Wong-Goodrich et al., 2010; Holmes et al., 2002).

We hypothesize that perinatal choline may be altering the developing microglial response to adult inflammatory events. Choline is anti-inflammatory (Pavlov et al., 2003; Mehta et al., 2010; Terrando et al., 2011), and can directly affect immune cells via the nicotinic acetylcholine receptors on macrophages (Báez-Pagán et al., 2015), microglia (Shytle et al., 2004), and astrocytes (Shen & Yakel, 2012; Liu et al., 2014). We have shown (chapter 2: aim 1, page 30) that prenatal choline supplementation can decrease neuroinflammation in the fetal hippocampus, but not in other brain regions. For this reason, we hypothesize that perinatal choline may be "priming" the neuroimmune system to react differently to an adult immune assault. In aim 2, we show that when mice consume a choline-supplemented diet for 3 weeks before and following tibial fracture surgery, they are protected against surgery-induced hippocampal-dependent memory deficits, hippocampal neuroinflammation, and alterations in DCX expression.

Post-operative cognitive dysfunction (POCD) occurs when increased neuroinflammation due to peripheral surgery leads to impaired cognition. Tibial fracture leads to increased macrophage number in the hippocampus, increased pro-inflammatory cytokines, and impaired hippocampal-dependent memory (Terrando et al., 2011). The resulting neuroinflammation is tied to the cognitive deficits seen in POCD

(Barrientos et al., 2002; Pugh et al., 2001; Terrando et al., 2011). Critically, both a bolus dose of a cholinergic agonist (Terrando et al., 2011) and 3 weeks of choline-supplemented diet (chapter 3: aim 2, page 68) prevented both the cognitive and neuroinflammatory impacts of tibial fracture surgery in the hippocampus. Because it is characterized by behavioral deficits directly tied to neuroinflammation, and because these effects can be prevented by alterations in the cholinergic system, we used the tibial fracture model to analyze the long-term developmental effects of cholinergic neuroprotection in this study.

Very little work has focused on perinatal choline supplementation with a neuroimmune lens. However, two studies support our hypothesis that perinatal choline supplementation is capable of priming the fetal neuroimmune system. Wu and colleagues analyzed the fetal neuroimmune impact of dietary maternal choline supplementation (Wu et al., 2015). After an immune challenge, maternal dietary choline supplementation protected the fetal brain from heightened IL-6. A second study analyzed microglial activation in an Alzheimer's disease model (Velazquez, Ferreira, Winslow et al., 2019). Prenatal choline supplementation reduced the characteristic microglial activation in adult mice due to Alzheimer's disease. Unlike the first study described here, Velazquez and colleagues analyzed microglia from adult mice - further indicating that prenatal choline supplementation can "program" the developing immune system to prevent excessive activation in adulthood. In addition to these

findings pertaining to microglia, there is some evidence that prenatal choline supplementation actually has an anti-inflammatory effect on astrocytes after adult status epilepticus (Wong-Goodrich et al., 2010). Currently there are no studies on perinatal choline and peripheral macrophage infiltration due to a peripheral immune assault. Far more work is required to understand the effect of perinatal choline supplementation on neuroimmune cells in the context of long-term POCD.

We hypothesize that perinatal choline supplementation may be altering neurogenesis after an immune assault as a possible mechanism of neuroprotection. One additional possible mechanism behind the hippocampal protection accompanied by perinatal choline supplementation is hippocampal neurogenesis, which has previously been closely tied to performance in hippocampal-dependent tasks (Alam et al., 2018). However, the findings regarding the effects of prenatal choline on neurogenesis have varied. Previous work has indicated that prenatally supplemented rats generate more young neurons in mid-life and old age than rats fed a standard diet (Glenn et al., 2007; 2008; McCall et al., 2015; Wong-Goodrich et al., 2010). In younger rats, when rates of neurogenesis are still very hard, this increase is harder to detect, likely due to a ceiling effect (Nickerson et al., 2017). One goal of this study is to determine whether early-life dietary choline supplementation is sufficient to protect mice from tibial fracture-induced memory loss and to explore the underlying neural mechanisms.

To further assess the underlying mechanisms behind long-term POCD and the possible lifelong protection by perinatal choline supplementation, we assessed hippocampal-dependent memory, neuroinflammation, and neuronal alterations 2 weeks after tibial fracture in male mice. Considering that a choline-rich diet is anti-inflammatory and neuroprotective in adulthood (chapter 3: aim 2, page 68), and considering the evidence of the fetal immune system reacting to maternal choline supplementation (chapter 2: aim 1, page 30), we hypothesize that neuroimmune cells can be "programmed" by maternal choline supplementation to prevent cognitive and neuroimmune effects of adult tibial fracture surgery. The hypothesis in this study is that perinatal choline supplementation alone will prevent the hippocampal cognitive impairments, neuroimmune activation, and alterations in DCX expression 2 weeks after peripheral tibial fracture surgery.

4.1 Methods

This experimental design was 2 perinatal dietary treatments (control diet, choline diet) X 2 surgical treatments (naïve, tibial fracture). Mice were behaviorally tested and sacrificed 2 weeks after surgery. This time point was chosen to further explore the mechanisms of long-term POCD. These experiments were conducted with the approval of the Duke University Institutional Animal Care and Use Committee.

4.1.1 Mice

C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, USA) were assigned to harem breeding groups (two females, one male). All mice were bred, raised, and housed in the Duke University vivarium. Once dams were confirmed to be pregnant by noting a significant increase in body weight, they were separated from the male to give birth and raise pups individually. Once weaned, male offspring were housed in groups of 2-5 with littermates.

Within a litter, a maximum of four males were used for the current experiments. Two pups were used for the behavioral experiments: one received tibial fracture, and the other remained naïve. Two other pups were used for immunohistochemistry experiments and received BrdU injections: one received tibial fracture, and the other remained naïve. Pups from 12 litters consumed the synthetic control diet until weaning, and pups from 14 litters consumed the choline-supplemented diet until weaning.

4.1.2 Dietary conditions

Once placed in breeding cages, dams were assigned to one of two *ad libitum* diet conditions a synthetic control chow (1.1 g/kg choline chloride in formula AIN-76A with choline chloride substituted for choline bitartrate, DYET #110098, Dyets, Inc., Bethlehem, PA, USA), or the same diet with 4.95 g/kg choline chloride (DYET #110210). The diet condition of each cage was randomly determined. Dams remained on these diets until offspring were weaned, at which time male offspring were transitioned to a standard

rodent chow (2.2 g/kg choline chloride; PicoLab Mouse Diet 5058, Lab-Diet, Philadelphia, PA, USA). Therefore, the mouse subjects had different diets throughout fetal and postnatal life until weaning at postnatal day 21.

4.1.3 Tibial fracture surgery

At 12 weeks of age, male offspring were assigned to either a naïve group (no anesthesia or surgery) or a tibial fracture group. Mice in the tibial fracture group were given 2.1% isoflurane in 0.30 FiO_2. Mice received a tibial fracture of the left hind paw with an intramedullary fixation as previously described (Xiong, Zhang et al., 2018). Briefly, the left hind paw was coated in Nair (Church & Dwight Co., Inc., Ewing, NJ, USA) to remove fur for 30 seconds, after which the Nair was cleaned off. The paw was disinfected with povidone iodine (Dynarex, Orangeburg, NY, USA) while on a heating pad. Mice were then subcutaneously injected with buprenorphine-SR (0.1 mg/kg; ZooPharm, Laramie, WY, USA). Bupivacaine (0.25%; McKesson, Irving, TX, USA) was administered directly to the incision site just prior to surgery. A median incision on the left hind paw was performed. A 0.38 mm pin was inserted into the intramedullary canal. Then, the periosteum was stripped, and the tibia was fractured using wire cutters. Post-fracture, skin was sutured. Mice were allowed to recover from anesthesia alone in an empty clean cage under observation, and were carefully observed twice daily during recovery.

4.1.4 Behavioral testing

Mice in the behavioral experiments were tested in an open field and novel object recognition task 2 weeks after surgery. Novel object recognition (NOR) testing was administered using a modified version of a previously published protocol (Leger et al., 2013). This task is dependent on the hippocampus when the inter-trial interval is more than 10 minutes (Cohen & Stackman, 2015). This task was chosen because it does not elicit a stress response, and can be refined to assess subtle differences in hippocampal-dependent pattern recognition ability (Tognoni, 2014). Mice in all diet and surgery groups were allowed to habituate for 5 minutes in an empty plexiglass arena covered on the outside with white paper one day before testing. For a half hour before habituation, mice were placed in individual cages identical to their home cages with corncob bedding in a quiet, dark anteroom. These mice had *ad libitum* rodent chow and water bottles. The testing room itself was kept in dim light, in which the ceiling light bulbs were distributed to ensure even light distribution. Light bulbs were a warm white light with 3000 kelvin color temperature. The testing room was kept entirely quiet during testing. A plastic tube was used to transport mice from room to room to minimize stress (Hurst & West, 2010). Mice then underwent 4 trials, each consisting of 5 minutes of automated motion tracking (HVS Image, Buckingham, UK) and video recording in a 406.4mm L x 203.2mm W x 254mm H Plexiglass arena on a white table 762mm above the ground. A tubular spirit level was utilized at the beginning of each testing day to ensure the table

and arena were not slanted. The arena walls were opaque to prevent possible distractions and navigational cues. Visible through the bottom of the arena was a grid dividing the floor into eighteen 63.5mm squares. Objects were secured to the floor with double-sided tape. Between each trial, the arena and objects were disinfected with 70% ethanol. Inter-trial interval was approximately 30 minutes. The four trials were habituation [in an empty arena, during which open field (OF) data was collected], training 1 (with two identical objects, Figure 17C), training 2 (with the same identical objects), and test (with one of the objects replaced with a novel object). Objects were no taller than 50 mm and were counterbalanced within each group. Objects were wooden and painted high-contrast colors, such as napkin rings and miniature candlestick (Figure 17C). Data for the open field was quantified as the distance traveled (activity), and percent of time spent in the inner 4 squares of the arena grid (anxiety-related behavior, reviewed in Prut & Belzung, 2003). Data for the NOR was quantified as percent of time spent in each third of the arena (i.e., area with or without objects) and was verified for accuracy using video recordings.

4.1.5 BrdU injection

In order to label dividing cells, all mice used in the immunohistochemistry experiments were given two bromodeoxyuridine (BrdU; ThermoFisher Scientific, Waltham, MA, USA) injections: 1 hour and 10 hours after surgery. Each injection contained 200 mg/kg of BrdU in 0.9% saline (Balu et al., 2009).

4.1.6 Immunohistochemistry

Mice were sacrificed 2 weeks after surgery. Under anesthesia, the thoracic cavity was opened, and the right atrium of the heart was cut. A solution of ice-cold 0.1M phosphate-buffered saline (PBS) was perfused into the left ventricle of the heart. The brains were harvested and fixed in 4% paraformaldehyde for 3 days, then cryoprotected in 30% sucrose for 3 more days. Then, brains were freeze-mounted in Tissue-Tek optimum cutting temperature (OCT) compound (VWR, Radnor, PA, USA), and sliced on a Leica CM 1850 cryostat (Leica Biosystems, Buffalo Grove, IL, USA) at -20°C in 40μm sections in a series of four. Free-floating sections were in PBS with 0.1% sodium azide preservative (VWR).

Sections that included the dorsal dentate gyrus (DG) of the hippocampus (bregma -1.28 through -2.12; Rosen et al., 2000) were washed with phosphate buffered saline (PBS) before quenching with a solution of 50% methanol (VWR) and 3% hydrogen peroxide (VWR) for 30 minutes. Sections were washed again in PBS and blocked in a solution of 5mL 0.01M PBS, 150μL normal donkey serum (Jackson ImmunoResearch, West Grove, PA, USA), and 50μL TX100 (Sigma-Aldrich, St. Louis, MO, USA) at 20°C for one hour and rinsed again in PBS. Sections were incubated at room temperature in one of four groupings of primary antibodies:

1. 1:1000 concentration of goat anti-Iba1 (Novus Biologicals, Littleton, CO, USA), a 1:2000 concentration of rabbit anti-P2Y12 (Anaspec Inc., Fremont, CA, USA), and a 1:500 concentration of rat anti-cluster of differentiation 68 (CD68; BioLegend, San Diego, CA, USA);
2. 1:500 concentration of rabbit anti-glial fibrillary acidic protein (GFAP; Santa Cruz Biotechnology, Inc., Dallas, TX, USA); or
3. 1:200 concentration of rabbit anti-doublecortin (DCX; Cell Signaling Technologies, Danvers, MA, USA);
4. 1:500 goat anti-bromodeoxyuridine (BrdU; Abcam, Cambridge, UK).

The next day, sections were washed in PBS and incubated for two hours in secondary antibody of donkey anti-rabbit Alexa 647 (ThermoFisher), donkey anti-goat 488 (ThermoFisher), and/or donkey anti-rat 547 at a 1:200 concentration. Sections were washed in PBS before they were mounted onto slides. Slides were coverslipped using a fluorescent medium (Vectashield; Vector Labs, Burlingame, CA, USA), and sealed with nail polish.

Images were collected on a Zeiss SP8 microscope (Zeiss, Oberkochen, Germany) in the Duke University Light Microscopy Core Facility. Leica LAS AF Lite software (Leica Biosystems, Buffalo Grove, IL, USA) was used to visualize images. Images were taken as 150 μm x 150 μm "tiles," which were then merged together to view the entire

hippocampus or cortex. The quantification procedures were: exhaustive manual cell counting (P2Y12-/Iba1+ cells, DCX+ cells in the hilus, BrdU+ cells); systematic random sampling of tiles, with a section interval of 4 (microglial morphology, DCX+ cells in the subgranular zone); or densitometry (GFAP antigen density, as previously described in chapter 3: aim 2 and Bilbo & Tsang, 2010) using ImageJ software (NIH, Bethesda, MD, USA).

4.1.7 Statistical analysis

Data were analyzed using a repeated measures, one- or two-way ANOVA and Tukey HSD post-hoc comparisons using JMP Pro version 12.2 (SAS Institute, Cary, NC, USA), where appropriate. All graphs were generated using Prism version 8.3.0 (GraphPad, San Diego, CA, USA) using XY (body weight) and grouped (all other metrics) table formats.

4.2 Results

4.2.1 Maternal weight and consumption

There were no group differences in maternal weight gain throughout pregnancy ($F_{1,34} = 0.84$, $p > 0.05$, Figure 30). Interestingly, there was a significant difference in food consumption; in a one-way ANOVA, choline-supplemented dams ate more food than dams on the synthetic control diet ($F_{1,474} = 11.34$, $p < 0.01$, Figure 31).

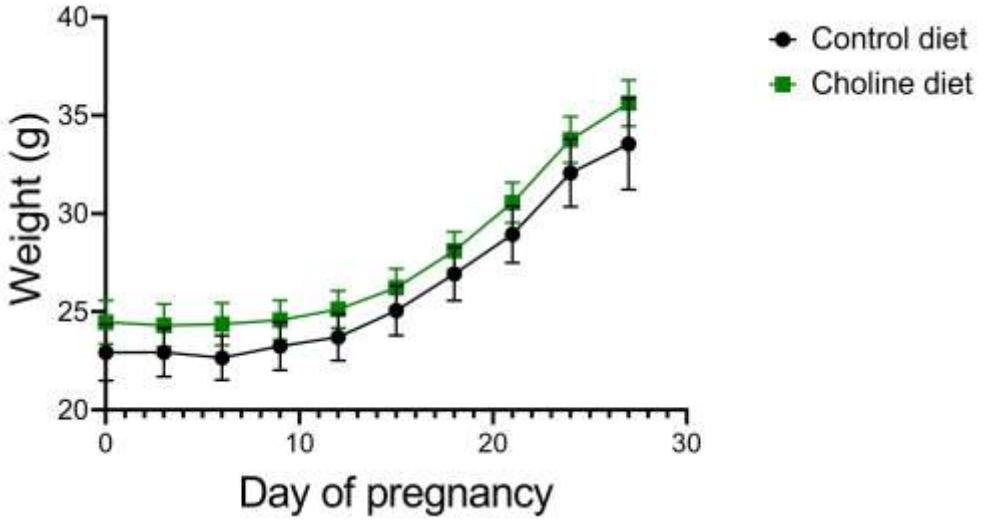


Figure 30: No differences of choline supplementation on maternal weight gain during pregnancy were observed. N = 13-15 pregnancies per group. Each value represents the mean ± SEM.

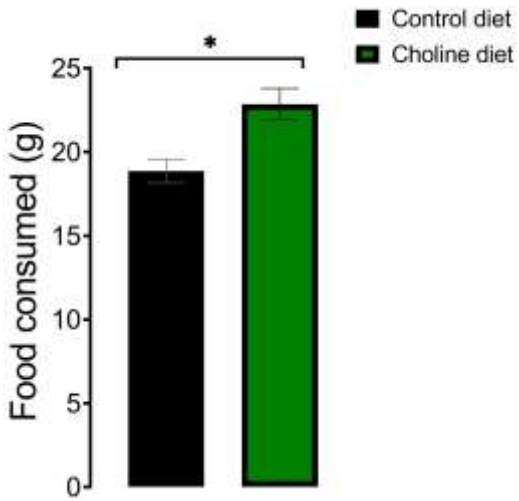


Figure 31: Choline-supplemented dams consumed significantly more food than dams on the synthetic control diet. * $p < 0.05$, one-way ANOVA. Each value represents the mean ± SEM.

4.2.2 Activity and anxiety-related behavior in an open field

Activity was measured by the total distance traveled in meters during the 5 minute trial in an empty arena. No differences were found in a two-way ANOVA due to perinatal diet ($F_{1,41} = 0.56$, $p > 0.05$, Figure 32), surgery ($F_{1,41} = 0.004$, $p > 0.05$), or a diet x surgery interaction ($F_{1,41} = 0.23$, $p > 0.05$). Consistent with findings in aim 2, no differences in activity due to tibial fracture were observed. As well, perinatal choline supplementation did not alter activity.

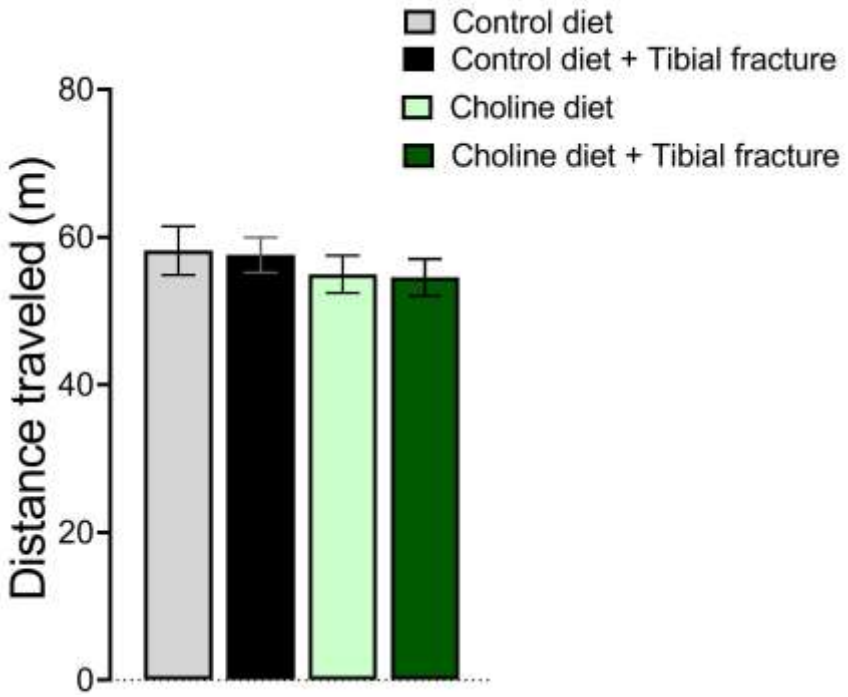


Figure 32: Tibial fracture surgery and perinatal choline diet exhibited no effects on activity in an open field. Histogram showing the distance traveled in meters over the 5 minute trial. N = 10-13 per group. Each value represents the mean ± SEM.

Anxiety-related behavior was assessed by the percent of time spent in the center of the arena during the open field trial. No significant effects were found due to

perinatal diet ($F_{1,41} = 0.005$, $p > 0.05$, Figure 33), surgery ($F_{1,41} = 0.001$, $p > 0.05$), or a diet x surgery interaction ($F_{1,41} = 1.85$, $p > 0.05$) in a two-way ANOVA. In contrast to the significant decrease in percent time spent in the center of the arena due to tibial fracture in aim 2, no differences in anxiety-related behavior were observed. Notably, the largest difference between the two aims in this measure is that mice given prenatal control diet and left naïve exhibit more anxiety-related behavior than mice given adult control diet and left naïve.

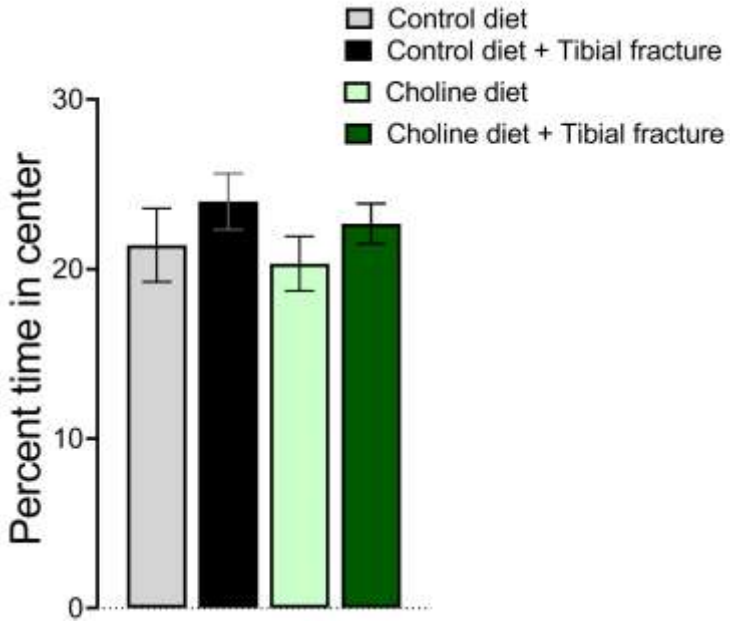


Figure 33: Tibial fracture surgery and perinatal choline diet did not alter the percent time spent in the center of the arena. Histogram showing the percent of time spent in the center of the arena during the 5 minute trial. N = 10-13 per group. Each value represents the mean ± SEM.

4.2.3 Novel object recognition

To assess hippocampal-dependent memory, a novel object recognition (NOR) paradigm was administered 2 weeks after surgery (Figure 34A). The discrimination index (DI: (Novel object time – Old object time)/(Novel object time + Old object time)) was used to assess whether mice spent more time with the novel object (Ennaceur & Delacour, 1988). A two-way ANOVA showed a significant main effect of diet ($F_{1,37} = 6.36$, $p < 0.05$, Figure 34). Perinatally choline-supplemented mice performed significantly better on the NOR task. Though no differences were found due to surgery ($F_{1,37} = 2.27$, $p > 0.05$), a significant interaction of diet and surgery was found ($F_{1,37} = 6.33$, $p < 0.05$). Tukey HSD post-hoc comparisons revealed that both choline-supplemented groups had significantly higher DI's than the control diet + tibial fracture group (Figure 34). Perinatal choline supplementation significantly blunts the memory impairment caused by tibial fracture.

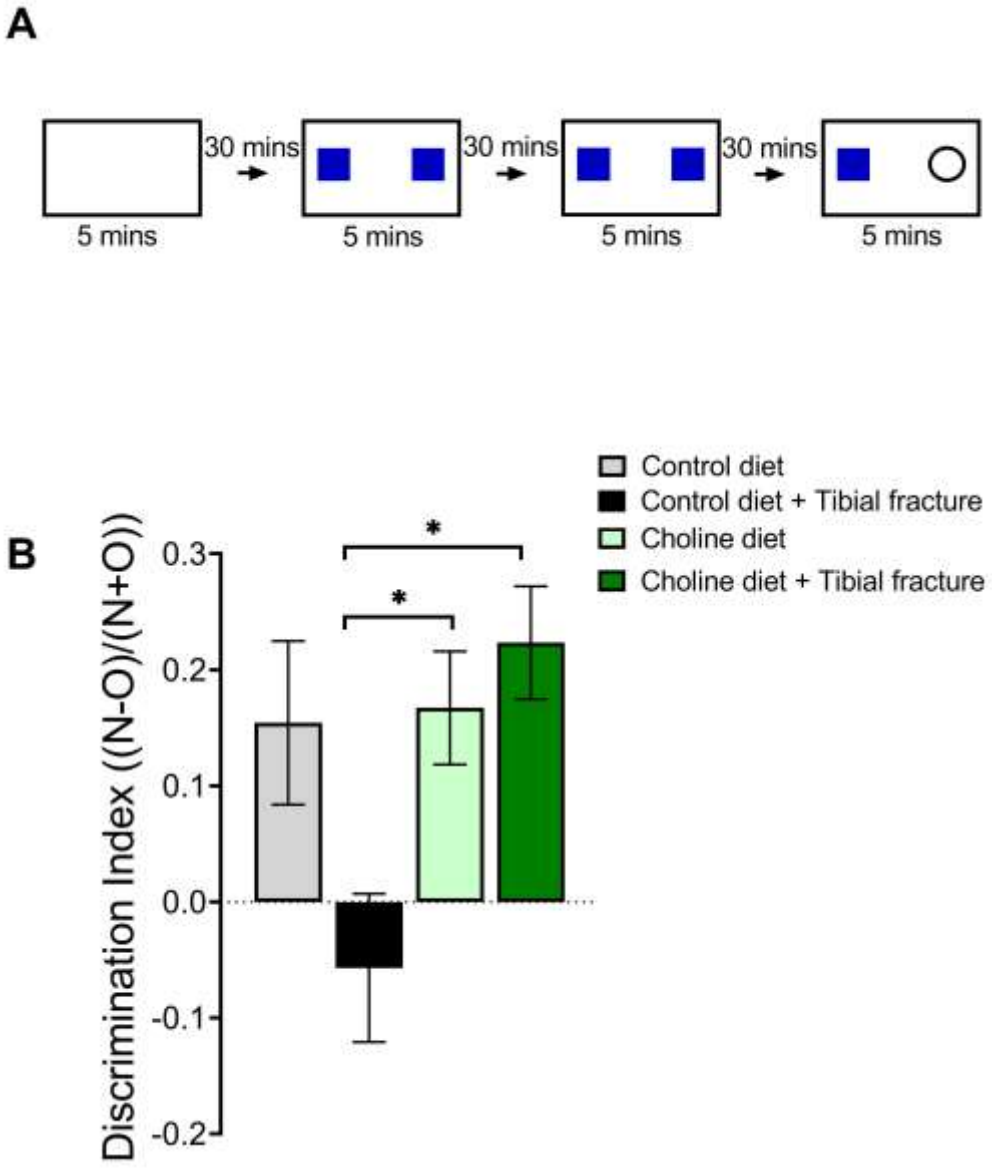


Figure 34: Tibial fracture and perinatal choline supplementation effects on discrimination index in a novel object recognition (NOR) test. (A) Schematic indicating NOR protocol. (B) Histogram showing discrimination index ((Novel object time – Old object time)/(Novel object time + Old object time)) 2 weeks after surgery. N = 8-12 per group. * $p < 0.05$, Tukey HSD. Each value represents the mean ± SEM.

4.2.4 Microglial activation and number

4.2.4.1 Dentate gyrus

Microglial activation was quantified as the percent of total microglia quantified as "round" or "stout." For microglial and macrophage analysis, the choline diet + naïve group was excluded due to the lack of effects in GFAP, the most likely candidate mechanism from aim 2, as seen on page 151. A one-way ANOVA revealed no significant effect of treatment ($F_{2,17} = 0.10$, $p > 0.05$, Figure 35) on the percent of activated microglia. In addition to corroborating the lack of microglial activation differences at this time point from aim 2, the pattern of activation is similar as the findings in aim 2.

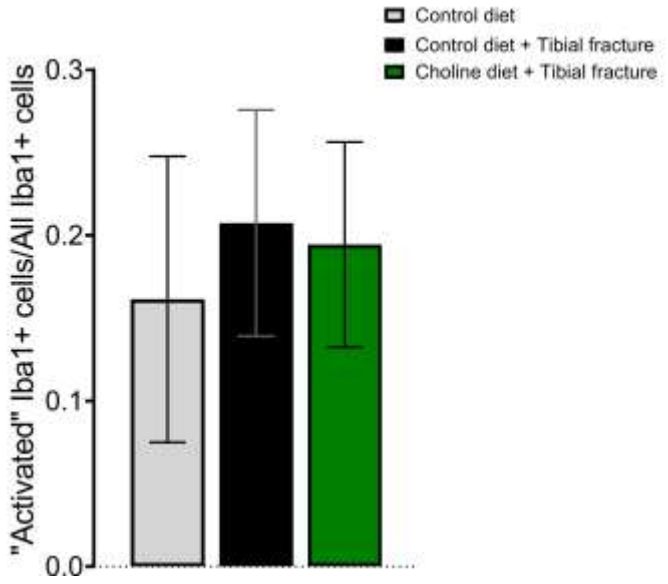


Figure 35: Tibial fracture and perinatal choline supplementation effects on the percent of total microglia in "round" and "stout" activation states. Histogram showing the percent of activated microglia in the dentate gyrus of the hippocampus. N = 6-8 per group. Each value represents the ± SEM.

Total microglial number was analyzed using immunohistochemical staining for Iba1 in the DG. A one-way ANOVA revealed a marginal effect of treatment on total microglia in the dentate gyrus ($F_{2,17} = 2.67$, $p = 0.10$, Figure 36). Again, the lack of difference in total microglia is consistent at the 2 week post-surgery time point from aim 2.

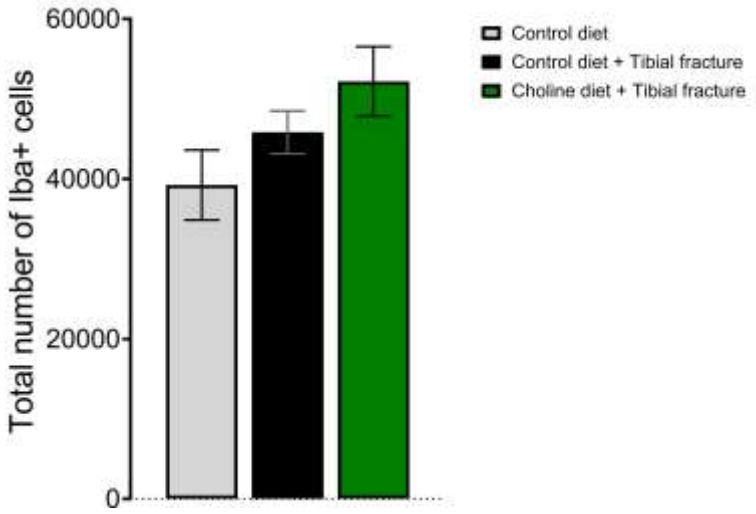
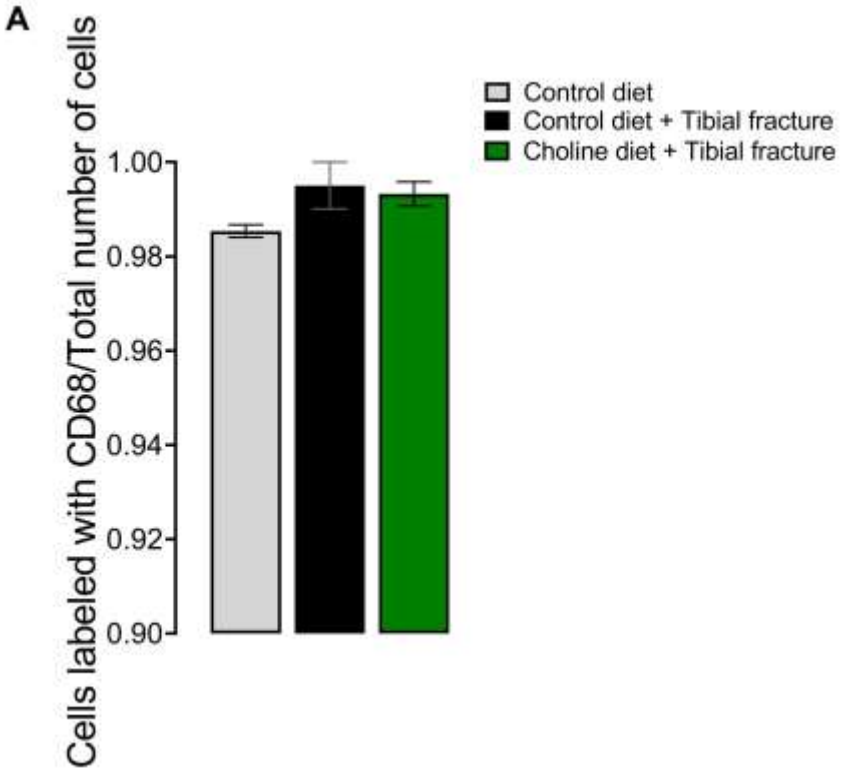


Figure 36: Tibial fracture and perinatal choline supplementation effects on total number of microglia. Histogram showing the number of total microglia in the dentate gyrus of the hippocampus. N = 6-8 per group. Each value represents the ± SEM.

An additional measure of microglial activation was analyzed by staining for the macrophage activation marker CD68. The percent of total stained cells that were colocalized with Iba1 and CD68 was quantified. A one-way ANOVA revealed no significant differences in this measure of microglial activation ($F_{2,15} = 2.29$, $p > 0.05$, Figure 37).


A
Control diet
Control diet + Tibial fracture
Choline diet + Tibial fracture
Cells labeled with CD68/Total number of cells
1.00
0.98
0.96
0.94
0.92
0.90

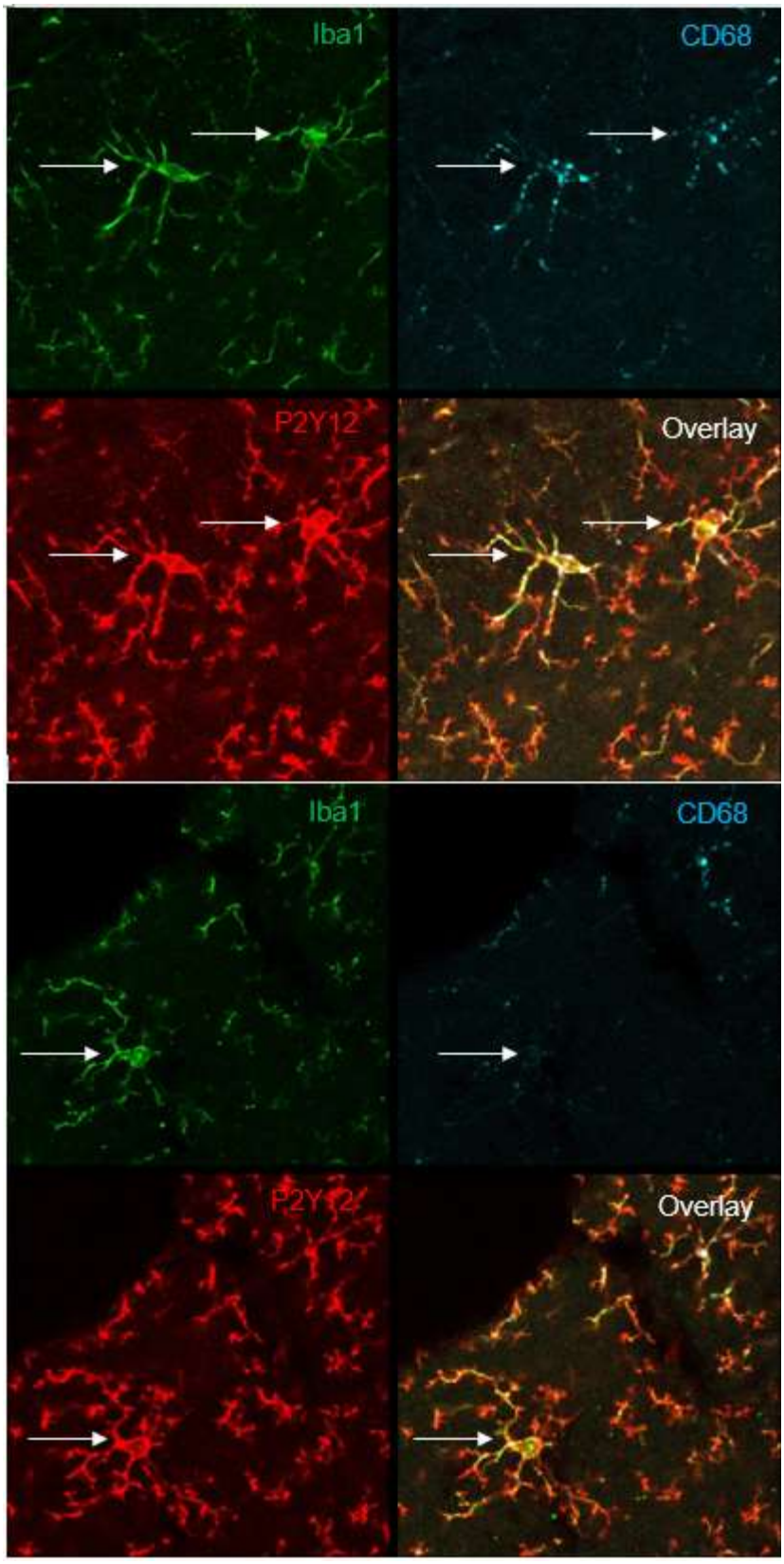

B
Iba1
CD68
P2Y12
Overlay
C
Iba1
CD68
P2Y12
Overlay

Figure 37: Tibial fracture and perinatal choline supplementation effects on the colocalization of Iba1 and CD68, a marker of activation. (A) Histogram showing the percentage of cells labeled with CD68 in the dentate gyrus of the hippocampus. N = 5-8 per group. (B) Representative images of cells colabeled with CD68 (white arrows) and (C) cells not colabeled with CD68 (white arrows). Each image is 150 μm x 150 μm. Each value represents the ± SEM.

4.2.4.2 Cortex

To assess the hippocampal specificity of the lack of microglial differences, we quantified microglial activation, total microglial number, and CD68 colocalization in the retrosplenial cortex. This region is involved in spatial navigation and episodic memory (reviewed in Vann et al., 2009), and also receives cholinergic input (Gage et al., 1994). To further isolate the actions of tibial fracture and dietary choline supplementation to the hippocampus, we assessed whether these variables impacted a distinct region with many hippocampal connections (reviewed in Wyass & Van Groen, 1992).

No differences due to condition were observed in one-way ANOVAs in any measure: percent of microglia in an "activated" state ($F_{2,17} = 1.22$, $p > 0.05$, Figure 38), total microglia ($F_{2,17} = 1.13$, $p > 0.05$, Figure 39), or CD68 colocalization ($F_{2,17} = 0.22$, $p > 0.05$, Figure 40). No significant differences were observed in any hippocampal microglia quantification, indicating that, at this time point, microglia are not the mechanism of long-term POCD.

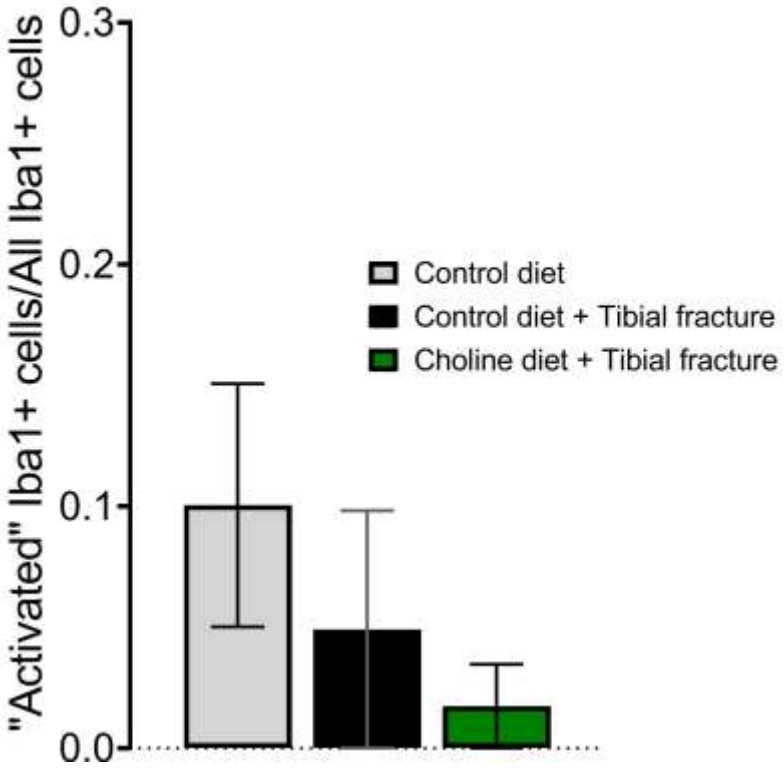


Figure 38: Tibial fracture and perinatal choline diet effects on microglial activation in the cortex. Histogram showing the percentage of "round" + "stout" Iba1+ cells in the retrosplenial cortex 2 weeks after surgery. N = 6-8 per group. Each value represents the mean ± SEM.

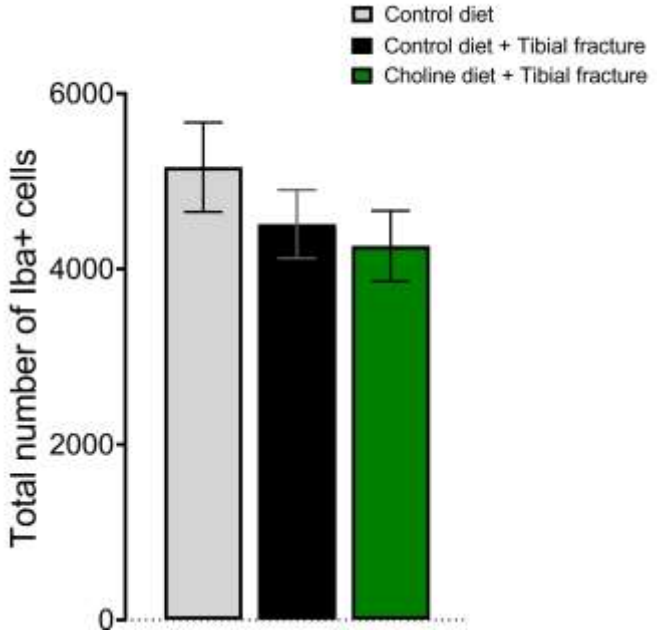


Figure 39: Tibial fracture and perinatal choline diet effects on microglial number in the retrosplenial cortex. Histogram showing the number of Iba1+ cells in the retrosplenial cortex 2 weeks after surgery. N = 6-8 per group. Each value represents the mean ± SEM.

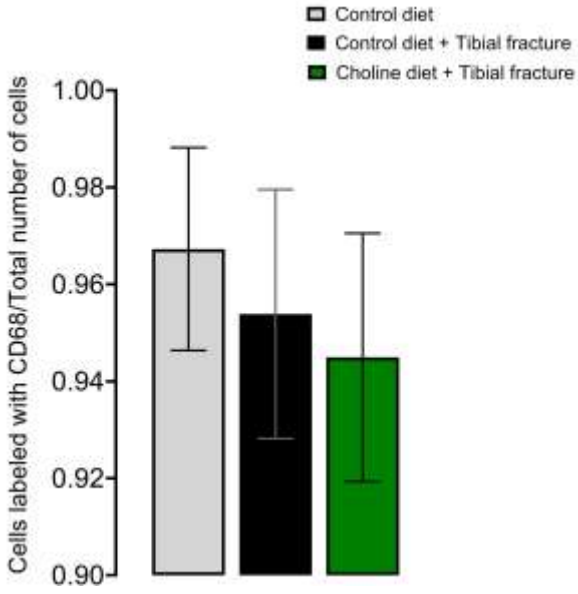
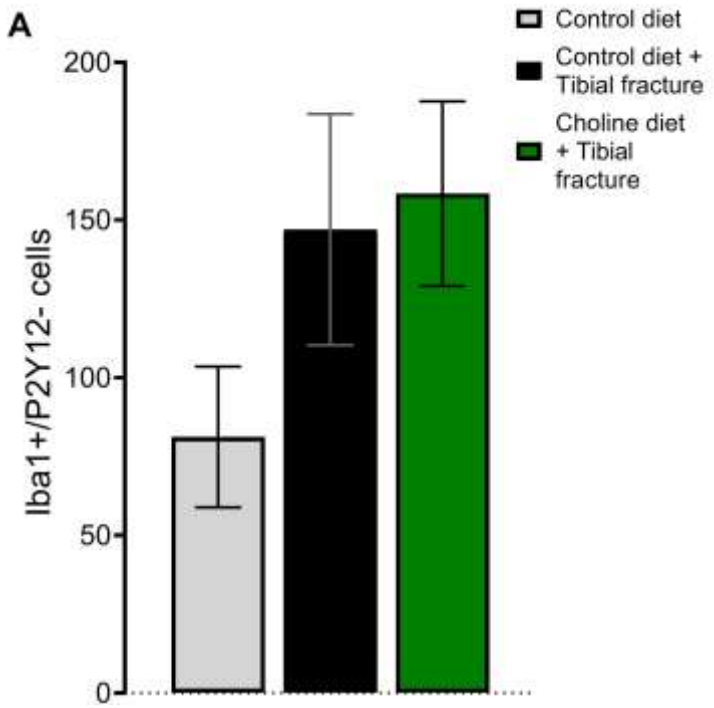


Figure 40: Tibial fracture and perinatal choline diet effects on the percent colocalization of CD68 and Iba1 in the retrosplenial cortex. Histogram showing the

percentage of cells labeled with CD68. N = 5-8 per group. Each value represents the mean ± SEM.

4.2.5 Macrophage infiltration

Macrophage infiltration was quantified by analyzing the number of cells that were Iba1+/P2Y12- in the DG after sacrifice 2 weeks after surgery, as described in aim 2. A one-way ANOVA showed no differences in non-microglial macrophages due to treatment ($F_{2,16} = 1.61$, $p > 0.05$, Figure 41A). The lack of differences in this measure is consistent with the same finding at this time point in aim 2.



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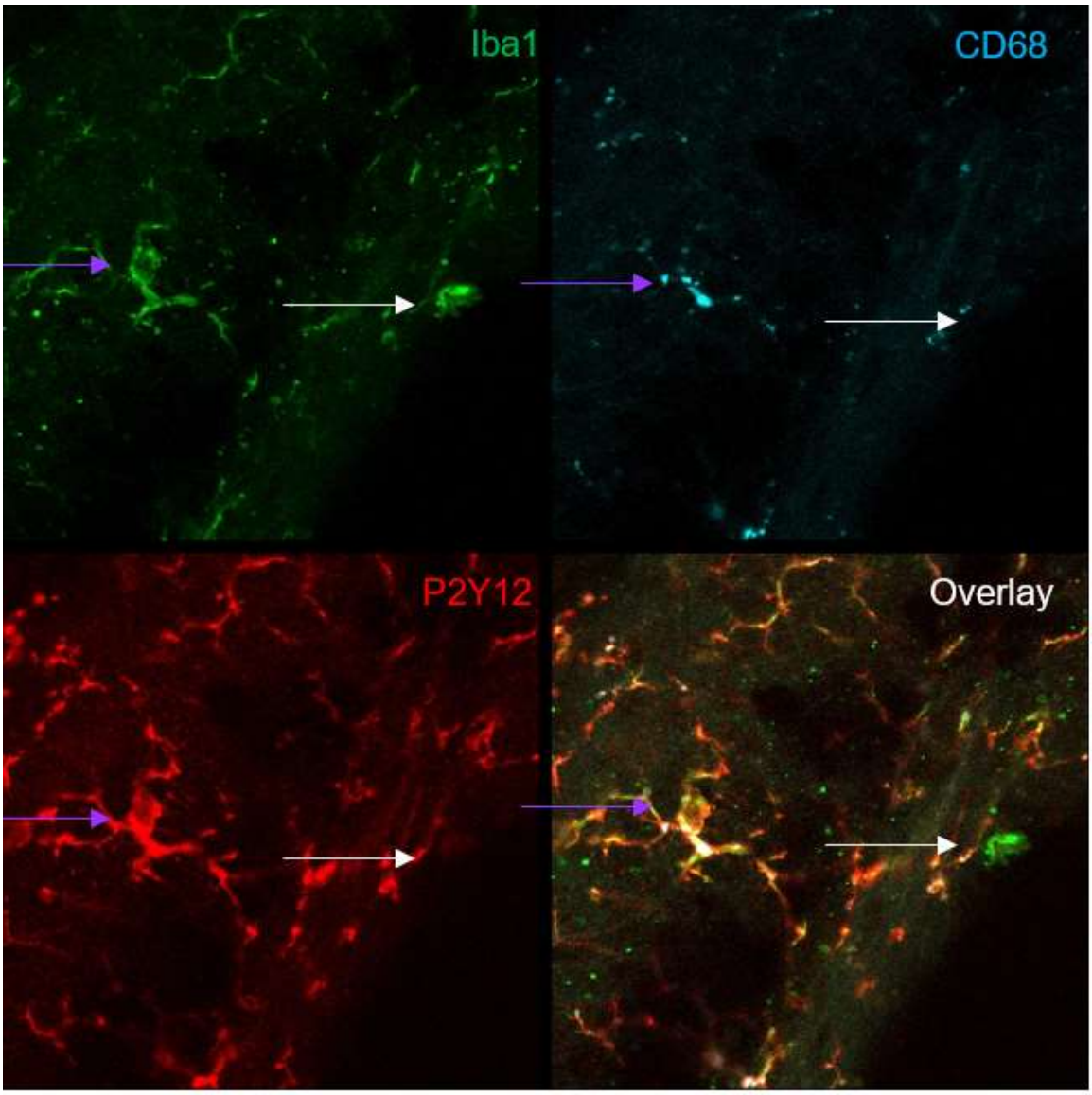

Figure 41: Tibial fracture and perinatal choline supplementation effects on the infiltration of peripheral macrophages in the dentate gyrus. (A) Histogram showing the number of Iba1+/P2Y12- cells in the dentate gyrus of the hippocampus. N = 5-8 per group. (B) Representative image of an Iba1+/P2Y12+ cell (purple arrows) and an Iba1+/P2Y12- cell (white arrows). Sections were stained with Iba1 (green), CD68 (cyan), and P2Y12 (red). The fourth panel is an overlay of all 3 channels. Each panel is 150 μm x 150 μm. Each value represents the mean ± SEM.

Macrophage infiltration was quantified in the retrosplenial cortex to assess brain region differences. No significant differences due to treatment were found in a one-way ANOVA ($F_{2,17} = 1.23$, $p > 0.05$, Figure 42). Though no significant differences were observed in infiltrating macrophages in the retrosplenial cortex, there is a non-significant increase due to tibial fracture that is blunted by perinatal choline supplementation. The lack of differences observed in the retrosplenial cortex 2 weeks after surgery is consistent with the same finding in aim 2.

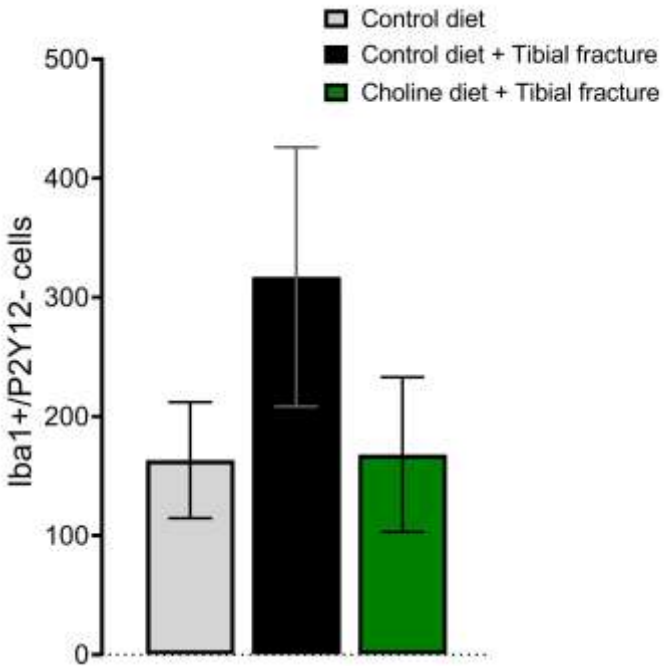

Figure 42: Tibial fracture and perinatal choline supplementation effects on the infiltration of peripheral macrophages in the retrosplenial cortex. Histogram showing the number of Iba1+/P2Y12- cells in the retrosplenial cortex. N = 5-8 per group. Each value represents the mean ± SEM.

4.2.6 Astrocytic density

Astrocytic density was analyzed using immunohistochemical staining for glial fibrillary acidic protein (GFAP) in the DG after sacrifice 2 weeks after surgery. A two-way ANOVA revealed a significant main effect of diet ($F_{1,21} = 5.61$, $p < 0.05$, Figure 43). Mice that received perinatal choline supplementation overall exhibited decreased GFAP antigen density. As well, a significant effect of surgery was observed ($F_{1,21} = 7.32$, $p < 0.05$). Mice that received tibial fracture surgery exhibited significantly higher GFAP antigen density than naïve mice. However, no prenatal diet X surgery interaction was observed ($F_{1,21} = 1.52$, $p > 0.05$). Tibial fracture led to astrocytic activation, which was blunted by perinatal choline supplementation. Consistent with findings in aim 2, astrocytic activation is activated by tibial fracture, and mitigated by choline supplementation – here, perinatal choline supplementation mitigates the increases in astrocytic density due to adult tibial fracture. In contrast with aim 2, however, differences due to surgery were observed 2 weeks post-surgery. However, comparing the Y-axes between two astrocytic density findings, the increase due to tibial fracture surgery 1 day after surgery is much larger than the increase due to surgery observed in the current aim 2 weeks after tibial fracture surgery. Though significant differences were found in the current aim, these more subtle differences may have emerged because the current aim exclusively focused on the 2 week post-tibial fracture time point. In the

absence of the dramatic spike in GFAP density 1 day after tibial fracture, perhaps significant differences can emerge.

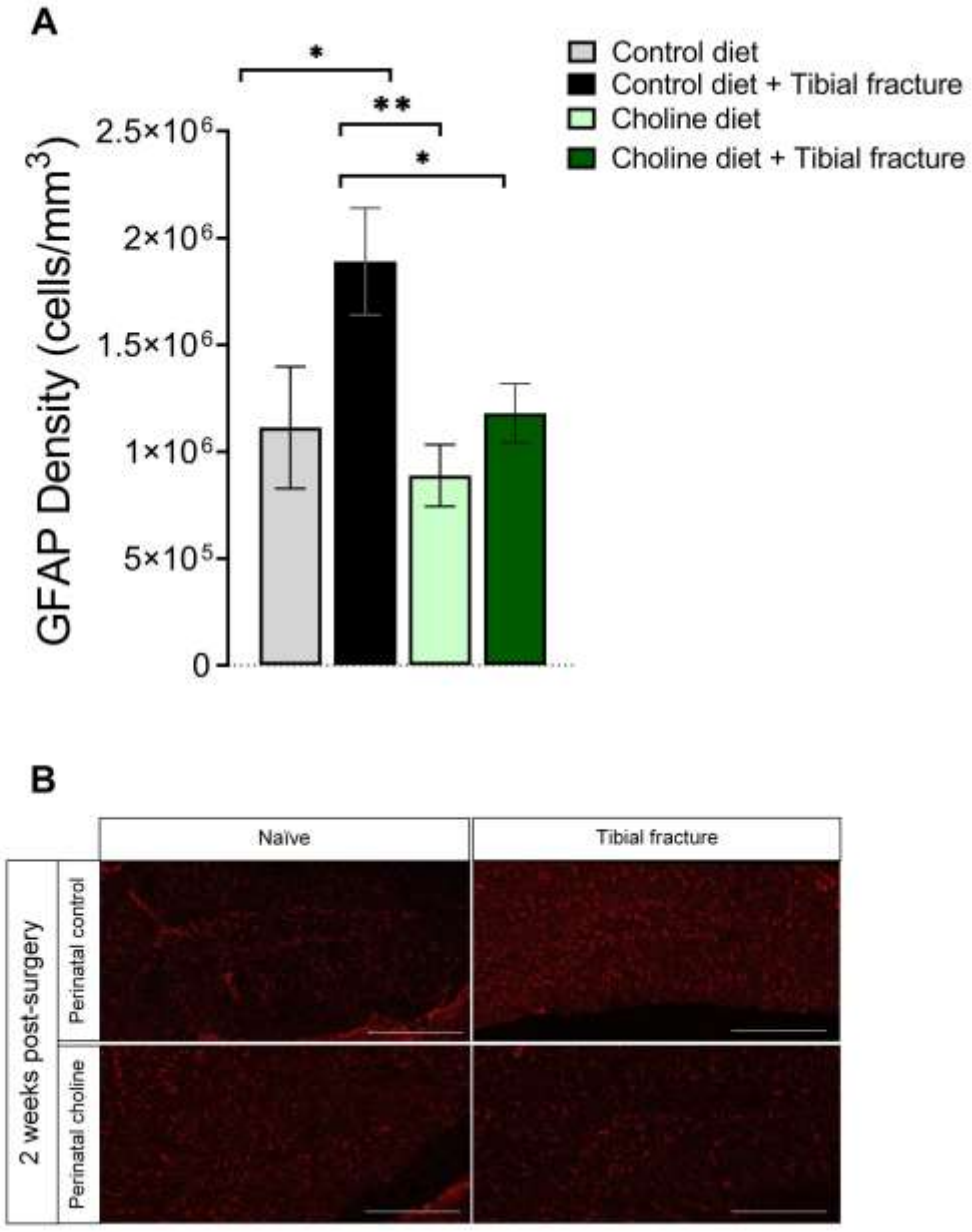

Figure 43: Tibial fracture and perinatal choline supplementation effects on astrocytic density. (A) Histogram showing the antigen density of GFAP in the dentate gyrus of the hippocampus. N = 4-8 per group. (B) Fluorescent micrographs of the

dentate gyrus at a 10x objective taken two weeks after surgery, immunostained with GFAP in mice given perinatal control diet and left naïve (upper left), given perinatal control diet and administered a tibial fracture (upper right), given perinatal choline supplementation and left naïve (bottom left), or given perinatal choline supplementation and administered a tibial fracture (bottom right). Scale bar: 200 μm. * $p < 0.05$, ** $p < 0.01$, Tukey HSD. Each value represents the mean ± SEM.

4.2.7 Cell survival

The number of cells that were proliferating shortly after surgery that survived until 2 weeks after surgery was analyzed using immunohistochemical staining for BrdU after sacrifice. A two-way ANOVA showed no significant effects due to perinatal diet ($F_{1,16} = 0.33$, $p > 0.05$, Figure 44), adult tibial fracture ($F_{1,16} = 0.08$, $p > 0.05$), or an interaction between perinatal diet and tibial fracture ($F_{1,16} = 0.57$, $p > 0.05$). Similar to findings in aim 2, no differences in BrdU+ cells 2 weeks after tibial fracture surgery were observed. No significant differences were found in cell survival 2 weeks after tibial fracture.

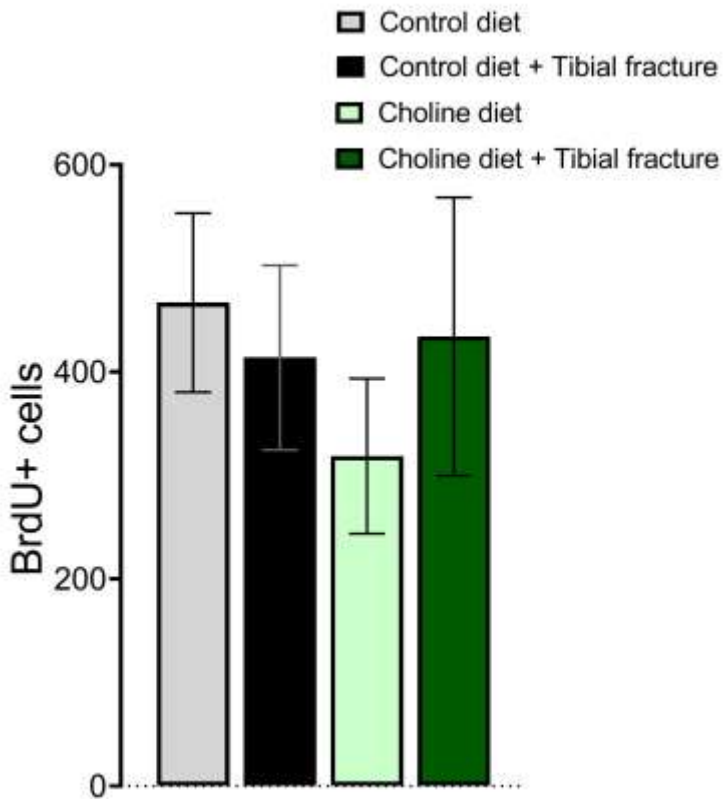

Figure 44: Tibial fracture surgery and perinatal choline supplementation effects on cell survival. Histogram showing the number of BrdU+ cells in the dentate gyrus of the hippocampus. N = 3-6 per group. Each value represents the mean ± SEM.

4.2.8 Young neurons in the SGZ

The number of young neurons in the SGZ was analyzed using immunohistochemical staining for doublecortin (DCX) after sacrifice 2 weeks after tibial fracture surgery. Perinatal diet did not affect the number of young neurons in the SGZ of the DG ($F_{1,17} = 1.31$, $p > 0.05$, Figure 45). However, mice that received tibial fracture exhibited more new neurons in the SGZ than mice that were left naïve ($F_{1,17} = 5.67$, $p < 0.05$). This is consistent with the reactive increase in DCX+ cells observed in aim 2 two weeks after surgery. No perinatal diet X surgery interaction effects on young neurons in

the SGZ were observed ($F_{1,17} = 0.04$, $p > 0.05$). Tibial fracture increases DCX+ cells, indicating reactive neurogenesis and/or a delay in neuronal maturation, and this effect was not rescued by perinatal choline supplementation.

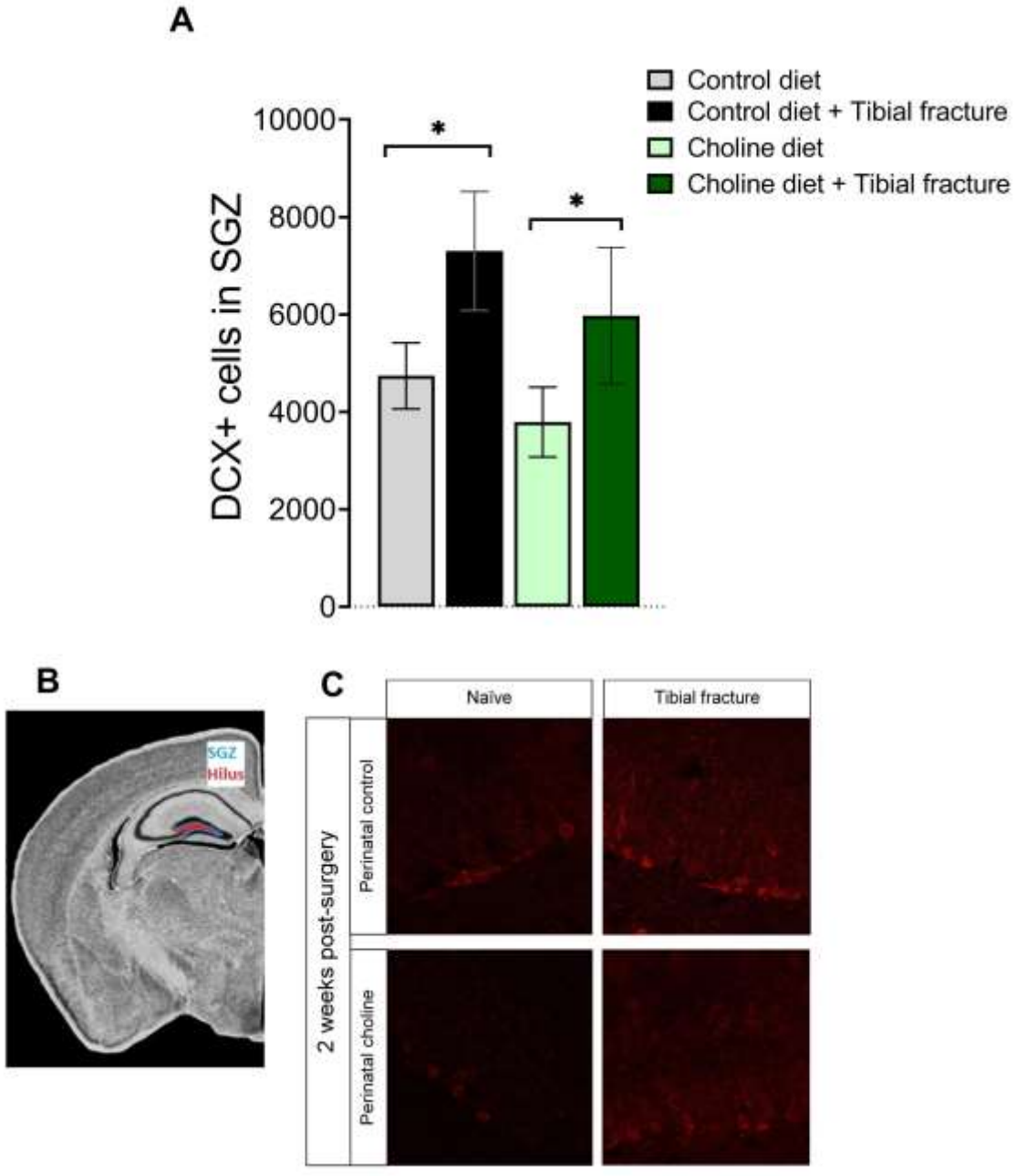

Figure 45: Tibial fracture surgery and perinatal choline supplementation on young neurons in the subgranular zone (SGZ) of the dentate gyrus. (A) Histogram showing the number of DCX+ cells in the SGZ. N = 4-6 per group. (B) Image from mouse atlas (Bregma: -1.64, Rosen et al., 2000) detailing the counting parameters. SGZ: blue, hilus: red. (C) Fluorescent micrographs of a portion of the SGZ at 40x objective taken two weeks after surgery, immunostained with DCX in mice given perinatal

control diet and left naïve (upper left), given perinatal control diet and administered a tibial fracture (upper right), given perinatal choline supplementation and left naïve (bottom left), or given perinatal choline supplementation and administered a tibial fracture (bottom right). Each image shows an area that is 150 μm x 150 μm. * $p < 0.05$, Tukey HSD. Each value represents the mean ± SEM.

4.2.9 Aberrant neuronal migration

Hilar neurons were analyzed using immunohistochemical staining for DCX in the hilus of the DG after sacrifice 2 weeks after surgery. Similar to the results in the SGZ of the DG, only a surgery effect was observed ($F_{1,17} = 5.81$, $p < 0.05$, Figure 46). Only mice that received tibial fracture showed an increase in hilar DCX+ cells, consistent with the findings in aim 2. No perinatal diet ($F_{1,17} = 1.57$, $p > 0.05$) or perinatal diet X surgery interaction ($F_{1,17} = 0.03$, $p > 0.05$) effects were observed. Tibial fracture induces increased numbers of hilar neurons, which was not affected by perinatal choline supplementation.

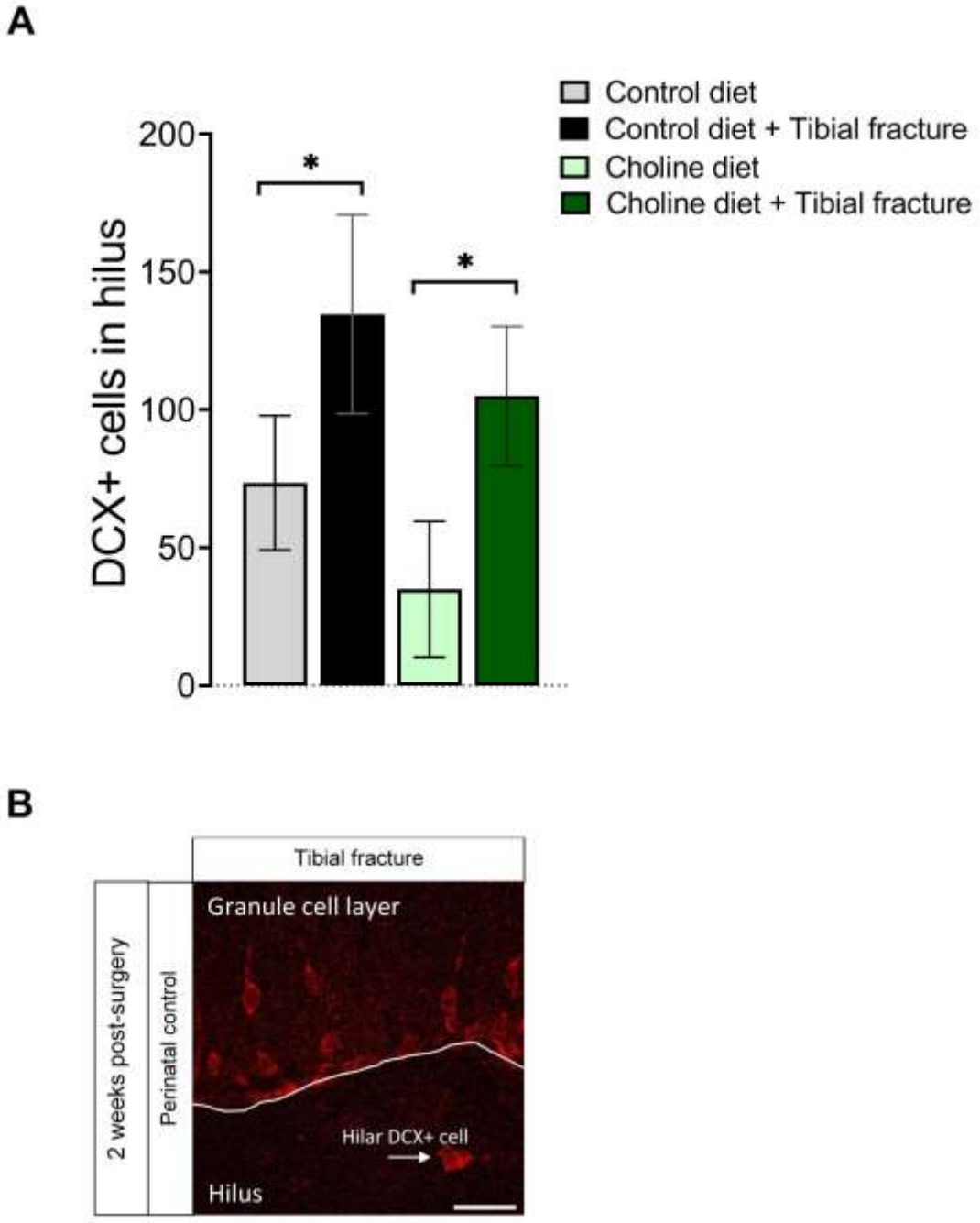

Figure 46: Tibial fracture surgery and perinatal choline supplementation effects on hilar neurons. (A) Histogram showing the number of DCX+ cells in the hilus of the dentate gyrus. N = 4-6 per group. (B) Fluorescent micrograph of a hilar neuron from a perinatal control diet + adult tibial fracture animal, immunostained

with DCX. Scale bar: 25 μm. * $p < 0.05$, Tukey HSD. Each value represents the mean ± SEM.

4.3 Discussion

Tibial fracture induces behavioral, astrocytic, and neuronal changes specific to the hippocampus two weeks after surgery as we have reported previously (chapter 3: aim 2, page 68). In addition, perinatal choline supplementation, much like choline supplementation in adulthood, blunts tibial fracture-induced alterations in behavior and neuroimmune measures. However, unlike the protective effects of adult choline supplementation, perinatal choline supplementation alone does not guard against the neuronal changes which occur following tibial fracture surgery. Because the post-operative cognitive decline was alleviated by perinatal choline supplementation despite the persistence of neuronal changes, astrocytic neuroinflammation may be the mechanism behind hippocampal-dependent memory impairments in the tibial fracture model.

We found that tibial fracture surgery impaired performance of a novel object recognition task in adulthood, consistent with our results reported in aim 2 (68). Additionally, perinatal choline supplementation prevented this impairment. Though cholinergic manipulations in adulthood have been shown to prevent POCD (chapter 3: aim 2, page 68; Terrando et al., 2011), we report here that perinatal choline supplementation alone is sufficient to provide protection against POCD following tibial fracture surgery in adulthood. These data extend previous work demonstrating that

perinatal choline supplementation improves novel object recognition (NOR) in aged and adult rats (Glenn et al., 2008; Kennedy et al., 2014), and prenatal choline deficiency impairs NOR performance in mice (Jadavji et al., 2015). Additionally, the behavioral impairment due to tibial fracture surgery is specific to hippocampal-dependent memory: no differences in anxiety-related behavior were observed. The current findings add to the growing literature supporting the view that added choline to the diet during early development provides resilience against assaults experienced in adulthood, such as Alzheimer's disease (Mellott et al., 2017), age-related memory decline (Meck et al., 2008; Glenn et al., 2008), and seizure (Wong-Goodrich et al., 2008; 2010; Holmes et al., 2002). It is likely that choline supplementation, possibly through epigenetic modifications, "programs" both neurons and immune cells such that it is more resilient.

Perinatal choline supplementation blunted the increase in astrocytic activation after adult tibial fracture surgery, suggesting an anti-inflammatory "programming" effect of choline on immune cells. Consistent with previous findings in adult suggesting that astrocytes are necessary for ACh to prevent interference in memory (Pabst et al., 2016), the current findings show that astrocytic dysregulation is correlated with impaired memory of a previously-seen object, which is mitigated by perinatal choline supplementation. Previous work has analyzed GFAP protein and mRNA expression in the hippocampus after prenatal choline supplementation and indicate that choline "programs" astrocytes. In an Alzheimer's disease model, prenatal choline

supplementation prevents the increase in GFAP protein in the hippocampus in male mice at both 9 months and 12 months of age (Mellott et al., 2017). Prenatal choline prevented the astrocytic upregulation due to seizure, and this prevention was long-term. Prenatal choline prevented the increase in GFAP mRNA, but not protein, 16 days after seizure (Wong-Goodrich et al., 2008), and prevented the increase in both GFAP mRNA and protein 11 weeks after seizure induction (Wong-Goodrich et al., 2010). The current work was the first to analyze astrocytic activation by analyzing the antigen density of GFAP in the context of early dietary choline supplementation, and has revealed significant neuroprotection of perinatal choline supplementation in a tibial fracture model. Previous differences - or lack of them - may depend on the model or on the measure quantified. In this specific tibial fracture model, perinatal choline supplementation prevents the upregulation in GFAP antigen density. This suggests a neuroimmune "priming" of astrocytes to not overreact in adulthood with an additional immune challenge.

We found that tibial fracture surgery slightly increased the number of new neurons, consistent with our results in aim 2 (page 68). However, perinatal choline supplementation did not prevent this increase. This is in contrast to the finding in aim 2 that adult choline supplementation prevented surgery-induced increases in young neurons.

Previous work has been devoted to the role of prenatal choline supplementation in protecting against neuronal loss, not increases in young neurons (Nickerson et al., 2017; Velazquez et al., 2013). However, as previously posited (aim 2), findings in a seizure model more closely corroborate with the observed findings in the tibial fracture model. After kainic acid-induced seizure, prenatal choline supplementation does not protect against increases in DCX+ cells in the SGZ and hilus 16 days after seizure (Wong-Goodrich et al., 2008). Hence, prenatal choline does not protect against seizure-induced increases in DCX+ cells, consistent with the current findings. Critically, hippocampal-dependent memory deficits were seen both short- (1 week post-seizure) and long-term (10 weeks post-seizure), and were attenuated in rats with prenatal choline supplementation (Yang et al., 2000; Wong-Goodrich et al., 2010). ln mice, reactive neurogenesis due to seizure has been tied to hippocampal-dependent memory deficits, not rescue (Cho et al., 2015). Reactive neurogenesis may not be the only mechanism by which memory is impaired in seizure-induced rats: because these rats did not exhibit mitigation of DCX+ cells by prenatal choline in either the SGZ or hilus, but memory was attenuated (Wong-Goodrich et al., 2008), alterations of DCX+ cells cannot be the only mechanism.

Similarly, in the present study, hippocampal-dependent memory in a NOR task was impaired after tibial fracture despite the lack of effect on DCX+ cells. This suggests that that this is not the mechanism of behavior in this specific model. Consistent with

previous work in the tibial fracture model (Terrando et al., 2011), neuroinflammation is likely the mechanism behind the POCD.

As we reported for adult choline supplementation and adult tibial fracture, neither treatment altered microglial activation or macrophage infiltration in either the DG or retrosplenial cortex. The lack of surgery differences in microglial activation at this time point is consistent with aim 2: two weeks after surgery, no differences in microglial activation were observed. Infiltrating macrophages, however, present conflicting evidence between the current work and our prior study in aim 2: there was no significant group difference in infiltrating macrophages in the DG two weeks after surgery, contrary to findings in aim 2. Previous findings have shown an increase in CCR2+ cells (bone-marrow derived monocytes and macrophages) in the hippocampus one day after tibial fracture surgery, which was mitigated by a cholinergic agonist just prior to surgery as well as an anti-TNF antibody (Terrando et al., 2011). Another group assessed the decrease in the integrity of the blood-brain barrier, allowing more infiltrating macrophages to enter the brain, for one week following tibial fracture (Hu et al., 2014). A decrease in blood-brain barrier integrity was observed both 1 and 3 days after surgery, not 7 days later. Hippocampal-dependent memory deficits were also exclusively seen 1 and 3 days post-tibial fracture, not 7 days later. Evidence of the regeneration of the blood-brain barrier 7 days after surgery, correlating with the timeline of rescue of hippocampal-dependent memory, is corroborated in a splenectomy model

of POCD (He et al., 2012). The lack of differences 2 weeks after surgery in the present work is consistent with the regeneration of the blood-brain barrier 1 week after surgery. It is possible that, after tibial fracture surgery, infiltration macrophages did come into the brain, but retreated back into the periphery or died prior to examination 2 weeks post-tibial fracture surgery. The specific functional role of infiltrating macrophages in POCD is currently unclear. However, because no effects in infiltrating macrophages were observed due to tibial fracture 2 weeks after surgery, peripheral macrophage infiltration is not likely to be the mechanism of behavioral impairment in the NOR task at this time point.

Consistent with aim 2, there were no significant differences in hilar DCX+ cells at this time point. Previous work using a rat seizure model showed increases in hilar neurons. However, these increases were not impacted by prenatal choline supplementation (Wong-Goodrich et al., 2008; 2010), consistent with the findings presented here. However, in both the present work and seizure literature, perinatal choline supplementation does prevent impairments in hippocampal-dependent memory (Wong-Goodrich et al., 2010), again indicating that the increase in hilar neurons is not likely the mechanism of behavioral impairment that choline prevents.

Ultimately, the mechanism of behavioral impairment in the tibial fracture model of POCD, and its rescue by perinatal choline supplementation, is likely astrocytic. Perinatal choline supplementation likely programs neuroimmune cells in development,

not the production of young neurons. Though the molecular mechanism behind these cellular programming effects is not known, one possible candidate is CCL2, which is secreted by astrocytes. CCL2 is upregulated after tibial fracture surgery and is specifically involved in microglial activation and cognitive deficits (Xu et al., 2017). However, previous work analyzed astrocytic CCL2 in adulthood – future work can and should explore the changes in CCL2 over development as a function of perinatal influences on the neuroimmune system.

A limitation of this experiment is the lack of data 1 day after surgery. The choice to examine 2 weeks after surgery was made for two reasons. First, there is an extreme dearth of research at this time point. The current body of work is the first to find behavioral impairments at this time point in the tibial fracture model, and we wanted to further explore that phenomenon in the context of perinatal diet. Second, the increase in DCX+ cells in the SGZ after surgery in aim 2 (page 68) was a novel finding that we wanted to continue to explore. However, the limitation to this work is the lack of information on how perinatal dietary choline supplementation affects the more immediate neural impact of peripheral surgery. Though the current work sought to explore the underpinnings behind long-term POCD, assessing the short-term effects in the context of perinatal choline supplementation would allow a more detailed depiction of the developmental progression of short- and long-term POCD.

In humans, the temporal length of POCD varies widely (Monk et al., 2008). The findings presented here may explain why some people are at higher risk for a longer incidence of POCD: in addition to the risk factor of age at the time of surgery, perinatal diet can also significantly impact POCD two weeks after surgery. By increasing awareness of the importance of perinatal choline in yet another model of adult protection, the critical importance of this nutrient early in life can be further emphasized. These findings, in addition to the observed neuroprotection in other models, will hopefully translate to more women achieving optimal choline intake during pregnancy.

5. Conclusion

The cholinergic system is heavily involved in hippocampal-dependent memory. Though the neuronal circuitry of this involvement has been characterized (Hasselmo, 2006), few have analyzed the cognitive impact of cholinergic manipulations on the hippocampal neuroimmune system. Because previous work has implicated the anti-inflammatory actions of non-dietary choline in protection of hippocampal-dependent memory (Terrando et al., 2011), the present body of work aims to converge two observed phenomena to explore an alternate mechanism of hippocampal memory protection. From a translational standpoint, dietary choline is a likely way that humans can undergo cholinergic modification in the absence of drugs. Because one underlying goal of this research is to provide evidence for a cheap, drug-free, and widely accessible means of protection from common inflammatory assaults, the overall question explored here is, "Can dietary choline protect against neuroinflammation and its behavioral and neuronal consequences throughout the lifespan?"

The first aim of this dissertation research was to examine the combined effects of maternal dietary choline supplementation and maternal diesel exposure on fetal microglial activation. Diesel exposure is one commonly-encountered experience that induces fetal and long-term changes in the offspring neuroimmune system (Bolton et al., 2012; Bolton, 2015). By mitigating early neuroinflammation, a choline-supplemented diet could prevent these long term alterations. Aim 1 (page 30) utilized a mouse model of

maternal diesel exposure and extracted fetal brains at embryonic day 18. Of the four brain regions analyzed, three showed microglial alterations due to maternal diesel exposure. As well, these effects were at least partially mitigated by maternal choline supplementation. Previous work has shown differences in body weight, insulin, and consumption of a palatable diet due to maternal diesel exposure in male offspring (Bolton et al., 2012), which could possibly be due to altered microglial reactions in the hypothalamus. The upregulation of microglial activation that was mitigated by maternal choline supplementation in the dentate gyrus of the hippocampus may point to an ability of dietary choline to prevent hippocampal inflammation and cognitive deficits as well. The implications of this aim are wide – women who are pregnant or may become pregnant in areas of high pollution may be able to prevent neuroinflammation and behavioral alterations in male offspring with a dietary change.

Because of the findings in aim 1 indicating an acute anti-inflammatory effect of dietary choline supplementation, the next direction in the current dissertation work analyzed the acute effects in adulthood of dietary choline supplementation and a peripheral immune assault. A different inducer of neuroinflammation was chosen for subsequent work because of previous work specifically implicating i.p. administration of choline in mitigating the inflammatory and behavioral deficits in the tibial fracture model (Terrando et al., 2011). Additionally, POCD is another real-world phenomenon in which people may be able to prevent excess inflammation and cognitive deficits with a

simple dietary change. For this reason, aim 2 (page 68) explores this interaction in the context of hippocampal-dependent behavior, neuroimmune cellular changes, alterations in cell division and survival, and alterations in young neurons. These differences were assessed at both 1 day and 2 weeks after surgery. Broadly, differences due to peripheral surgery were widespread, but dependent on the time point analyzed. Every difference due to surgery was at least partially mitigated by dietary choline supplementation except for anxiety-related behavior. Surgery also induced an increase in hilar granule cells, a phenomenon previously only seen after status epilepticus. Though differences were found in both neuroimmune cells and neurons, currently the specific mechanism for the behavioral impairment and rescue is not known. However, with the addition of a high-choline diet, people may be able to avoid the neuroinflammation, neuronal changes, and hippocampal-dependent memory deficits due to peripheral surgery.

To analyze whether there is a "programming" effect in addition to acute effects at different developmental time points, aim 3 (page 124) sought to examine the possible neuroprotection of perinatal choline supplementation against the varied consequences of tibial fracture surgery in adulthood. This aim sought to contextualize the already-known protective effects of perinatal choline supplementation with a neuroimmune lens. Particularly astonishing was the ability of maternal diet to protect dramatically against hippocampal-dependent deficits in the novel object recognition task two weeks after surgery. These findings suggest a long-term behavioral programming consistent

with the neuroprotection due to perinatal choline supplementation in other models of adult cognitive dysfunction (Blusztajn & Mellott, 2013; Wong-Goodrich et al., 2010). Though perinatal choline supplementation is already known to be neuroprotective across the lifespan, the present findings indicate yet another way in which perinatal choline is good for offspring: through protection against an immune assault in adulthood.

Future directions of this work can be used to pinpoint molecular mechanisms behind this protection. Specifically, this body of work suggests that neuroimmune cells and neurons interact, and that the immediate neuroinflammation due to peripheral surgery leads to long-lasting neuronal changes. How do these changes come about? Do neuroimmune cells release pro-inflammatory cytokines, which then cause neuronal increases and/or a delay in neuronal maturation? Does dietary choline mitigate the increase in DCX+ cells by preventing cytokine release, as it does in the cholinergic anti-inflammatory pathway? These are all questions that remain to be answered that stem from the current findings.

Taken together, this work analyzes dietary choline supplementation as a preventative measure against the deleterious cognitive effects of neuroinflammation across the lifespan. Though specific molecular mechanisms behind the interaction of these two variables remains to be solidified, the present work indicates that dietary choline is protective of memory across the lifespan within neuroinflammatory models as

well as models of status epilepticus and Alzheimer's disease (Wong-Goodrich et al., 2010; Velazquez, Ferreira, Winslow et al., 2019). The behavioral protection after peripheral surgery is long-lasting. The effect of this may be due to neuronal alterations, neuroimmune activation, or very likely a combination of both. The definitive implication of the present work is that dietary choline, whether administered just prior to an immune assault or perinatally, can protect offspring from cognitive deficits.

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Biography

Sara Victoria Maurer graduated from Upper Saint Clair High School in Upper Saint Clair, Pennsylvania, in May 2009. She attended Saint Vincent College in Latrobe, Pennsylvania. She graduated in May 2013 cum laude with a major in psychology and a minor in biology. She became a Doctor of Philosophy in May of 2020, graduating from the Systems and Integrative Neuroscience Program in the department of Psychology and Neuroscience at Duke University. She next intends to complete an NIH-funded postdoctoral fellowship at the University of Iowa beginning in July of 2020, where she will continue exploring the neuroimmune system in development.

Publications

Maurer, S. V., & Williams, C. L. (2017). The Cholinergic System Modulates Memory and Hippocampal Plasticity via Its Interactions with Non-Neuronal Cells. *Frontiers in Immunology, 8,* 1489.

Rivardo, M. G., Brown, K.A., Rodgers, A. D., Maurer, S. V., Camaione, T. C., Minjock, R. M., & Gowen, G. M. (2011). Integrating inattentional blindness and eyewitness memory. *North American Journal of Psychology, 13,* 519-538.

Honors and awards

- Graduate Travel Award sponsored by the Charles Lafitte Foundation Program for Research in Psychology & Neuroscience, 2018 & 2019
- Bass Connections Award for Outstanding Mentorship, 2018
- Duke Graduate School Conference Travel award, 2017, 2018 & 2019
- NSF GRFP Honorable mention, 2015
- Claire Hamilton Graduate Studies Conference Travel Award, 2015
- Saint Vincent College Psychology Scholars Program member, 2013
- "Excellence in Research and Service" Awardee, Saint Vincent College Psychology Department, 2013
- NSF-REU award, Duke University Mechanisms of Behavior program (2012)

Professional memberships

- Society for Neuroscience (SfN)
- Organization for the Study of Sex Differences (OSSD)