MICROGLIA ACTIVITY IN THE MOUSE BRAIN LACKING PROSTAGLANDIN E2- CONNECTION TO AUTISM

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Abstract

Prostaglandin E2 (PGE2) is a signaling molecule produced by cyclooxygenase–2 (COX-2) from cell membrane phospholipids and is important in the development of the healthy brain. PGE2 signaling can also regulate microglia activation during inflammation. Anomalies in the COX-2/PGE2 pathway due to genetic or environmental factors have been linked to Autism Spectrum Disorders (ASD). Our previous studies found that the COX-2 deficient (COX-2^{-}KI) mouse has molecular and behavioral traits that are autism-related, occurring in sex-dependent manners. Here, we aim to further determine the impact of abnormal signaling of the COX-2/PGE2 pathway through its effect on microglial density and morphology in the developing brain. Microglia are known to transition between one of two morphologies depending on their surroundings, known as active or ramified, which are visibly distinct from one another. Previous literature shows the importance of microglia in the development and maintenance of the healthy brain, having roles in synaptogenesis, synaptic pruning, and in the phagocytosis of cell debris. In this study we use COX-2^{-}KI mice to examine five regions in the brain, all previously implicated in ASD, in order to evaluate the role of COX-2/PGE2 signaling in the regulation of microglia activation in the developing brain. Using immunohistochemistry technique, we demonstrate that at embryonic day 19 (E19) and postnatal day 25 (P25) COX-2^{-}KI mice had sex and regional specific differences in microglial density, activation state, branch length, and branching networks. We discovered that at E19 the COX-2 KI animals had increased microglial density, altered percentage of active and ramified morphology, altered branch length, and decreased branching networks in a region and sex specific manner. When examining the P25 mouse brain we also found greater microglial density, and lower percentages of active microglia, which was complemented by increased branch counts and length compared to sex and age matched controls.

This study shows the differences in developmental trajectories between the COX-2 deficient mice and healthy controls in the microglial measurements tested. We show for the first time that these trajectories were influenced not only by the COX-2 deficiency, but also by sex, brain region, and developmental stage. These novel findings provide additional evidence that abnormal COX-2/PGE2 signaling can result in a disruption of microglial density and morphology during development and contribute to brain pathologies that result in ASDs.

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Abbreviation list

AA- Arachidonic acid
ALA- α-linoleic acid
ASD- Autism spectrum disorder
ACC- Animal care committee
AD- Alzheimer's disease
APC- Adenomatous polyposis coli
BMP- Bone morphogenetic protein
BDNF- Brain derived neurotrophic factor
CAPASE- Cysteine-dependent aspartate-directed protease
CB- Cerebellum
CDC- Center for disease control
CNS- Central nervous system
CK1- Casein kinase 1
COX- Cyclooxygenase
COX-2^{-}KI- Cyclooxygenase 2 knock in
DAB 3-3'- Diaminobenzidine
DHA- Docosahexaenoic acid
DHCR7- 7-dehydrocholesterol reductase
E2- Estradiol
E-9.5,-16,-19- Embryonic day -9.5, -16, -19
EN2- Homeobox protein engrailed-2
EP- E-Prostanoid
EPA- Eicosatetraenoic acid
FMR1- Fragile X mental retardation 1
FZD- Frizzled
Gsk-3β- Glycogen synthase kinase 3 beta
HPC- Hippocampus
IBA-1-Ionized calcium binding adaptor molecule 1
IL-1β, -6, -10, - Interleukin -1β, -6, -10,
IL2R- Interleukin 2 receptor
IHC- Immunohistochemistry
IFNγ- Interferon gamma
LA- linoleic acid
LEF- Lymphoid enhancer factor
LPS- Lipopolysaccharide
MIA- Maternal immune activation
mPGES- Microsomal prostaglandin synthase
MECP2-Methyl CpG binding protein
NSAIDS- Nonsteroidal anti-inflammatory drug
NE-4C- Neuroectodermal stem cells
NFKB- Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF- Nerve growth factor
NO- Nitric oxide
NRXN- Neurexin
OB- Olfactory bulb

P-4,-8,-21,-25,-30,-60- Postnatal day -4, -8, -21, -25, -30, -60
PBS- Phosphate-buffered saline
PFA- Paraformaldehyde
PFC- Prefrontal Cortex
PGE2- Prostaglandin E2
PI-3K- Phosphatidylinositide 3-kinase
PKA- Protein kinase A
PLA_2- Phospholipase A2
PTGS2- Prostaglandin-endoperoxide synthase 2
PUFA- Polyunsaturated fatty acids
PTCHD1-Patched domain containing 1
ROS- Reactive oxygen species
SHH- Sonic hedgehog
SFARI- Simons found autism research initiative
SHANK- SH3 and multiple ankyrin repeat domains
TCF- T-cell factor
TGF-β-Transforming growth factor beta
TNF-A- Tumor necrosis factor alpha
TNFR2- Tumor necrosis factor receptor 2
TH-Thalamus
TLR- Toll-like receptor
UBE3A- Ubiquitin protein ligase E3A
WNT- Wingless
WT-Wild type

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Introduction

1.1 Autism Spectrum Disorders - Overview

Autism is a neurodevelopmental disorder that manifests in the first three years of life. It belongs to a spectrum known as Autism Spectrum Disorders (ASDs) that are classified by three core characteristics, including deficits in communication, restrictive behaviors and interests, and impairments in social interactions (Marshall et al, 2008; Tamiji and Crawford, 2011). The rate of autism diagnosis has been increasing over recent years at an alarming rate, and as of 2014 was estimated by the Center for Disease Control (CDC) that one in every 59 children will be identified with ASD (Baio et al, 2018). It has been argued that increased rates of diagnosis are due to an increase in awareness or improvements of diagnostic methods concerning ASD, but these increased rates cannot be solely explained by these factors (King and Bearman, 2009; Hertz-Picciotto and Delwiche, 2009; Matson and Kozlowski, 2011). Clinical and molecular research worldwide has provided convincing evidence that ASDs neuropathology results from the effect of environmental risk factors on genetics, which will be discussed below.

In addition, autism has a diagnosis rate for males approximately four times higher than in females, making it one of the most extreme cases of sex bias in neurodevelopmental disorders (Werling and Geschwind, 2013). Despite being such a striking difference only in recent years have sex differences concerning autism become an area of focus for many researchers. Even with the increased attention to autism etiology in recent years the underlying causes to this disorder and the sex differences seen remain complex and not fully understood.

Many regions of the brain have been implicated in ASDs, including the cerebellum, hippocampus, olfactory bulb, thalamus and prefrontal cortex.

The cerebellum has been found to be functionally and structurally abnormal in patients with autism, resulting in impairments in motor control, as well as non-motor abnormalities via its connections with the cerebrum (Fatemi et al, 2012; Bracke-Tolkmitt et al, 1989; Becker and Stoodley, 2013; Rogers et al, 2013). In children with ASD it has been found that neurons within this region are unusually large and bountiful, relating to the trend of cerebellum overgrowth with the disorder (Kemper and Bauman, 2002). As well its commonly seen within humans that a decreased amount of Purkinje cells is found within ASD patients (Rogers et al, 2013).

The hippocampus has been implicated in ASDs based on human studies showing that the area was enlarged in children diagnosed with the disorder compared to controls; as well an increased number of neurons was found in ASD patients in this study (Barnea-Goraly et al, 2014). DeLong (1992) has referred to ASD as "the developmental syndrome of hippocampus dysfunction" indicating its importance in the disorder.

Oddities such as impaired odor perception, as well as decreased activity in the olfactory bulb has been noted in those with autism (Koehler et al, 2018; Ashwin et al, 2014). Current literature shows that children with ASD are far less likely to react to unpleasant odors, such as rotten milk and fish (Rozenkrantz et al, 2015). For this reason, the authors suggest that a child's reaction to smell may be a potential early marker for ASD, implicating the importance of the olfactory bulb in the disorder.

As well abnormal synaptic connectivity has also been noted within the prefrontal cortex, which is involved in the regulation of many complex cognitive behaviors such as personality and social expression (Diamond, 2000; Miller and Cohen, 2001; Shalom, 2009). Interestingly the prefrontal cortex was found to have an increased volume in human ASD patients compared to controls (Carper and Courchesne, 2005).

The thalamus is another region of the brain seen to be abnormal in volume in ASD patients compared to controls (Tamura et al, 2010). Abnormalities in synaptic connectivity of the thalamus have been noted in ASD; this is of importance as this region is known to regulate sociocommunicative, cognitive, and sensorimotor functions- all of which are implicated in the disorder (Nair et al, 2013; Woodward et al, 2017).

Although the molecular basis that leads to brain pathology in ASD is still being investigated by many research labs, there is a consensus in the scientific community that the disorders arises from a contribution of genetics, environmental and immunological factors (DiCicco-Bloom et al, 2006; Yoo, 2015; Modabbernia et al, 2017; Rossignol et al, 2014; Gladysz et al, 2018; Meltzer and Van de Water, 2018).

1.2 Genetic Influence on Autism

Genetics has been suggested to play a strong component in the etiology of autism. For example, the human database called SFARI Gene (2019), has already identified over 1000 ASD risk genes from various research studies. Along with the great number of ASD risk genes, the importance of genetics in this disorder has been demonstrated through heritability estimations in twins. Monozygotic twins, which share an almost identical genetic profile and begin as one fertilized egg, have a 60-92% chance of concordance (Muhle et al, 2004; Tick et al., 2016). Comparatively dizygotic twins, each being derived from a separate ovum, have a much smaller concordance rate of 0-10%. These differences in concordance rates between monozygotic and dizygotic twins is one of the main reasons autism is considered one of the most heritable neurodevelopmental disorders (Hallmayer et al, 2011). As well within the general population a concordance rate of 0.1-0.15% further emphasizes the importance of genetics within ASDs (London and Etzel, 2011).

Within the ASD population it has been shown that there are a large number of genes that are altered which have important roles in the healthy brain. Casanova and Casanova (2014) found that approximately 80% of genes noted as ASD-risk genes are involved in later phases of neurodevelopment and regulate processes such as synapse formation and neurite growth. A more recent focus comes to the attempt to converge these gene abnormalities into common pathways that may drive the development of core ASD characteristics (Oron and Ellior, 2017). This has led to an understanding that there are a variety of important pathways for healthy brain development that are influenced by autism-associated mutations.

Synaptopathology is commonly recognized in ASD. Recent studies have reported a wide variety of genes that have been proven to be mutated within individuals with the disorder, which may contribute to the synaptic disfunction (Guand et al, 2018).These mutations include genes that are involved in a variety of key processes at the synapse, such as *NRXN, SHANK,* and *MECP2* (Guand et al, 2018). These mutations resulting in synaptic dysregulation can result in functional and cognitive impairments associated with ASD (Guand et al, 2018).

Genes associated with immune system function have also been found to be mutated within ASD individuals (Garbett et al, 2008). A variety of these mutations were related to pathways in inflammation (NFKB, IL6), cell death (CASPASE, TNFR2), and antigen-specific immune response (TOLL, IL2R) (Garbett et al, 2008). We discuss immune abnormalities further in section 1.5.

Neurodevelopment pathways have been implicated in ASD, with dysregulation of several key signaling pathways, such as sonic hedgehog (SHH), and bone morphogenetic protein (BMP) (Kumar et al, 2019). SHH signaling plays crucial roles in the organization of the brain, with a wide variety of roles that drive axonal targeting, proliferation and specification. Genes including

PTCHD1, DHCR7 and *EN2* which are known to interact with the SHH pathway have been mutated within ASD, typically resulting in the downregulation of the pathway (Kumar et al, 2019). BMP is critical in the development of the nervous system, which has been found to be dysregulated in ASD (Bond et al, 2012) Mutations in genes such as *FMR1* and *UBE3A* which influence the BMP pathway have been commonly found in those with ASD as well (Kumar et al, 2019).

1.3 Environmental Influence on Autism

Despite the amount of evidence implicating genetics in autism etiology, it does not alone explain the development of the disorder. Within families, as well as monozygotic twins, there can be wide-ranging phenotypes which suggest other factors to the etiology than genetics (London and Etzel, 2011). Recent evidence heavily suggests that various environmental factors can increase the risk of autism development to those susceptible to the disorder (based on their genetics) if exposed prenatally to these factors (Karimi et al, 2017). Prenatal brain development is a critical period in which involves many biological processes such as neurogenesis, migration, differentiation or synaptogenesis driven by the expression of specific developmental genes. These events have been shown to be influenced by environmental imbalances or insults.

Common environmental risk factors include the particulates in diesel exhaust and herbicides, have been shown to result in autism-like symptoms in mouse offspring exposed prenatally and in early life to the substances (Chang et al, 2018; Zablotsky et al, 2015; Roberts et al, 2013; Samsel & Seneff, 2015). Diesel exhaust has been found to activate and prime microglia, resulting in a pathogenic state; the importance of activated microglia are explained in section 1.6 (Levesque et al, 2011). As well Sugamata et al (2006) found that pregnant mice who

inhaled diesel exhaust gave birth to offspring having damages in the brain tissues of the cerebral cortex and hippocampus. Further investigation of these offspring showed that the maternal exposure to diesel exhaust resulted in an increase of apoptotic cells within these areas. To emphasize the significant impact of these particulates a recent study showed that residential proximity (<309m) to a major freeway resulted in a near doubling of the chance of having a child with ASD, compared to living further distances away (Volk et al, 2011)

Another common environmental factor that has been recently receiving attention for its contribution to ASDs is perfumes and synthetic fragrances. Fragrance producers are protected from having to disclose the ingredients of perfumes based on the Federal Fair Packaging and Labeling Act of 1973, and thus do not make the information available that often highly mutagenic, neurotoxic and neuromodulatory chemicals are within the product (Bagasra et al, 2013). Phthalates are an example of one of the chemicals which can be found within perfumes and not disclosed to the general public; they are known to obstruct normal development in the brain (Kougias et al, 2018). Using male and female human fetal brain neurons it was see that there was a distinct reduction of in viability when exposed to three commercially available fragrances, with a particular effect on male neurons (Sealey et al, 2015).

1.4 Immune system dysfunction and Autism

As of recent years, studies have begun to show a link between abnormal immune function and altered behavioral traits. Several studies have shown within humans an active role of neuroinflammation in ASD which has been supported by analysis of both brain tissue and cerebrospinal fluid analysis (Pardo et al, 2009; Gladysz et al, 2018). A variety of independent post-mortem studies have found dysregulated levels of immune system related genes in a variety of brain regions of those with autism (Garbett et al, 2008; Voineagu et al, 2011; Gregg et al,

2008; Linras et al, 2012). As well, studies have seen that in 15-60% of children with autism have some form of abnormal immune activation which can include B-cell, T-cell, Natural-killer-cell, and microglial dysfunction as well as increased pro-inflammatory cytokines (Pardo et al, 2009; Masi et al, 2017; Han et al, 2017).

Maternal immune activation (MIA) has been indicated as a risk factor for the development of ASDs, which has been specifically associated to fetal exposure to maternal cytokines (Parker-Athill and Tan, 2010; Urakubo et al, 2001). Human studies have shown that mothers who reported illness during pregnancy had an increased risk of their child having ASD (Zerbo et al, 2015; Jones et al, 2017). These findings are complemented in animal model studies where maternal immune factors such as interleukin-1B (IL-1B), IL-6 and tumor necrosis factor α (TNF-α) were able to cross the placental barrier and lead to immunological and neurological disturbances within the developing fetus, resulting in ASD-like behaviors (Girard et al, 2010; Shi et al, 2003; Patterson, 2009; Malkova et al, 2012). Cytokines and chemokines have been shown to have critical roles in neurodevelopment, assisting in the differentiation and maturation of neurons (Jones et al, 2017). In general, it is suggested that the altered gestational immune environment of the mother may lead to alterations in the neurodevelopmental trajectory of the fetus, based on the irregular levels of cytokines and chemokines in the fetal environment. Currently, the exact mechanism how the increase in circulating maternal cytokines and chemokines, due to MIA, results in abnormal fetal neurodevelopment is unknown (Jones et al, 2017).

1.5 Lipids in the brain

Recent research, including studies from our lab, provides evidence that ASD may result from abnormal lipid metabolism in the brain during its critical time of development (Wong and

Crawford, 2014; Tamiji and Crawford, 2010). Lipids are extremely important for the maintenance of a healthy brain, composing approximately 60% of the brain's overall weight. Near 20% of the total brain is composed of the polyunsaturated fatty acids (PUFAs), docosahexaenoic acid (DHA), and arachidonic acid (AA), which are derivatives of the omega-3 α-linolenic acid (ALA) and omega-6 linoleic acid (LA) respectively (Bentsen, 2017). These PUFAs play important roles not only in building the brain structure, but also in neuroplasticity, signal transduction, and learning and memory (Su, 2010; Wu et al, 2008; Ferrucci et al, 2006).

The conversion of these lipids into their downstream products are extremely important in the healthy function and maintenance of the brain. Recently a derivative of AA called prostaglandin E_2 (PGE2) has been focused on for its potential role in autism etiology (El-Ansary and Al-Ayadhi, 2012; Wong et al, 2014). An enzyme known as phospholipase A2 (PLA_2), whose function is involved in the maintenance of membrane phospholipids, breaks the membrane phospholipid into AA. AA will then be metabolized by one of two forms of the cyclooxygenase (COX) enzyme, identified as COX-1 or -2. COX-1 is constitutively expressed in most tissues, while COX-2 which is inducible in the body and constitutive in the brain (Phillis et al, 2006). COX-1 and -2 subsequently convert AA to PGE2 as illustrated in figure 1.

1.6 The Implications of Lipids in Autism Etiology

Abnormalities in the COX-2/PGE2 metabolic pathways due to genetic causes or environmental influences have been implicated in ASD and are discussed in our previous review articles (Wong and Crawford, 2014; Tamiji and Crawford, 2010). For example, studies have found altered levels of AA, DHA and another PUFA known as eicosatetraenoic acid (EPA) in blood samples of ASD patients (Neggers et al, 2009; Mostafa and Al-Ayadhi, 2015; Wiest et al, 2009). To complement the importance of balance in the lipid pathway and its relation to autism,

omega-3 and omega-6 fatty acid supplements have been used as effective treatments for ASD symptoms (Keim et al, 2018; Nagwa et al, 2008).

Of interest, previous work has demonstrated that both high or low levels of PGE2 are related to ASD. PGE2 has many roles in the brain that include dendritic spine formation, pain transmission, refining of mature neuronal connections and synaptic plasticity (Akaneya, 2007; Scott et al, 2007; Tegeder, 2007; Chen et al, 2002; Yang and Chen, 2008). Once produced PGE2 acts on one of four E-Prostanoid (EP) receptors: EP1, EP2, EP3 and EP4 (Figure 1). Activation of the PGE2 pathway has also been heavily implicated in events such as the inflammatory response, which is known to be abnormal in those with autism (El-Ansary and Al-Ayadhi, 2012). Antagonists to the PGE2 pathway include Nonsteroidal anti-inflammatory drugs (NSAIDs) that result in the inhibition of the COX enzymes (Meek et al, 2010). Interestingly, previously discussed factors such as immune activation, air pollution such as diesel exhaust, and common consumer productions have been found to increase the PGE2 production pathway (Hofer et al, 2004; Ricciotti and FitzGerald, 2011). Wong et al (2015) summarized a variety of environmental risk factors previously mentioned that have been linked to autism result in a dysregulation of PGE2 production demonstrated below (Figure 1).

We have shown that altering the COX-2/PGE2 pathway results in different expression patterns of ASD related genes, with functions including cell migration, synaptic transmission and axon guidance (Wong et al, 2014). As well, behavioral testing has recently been done by Wong et al (2018) demonstrating characteristic behaviors of ASD such as high levels of anxiety, hyperactivity, and repetitive behaviors as well as decreased sociability in two PGE2 mouse models. One model having a mutation in the COX-2 gene called- Prostaglandin-endoperoxide synthase 2 (*Ptgs2)* resulting in low levels of PGE2, and the other with increased levels due a

maternal PGE2 injection. These mice were found to have ASD-like traits in the males and females of both PGE2 models compared to controls. The results from our lab in both animal and cell work have shown that altering normal levels of PGE2 result in molecular and behavioral changes that can be implicated back to ASD.

Microarray data from our lab using male brain tissue from embryonic day16 (E16) and E19 COX-2^{-}KI mice showed abnormalities in a variety of genes related to autism and neuronal functions (Rai-Bhogal et al, 2018). This work found 42 ASD genes within the COX-2^{-}KI model to be dysregulated, involved in pathways such as wingless (WNT) signaling, axon guidance, intracellular transport, and negative regulation of apoptosis. This complements previous findings from our lab indicating that PGE2 is able to alter WNT-regulated gene expression in a neuroectodermal (NE-4C) stem cell model (Wong et al, 2014; Wong et al, 2016). Wong et al (2014) was able to confirm for the first time that within NE-4C cells that PGE2 signaling can influence WNT-signaling via downstream effectors such as protein kinase A (PKA) and phosphatidylinositide 3-kinases (PI-3K) (Figure 2). The WNT signaling pathway is known as a crucial pathway for the development and organization in the brain, and its dysregulation has been correlated with autism, schizophrenia, Alzheimer's disease and various inflammatory diseases (Ferrari and Moon, 2006; Zimmerman et al, 2012). The WNT signaling pathway has also been recently tied to microglial viability and proliferation in neurological diseases such as Alzheimer's disease (Zheng et al, 2017). The role of activated microglia and their implications to the brain are discussed below.

Additional work from our lab done in NE-4C cells demonstrated that PGE2 has pivotal roles in cytosolic and growth cone calcium levels and in promoting stem cells to differentiate into neuronal-lineage cells (Tamiji and Crawford, 2010a; Wong et al, 2016).

Further implications for fatty acids and their derivatives in ASD come from studies involving misoprostol. Misoprostol is drug typically used for gastric ulcer treatments as well as to induce uterine contractions to induce early pregnancy termination in pregnant women. Pregnancies that were not properly terminated by this drug resulted in autistic-like characteristics in a pathology termed Moebius syndrome; this is of particular interest as misoprostol is a PGE2 analogue (Bandim et al, 2003). The findings from our lab and others clearly demonstrate the implications of the abnormal regulation to the COX-2/PGE2 pathway, resulting in higher or lower levels of PGE2 compared to healthy controls, and their possible connections back to ASD.

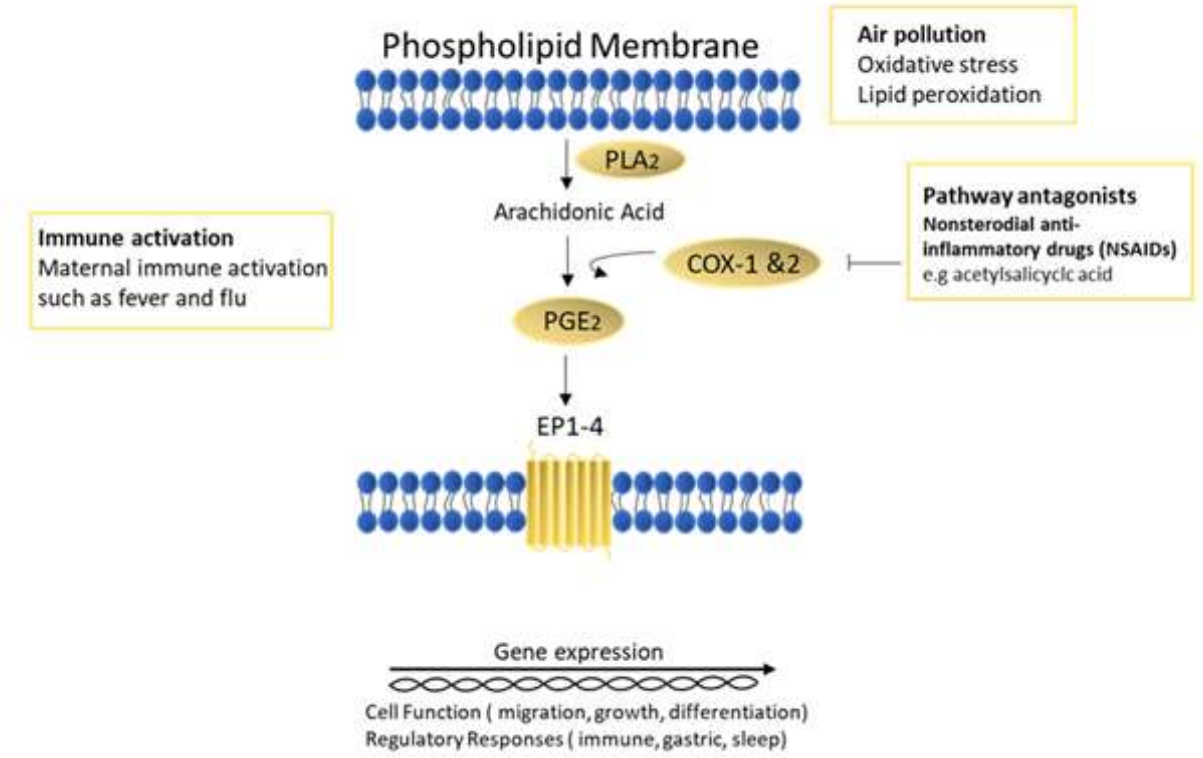


Figure 1: An overview of the prostanoid synthesis pathway and environmental interactions. AA is metabolized from the phospholipid membrane by PLA2, which will then be acted on by COX-1 and -2 for the production of PGE2. PGE2 will affect one of four receptors, prostaglandin receptor 1-4 (EP1-4), resulting in downstream gene expression. Pathway antagonists such as nonsteroidal anti-inflammatory drugs (NSAIDs) decrease PGE2 production through the disruption of the COX-1 and COX-2 enzymes. Other common factors previously shown to alter prostaglandin production include air pollution, consumer products, and products that influence hormone production. This image has been modified from previous work in Wong et al, 2015

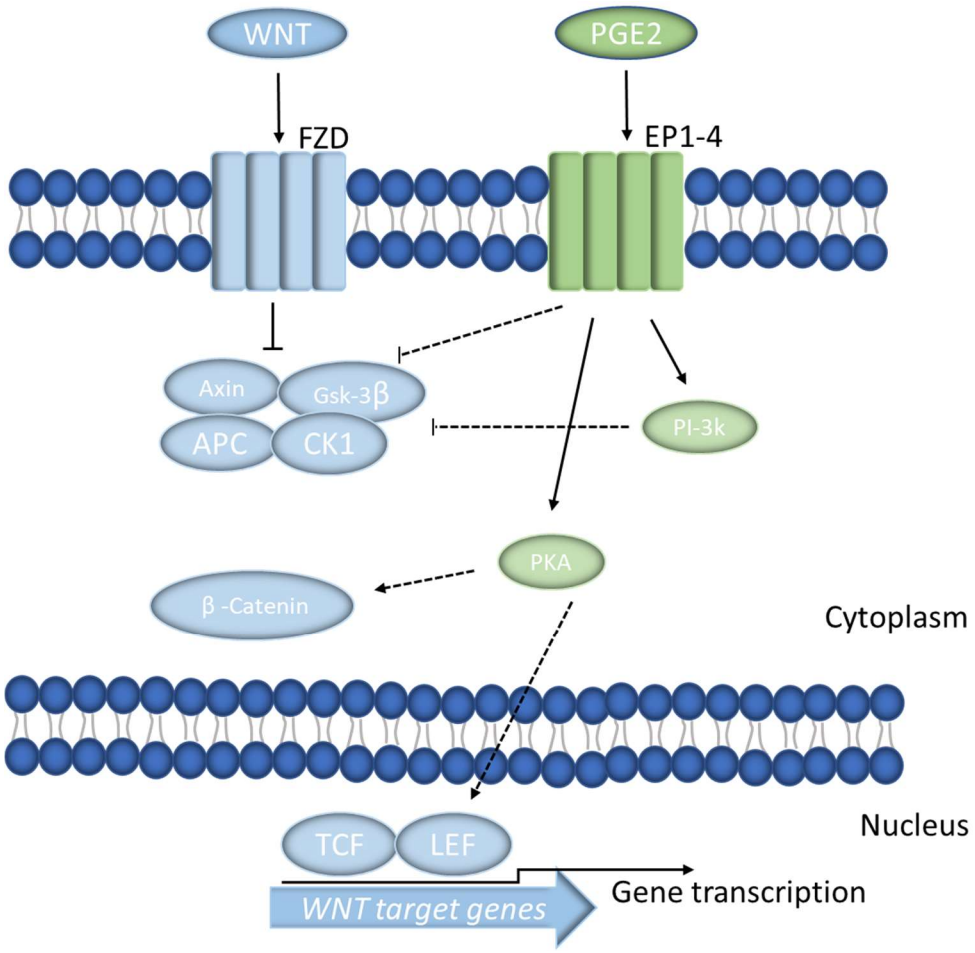


Figure 2: A proposed model of PGE2 and WNT pathway interactions. Adapted from Wong and Crawford (2014) this image demonstrates how PGE2 regulates the canonical WNT pathway. PGE2 interacts with EP1-4 receptors resulting in downstream protein kinase A (PKA), phosphatidylinositide 3-kinases (PI-3K) activity; these kinases are suggested to act as the link between the two pathways.

1.7 Microglia Overview

Microglia are a type of glial cell found within the central nervous system (CNS) accounting for as much 10-15% of the total cell population of the brain (Lawson & Gordon, 1992). They are known as the tissue-resident macrophage of the CNS but have a distinct lineage from macrophages. Microglial precursors are yolk sac derived and invade the brain during early development at E9.5 within the mouse brain, where they will migrate through the parenchyma and mature (Ginhoux et al, 2013). Various studies have shown that microglia that are a self-sustaining population that remain distinct from macrophages throughout life, repopulating through constant proliferation known as microgliosis (Reu et al, 2017). These cells are very sensitive to their environmental cues and as such are variable between different subregions of the brain in both overall density and activity due to the difference in gene expression within those regions (Grabert et al, 2016).

The driving force for microglial precursors to be drawn to the brain from the yolk sac is currently thought to be based on the brain's need for apoptotic cells to be cleared. "Find me" and "Eat me" signals, which are signals released from apoptotic cells, call phagocytotic cells like the microglial precursors to it for clearance (Du and Du, 2016). Some of these signals include adenosine triphosphate (ATP) and uridine diphosphate (UDP) which respectively bind to the P2Y12 and P2Y6 purinergic receptors of microglia, initiating the movement towards the area which the signal came (Koizumi et al, 2012).

The precursors will mature into adult microglia by P14, identifiable by their shape of a small cell body and large thin branches extending from it, known as a ramified morphology (Matcovitch-Natan et al, 2016). The roles of ramified microglia are discussed below.

Although the exact mechanism of microglial maturation is still debated it is generally thought that the environmental changes of the developing brain are what causes the microglia to

mature (Matovich-Natan et al, 2016). Microglia are incredibly functionally diverse being able to meet the needs of the developing brain, as well as the life-long maintenance of the healthy adult, which drives the concept that microglia gain their specialized functions tailored to the environment they are in. Indeed, as the brain develops it has been recently found that microglia will follow a stepwise pattern of development, each having characteristic gene expression and functional states (Matovich-Natan et al, 2016). From when the precursors enter the brain until they mature, they demonstrate morphologies and functions similar to the mature active morphology we discuss below.

Microglia have two main morphologically distinct states in the mature brain known as active or ramified (Figure 3). Ramified microglia were previously known as resting microglia, but there has been a recent change in nomenclature in the literature as these cells are anything but "resting". Ramified microglial cells have a distinct morphology of a small cell body with thin, far reaching, extensions (Young et al, 2018). These cells were previously believed to have very little influence on the overall health of the brain, essentially waiting to become functional by activation. It is now understood that while their somas remain sedentary the microglial branches are highly motile. These branches are continuously surveying the brain for possible insults or pathogen, as well as transiently contacting synaptic elements of the brain (Tremblay et al, 2010. Nayak et al, 2014). Studies examining the function of these branch connections have suggested ramified microglia play a role in synaptic pruning, indicated by pre- and post-synaptic puncta located in the cell. As well, ramified microglia have also been seen to assist in the maturation of synaptic elements through the release of growth factors such as brain derived neurotropic factor (BDNF) (Parkhurst et al, 2013).

When ramified microglia encounter a possible insult or pathogen, the cell begins to rapidly change into a state known as “active”. The long thin branches will begin to retract into the cell body, creating a distinct morphology of a large, rounded cell bodies with no little to no branching extensions (Young et al, 2018). In this state microglia are considered fully phagocytic and able to encapsulate harmful debris such as apoptotic or necrotic cells, bacteria and viruses (Janda et al, 2018). These cells are highly reactive and begin to express a variety of receptors and secrete factors distinct from the ramified morphology. As more research is completed, more is understood the complexity of microglia and its activation- leading us to understand that there are two forms of active microglia known as M1 and M2 (Orihuela et al, 2015; Ponomarev et al, 2007). While the M1 and M2 stage share a similar rounded morphology, based on the stimulus that resulted in their move from the ramified state, they will complete different roles.

M1 microglia, considered to be classically activated, are known as the pro-inflammatory active state typically induced by lipopolysaccharides (LPS) or interferon gamma (IFNγ) contact. M1 microglia have nuclear factor kappa-light-chain-enhancer of activated B cells (NF-KB) pathways activated which are heavily tied to inflammation; this pathway results in the expression of various cytokines and chemokines (Lawrenece, 2009; Liu et al, 2017). As well these M1 cells are linked to reactive oxygen species and nitric oxide production, all of these factors contribute to the inflammation process (Tang and Le, 2015). These cells exhibit an increased subset of receptors typically associated with phagocytic processes such as cluster of differentiation (CD)11b. CD11b binds to CD18 to form the Mac-1 receptor displayed on the surface of activated microglia, which is an essential receptor in microglial phagocytosis (Schuman and Weiss, 2005; Zhang et al, 2011). While the Mac-1 receptor is constitutively expressed within all microglia, it

is upregulated within the M1 phenotype, which grants it as a marker of microglial activation (Wang et al, 2013).

M2 cells are known as alternatively activated or acquired deactivated cells and have multiple anti-inflammatory properties that antagonize the M1 pro-inflammatory responses. Alternatively activated (M2) cells arise from ramified cells that have been stimulated by IL-4 and IL-13, while acquired deactivation cells are M1 cells that have been stimulated by IL-10 or transforming growth factor beta (TGF-β) and switch to the M2 anti-inflammatory state (Tang and Le, 2015). M2 secrete factors such as insulin growth factor 1 (IGF-1), arginase 1 (Arg1), and YM1 which assist in anti-inflammatory processes.

These diverse cells not only help with the maintenance of the healthy brain, but also assist in its formation during development. Although these cells are not required for the general assembly of the brain structures, they have been shown to be crucial in its fine tuning. Microglia in recent years have been found during development to be important for neuronal apoptosis, angiogenesis, synapse elimination, and axon growth and guidance (Pont-Lezica et al, 2011). Microglia are also able to play a large role in programmed cell death through the secretion of TNF-a, reactive oxygen species (ROS), and glutamate (Bessis et al, 2007). This is of importance as during vertebrae development the brain overgrows, resulting in the need for approximately half of all neurons born to undergo programmed cell death for a healthy brain (Vanderhaeghen and Cheng, 2010; Bang and White, 2000). As well, these dying cells will then need to be properly cleared from the locale, further implicating microglial importance in healthy development, based on their phagocytic properties.

1.8 Microglia, Sex, and Age Differences

Microglia are extremely sensitive to their local environment, including to the sex hormones appropriate to the sex of the organism they belong to, resulting in distinct differences between males and females. It has been shown for example that estrogen, the female sex hormone, has a general anti-inflammatory effect on microglia (Bruce-Keller et al, 2000; Villa et al, 2016; Vegeto et al, 2002). In line with this concept, a variety of studies have demonstrated male mice to have a greater inflammatory profile in mice 3 months old as well as at 15-17 months. It has also been shown that estradiol, the aromatized version of testosterone within the mouse brain, promotes a more active morphology within microglia which is a sign of inflammation (Villa et al, 2018; Rahimian et al, 2018; Lenz et al, 2013).

It has also been shown that both age and sex play a large role in microglia colonization and morphology across development. Schwarz et al (2012) examined for these differences within the developing and postnatal rat brain in one prenatal and 4 postnatal stages. They reported that males were seen to have higher densities at P4 within the hippocampus, amygdala and the parietal cortex. Interestingly during later stages, P30 and P60, females were then seen to have increased microglia within these same regions.

Microglia have also been found to be extremely important in the masculinization of the neonatal brain induced by the male gonadal activity (Lenz et al, 2013). The spark that originated the interest that microglia may play a role in masculinization originated from work completed by Gorski et al (1978) where they determined there were sex-based microglial differences within the preoptic area. This is of significance as this area of the brain is generally implicated in the control of male reproductive behavior. Further studies have examined the role of testosterone in male rodents and how it may influence the changes seen in microglia. In male mice there is a surge in testosterone during development that once it enters the brain is aromatized to a variety

of products including estradiol (E2). E2 was found by McCarthy (2008), using P4 mice to be the dominant masculinizing hormone within the rodent brain and interestingly was found to induce PGE2 production in microglia (Lenz et al, 2013). To determine if the microglia were the particular cell type being influenced by E2 that consequently influenced changes in the brain that resulted in male-like behavior Lenz et al (2013) completed an experiment using E2 and a microglial inhibitor. Newborn female mice were administered E2 alone, mimicking the naturally occurring gonadal surge in males, or E2 and a microglial inhibitor known as minocycline. Interestingly they found that females who received E2 alone were found to have masculine sexual behaviors, while those given the inhibitor did not. This implicates not only the importance of sex on microglia, but also the importance microglia have on the sexing of the brain.

1.9 Microglia, PGE2, and Autism

With all the important roles microglia play within the healthy brain the role microglia may play within pathologies has recently come into question. Deletions of key microglial transcription factors such as Hoxb8, which are only found in microglia within the CNS, resulted in excessive grooming behavior within animal studies (Greer and Capecchi, 2002; Chen et al, 2010). This was one of the first studies directly showing that abnormalities in microglia can have a direct effect on behavioral syndromes, although the exact method of how microglia induce these changes is currently unknown.

As previous mentioned animal models of ASD, as well as human studies, have shown that these individuals have increased expression of nitric oxides, chemokines and cytokines which is a clear sign of neuroinflammation (Goines et al, 2013). Another indication of inflammation is characterized by the pro-inflammatory active state of microglial cells (Rodriguez and Kern, 2011). Various post mortem studies have shown that in patients with ASD there was

abnormal microglial activation to controls, tying the cells further to the disease (Vargas et al, 2005; Morgan et al, 2010; Tetreault et al 2012). It is also well documented that ASD patients have abnormal brain synaptic connectivity shown through fMRI studies done in humans, which may relate to aberrant microglial activity (Rodriguez and Kern, 2011; Damarla et al, 2010; Ebisch et al, 2011). As well, the environmental and immune factors linked with ASD have strong ties with microglial abnormalities in activation state and density. As microglia regulate so many vital functions to healthy brain development and maintenance dysregulation of these cells has been implicated in many of the pathologies associated with ASD.

Some individuals with ASD were found to have increased levels of PGE2 compared to controls (El-Ansary and Al-Ayadhi, 2012). Interestingly, males have increased levels of PGE2 during development due to the elevated steroids associated with the masculinization of the brain. PGE2 has been associated with the masculinization of the brain, shown through increased dendritic spine density, a known trait of the male brain (Wright et al, 2008; Wright and McCarthy, 2017). PGE2 and microglia have been found to interact in a cyclical manner, beginning with PGE2 signaling being found to influence the activation of microglia through stimulation of EP2 receptors on the cell's surface (Quan et al, 2013)**.** Once the cell is activated, microglia produce an inducible protein called microsomal prostaglandin E2 synthase 1 (mPGES-1), which will further produce PGE2 downstream, leading to an exacerbation of the inflammatory process (Ikeda-Matsuo et al, 2005). The levels of mPGES-1 have been found to be higher in those with autism and have been noted as a marker to describe the severity of the disorder, indicating the important role in ASDs (Zhang et al, 2009).

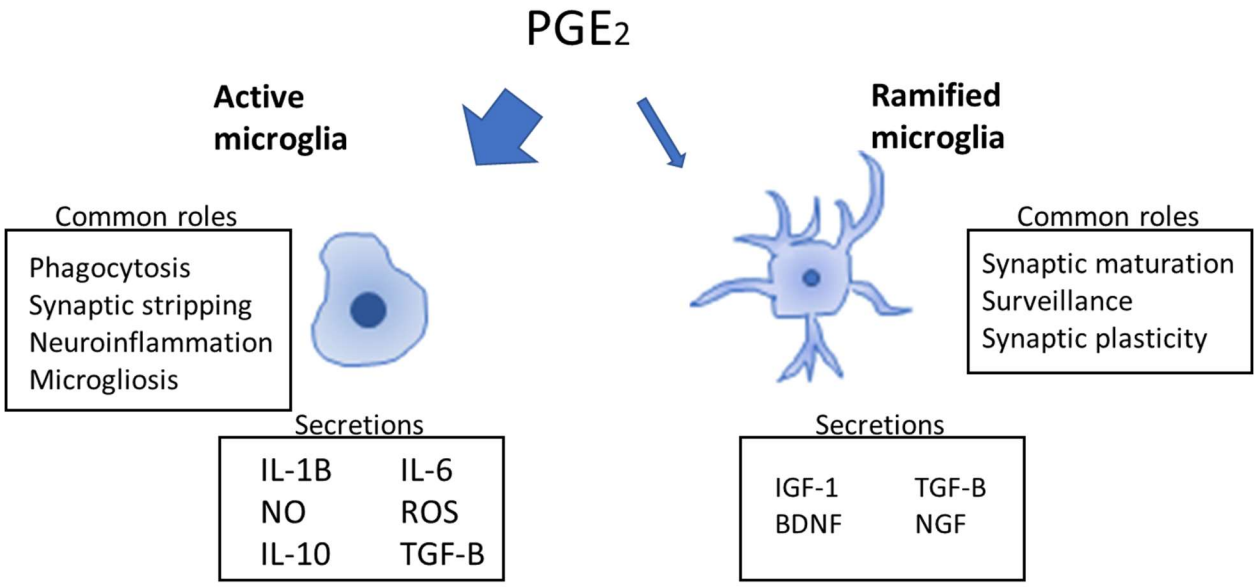


Figure 3: PGE2 effect on microglial morphology and common roles of the activation states. Active microglia are represented on the right, showing an amoeboid shape with a variety of roles and secretions revolving around inflammatory processes. Some of these secretions include interleukin-1β ,-6,-10 (Il-1β , Il-6, Il-10), nitric oxide (NO), reactive oxygen species (ROS), and transforming growth factor- β (TGF- β). Ramified microglia exhibit a smaller cell body with long extending branches with roles and secretions revolving around maintenance of the brain. They have been shown to secrete insulin growth factor-1 (IGF-1), brain derived neurotrophic factor (BDNF), nerve growth factor (NGF) and TGF-β.

2. Research Justification and Review

Microglia are now understood to play many diverse roles in the brain, not only during adulthood, but throughout development as well. These cells have a wide range of roles that influence the overall health of the brain that are heavily related to their morphologically distinct activation states. The balance of the two activation states, active or ramified, is a "goldilocks" situation where imbalances in the morphologies can result in neurological abnormalities- it needs to be just right in order to ensure a healthy brain. Previous studies have focused on the differences in select brain regions at a variety of time points to examine differences in microglial colonization and activity to gain insight into various diseases and disorders. As microglia are related to the immune system and synaptic pruning, two extremely important processes in the brain that are known to be dysregulated in those with ASD, naturally the relation of these cells to ASD began to spark (Rodriguez & Kern, 2011). Beyond a possible speculation, both gene and post-mortem studies have been completed that heavily tie together microglial involvement in ASDs (Pardo et al, 2009; Vargus et al, 2004; Morgan et al, 2010; Rodriguez & Kern, 2011; Smith et al, 2012).

PGE2 is a lipid signaling molecule known to influence the balance between active and ramified microglia, whose expression in autistic individuals has been found to be altered as well. Many studies completed in our lab have shown the importance of PGE2 to the brain, its connection to autism, as well as showing that the COX-2^{-} KI model serves as an ASD model. With this in mind Wong et al (unpublished) began to examine differences in microglial density, activity levels, and branching patterns within the P8 wild-type (WT) and COX-2^{-} KI mouse. The P8 stage is significant for autism studies as it is analogous to a human infanthood, which is when most autism behaviors become evident (Pressler and Auvin 2013). This work showed significant microglial differences within the COX-2^{-}KI mouse using the same criteria to be used in this

study, looking into five regions of that have been tied to ASD: the cerebellum, hippocampus, olfactory bulb, prefrontal cortex and the thalamus. Our lab has also completed gene expression analysis of P8 COX-2$^-$ KI male and female mice examining microglial genes such as *IL-1B, 1L-6,* and *CD11B*, which were all seen to be dysregulated within our COX-2$^-$ KI model (Wong et al, 2018).

My work will be completed in two developmental stages that, to our knowledge, have not been previously analyzed for microglial differences within the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and the thalamus. We will be examining all data for possible sex-based differences at E19 and P25, as well as differences in microglial density, activity and branching patterns based on the COX-2$^-$ KI condition. These novel findings will help to provide deeper insight into the effects of age, sex, and PGE2 on microglia, as well as eliciting to the potential roles of PGE2 and microglia in the manifestation of autism.

3. Research plan

3.1 General objective

The general objective of this study is to examine microglia activity in the brains of COX-2^{-}KI mice, which lack the COX-2 enzyme which produces PGE2, to provide further knowledge into the pathology of ASD. I will quantify microglial density, activity, and branching using a morphological analysis within E19 and P25 developmental stages. This study will also investigate for the first-time differences between male and female offspring in order to gain more insight into ASD sex-related differences at these developmental time points. Results from this study will add to our understanding of how abnormal signaling of the COX-2/PGE2 pathway influence microglial activity in the developing brain and how it may contribute to brain pathology that results in ASD.

3.2 Study 1: Microglial morphology and density in prenatal and postnatal brain of COX-2 deficient mice

Objective: In this study I aim to examine the density and morphology of microglia within five regions of the brain: the cerebellum, hippocampus, prefrontal cortex, olfactory bulb and thalamus. I will analyze morphological differences for activity while also examining their average branch length, primary branch counts as well as total branch counts. Using both male and female COX-2^{-}KI mice I will examine differences prenatally at E19 and postnatally at P25.

Hypothesis: PGE2 has been shown to be a classical microglia activator. Our previous work in the brains of COX-2^{-}KI offspring at P8 have already shown sex and region-specific decreased microglia activation, increases in microglial density and branching behaviors. This study stems from these findings and will evaluate these morphologies in development at the prenatal (E19) and postnatal (P25) stages. We hypothesize that in the COX-2-KI offspring we will observe age-

related differences in microglial density, activation (amoeboid vs. ramified), process length and numbers in two developmental ages (E19 vs P25) based on sex and brain region.

Methodology: I will visualize microglia using immunohistochemistry with well-established microglial marker anti-ionized calcium binding adaptor molecule 1 (Iba1) to examine morphological differences within five brain regions. I will analyze the microglial density, activity, and branching differences in two developmental stages while also examining for sex-dependent differences.

3.3 Experimental Model System

The COX-2- KI model obtained from Jackson Laboratory (stock number: 008101) were created by a targeted gene mutation of a Y385F substitution in *Ptgs2* (Queen's University, laboratory of C. Funk). COX-2- KI mice were back crossed with our wild-type model, 129S6/SvEvTac acquired from Taconic Laboratory, for at least five generations. COX-2- KI mice also known as $Ptgs2^{Y385F}$ founder mice and B6.129S6(FVB)-$Ptgs2^{tm1.1Fun}$/J, ultimately have complete inhibition of COX-2 activity (Yu et al, 2006). As homozygous females are infertile, heterozygous females were bred with homozygous or heterozygous males in order to obtain the required homozygous COX-2^{-} KI offspring used in this study. The wildtype strain 129S6/SvEvTac comes from Taconic Laboratory.

3.4 Contributions

All Immunohistochemical staining, microglial morphological analysis and statistics were conducted and completed by Sarah Wheeler. Mouse brains were extracted by Ravneet Rai-Bhogal and Sarah Wheeler.

4. Methodology

4.1. Generation of COX-2-KI and Wild Type Models

As homozygous COX-2^{-}KI females are infertile, we bred COX-2^{-}KI homozygous males with heterozygous COX-2^{-}KI females to collect homozygous COX-2^{-}KI offspring. Mice were housed at the York University animal facility according to the York University Animal Care Committee (ACC) ethics guidelines. Upon arrival to the facility mice were acclimatized for one week to the 12-hour light/dark cycle. Animals were maintained in under similar conditions with food and water available *ab libitum.* All breeding and experiments were approved by the Research Ethics Board of York University.

4.2 Genotyping

To determine the sex and the knockout zygosity gDNA was isolated from tail samples and ear punches that were homogenized in alkaline lysis reagent (25 NaOH) and then neutralized via Tris-HCl. PCR reactions to genotype the COX-2-KI offspring was completed using a primer sequence obtained from Jackson Laboratories specifying the COX-2 gene (*Ptgs2)* (Table 1). For prenatal stages, a PCR for the sex determining region Y (*SRY),* was used to examine the sex of the offspring (Table 1). The PCR reaction was carried out using a PCR master mix (2.5 mM dNTP, 10x Taq buffer Biolabs #M04481S, 5 units/µL Taq DNA polymerase Biolabs #M0480S, 20mm MgSO4), 4 µL of DNA and ddH20, and 10µM of primers (Table 1). The reaction was performed in an Eppendorf Mastercycler with PCR products resolved using a 1.2% agarose gel with SafeView (Abm, G108) and 6x-BB used as a loading buffer.

Table 1. PCR primer sequence for *Gapdh* gene, Sex and Genotype Analysis.

Primer	Name	Sequence (5'-3')	Length (bp)
Forward	Mus *Gapdh*	TTGTGATGGGTGTGAACCAC	20
Reverse	Mus *Gapdh*	GTCTTCTGGGTGGCAGTGAT	20
Forward	Mus *Sry*	TCCCAGCATGCAAAATACAGAGATCAGC	28
Reverse	Mus *Sry*	TTGGAGTACAGGTGTGCAGCTCTAC	25
Forward	Mus *Ptgs2*	ACCAGTCTCTCAATGAGTAC	20
Reverse	Mus *Ptgs2*	AGAATGGTGCTCCAAGCTCTAC	22

4.3 Brain Extraction

Brain samples of the COX-2^{-}KI model and WT were extracted at a prenatal and postnatal time points E19 and P25 respectively. Mice were rendered unconscious by placing them into a chamber of oxygen and isoflurane and were quickly decapitated. E19 was a selected time point for this study as it is the last possible embryonic point that could be collected before birth, allowing us to examine the brain at a period of growth and development. It also represents a time where the microglia population has recently invaded the brain; cells are anticipated to be found in an amoeboid shape with an increasing population based on increased rates of microgliosis (Figure 4). P25 mice were selected as they have fully formed regions of the brain such as the cerebellum. At this developmental point the microglial population is expected to be at the stable population seen throughout adulthood, with cells anticipated to be in a more ramified morphology (Figure 4).

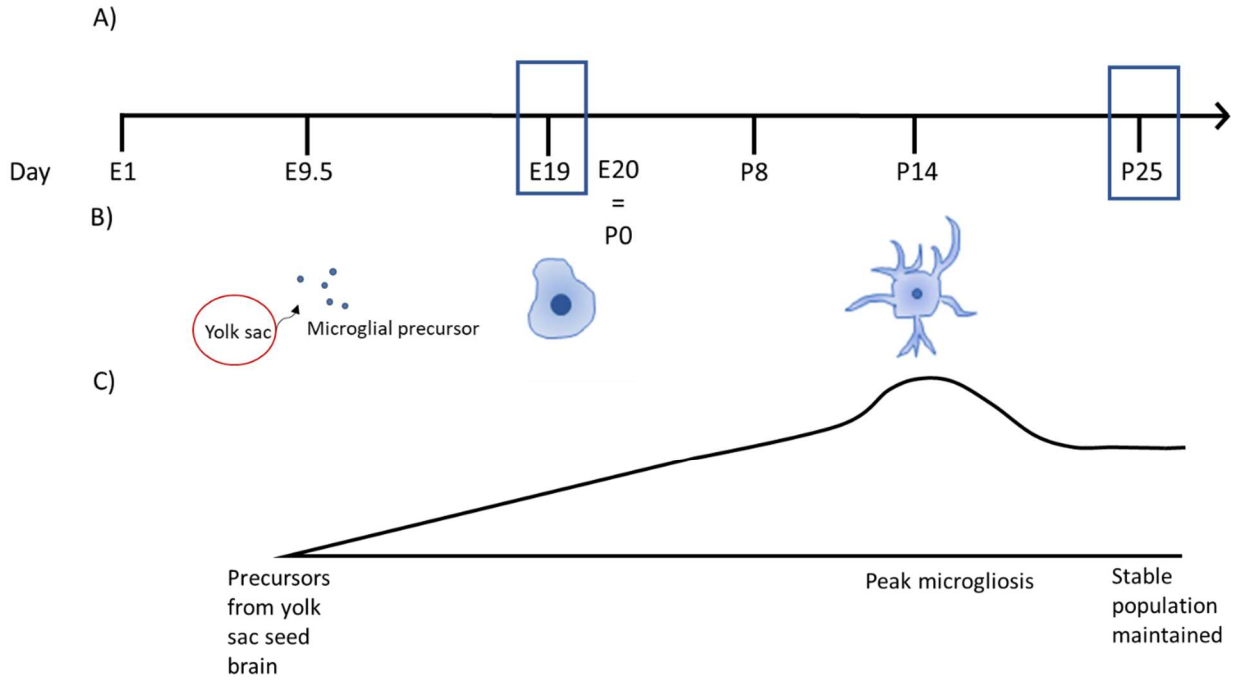


Figure 4: Timeline showing progression of microglia development in the mouse brain. (A) Key prenatal and postnatal developmental time-points including dates important for our study, (B) initiation of microglial development and morphology time-line of microglia maturation and (C) the progression curve of microglia density throughout development. Information for (B) and (C) is representative of what is known for the healthy mouse brain (Lenz and Nelson, 2018; Matcovitch-Natan et al, 2016). "E" stands for embryonic day and "P" for postnatal day. E1 represents the beginning of the embryonic stage, determined by females who have formed a plug, a sign of fertilization. E20 or P0 represents birth and the start of postnatal stages. Stages that have been outlined in a blue box represent time points of collection in this study.

4.4 Immunohistochemistry

Extracted brain samples were observed for any damages, abrasions or detached structures. Samples that were found to be intact and undamaged were subsequently washed in 4% paraformaldehyde (PFA) for one hour, then allowed to soak in a replenished 4% PFA solution for 48 hours. After which time they were transported to the Center for Phenogenomics (Toronto, Canada) to be embedded sagittally in paraffin and sliced at 4um. Samples had paraffin removed through washes in xylene and rehydrated in descending ethanol washes. Samples then were boiled in sodium citrate (pH=6) for 20 minutes for antigen retrieval. To prevent any possible endogenous peroxidase activity samples were incubated in 0.3% hydrogen peroxide in methanol for 30 minutes. Following this the samples were circled using a super PAP pen (Cedarlane); a hydration chamber was created and used for all following steps. Samples were blocked using phosphate-buffered saline (PBS), with 5% goat serum and 0.1% Triton-X for 30 minutes. Samples then left overnight at 4°C to incubate with the primary antibody Rabbit anti-Iba1 (1:1000, Wako Cat. #019-19741) diluted to 5% goat serum in PBS. Secondary antibody staining was done with an anti-rabbit IgG antibody (1:2000, Vector Laboratories) incubation for one hour at room temperature, followed by an incubated with Elite Reagent (Vectastain Elite Kit, Vector Laboratories) for an additional hour. 3-3'-Diaminobenzidine (DAB) was used to develop the immunostain through incubating slides with a DAB substrate solution (SIGMAFAST DAB tablets, D4168, Sigma) dissolved in Milli Q water and 0.3% ammonium nickel sulfate hexahydrate. Using ascending ethanol washes samples were dehydrated, followed by a xylene incubation. Permount mounting medium was used to fix coverslip slides to the samples. An example of this staining technique is demonstrated in figure 5.

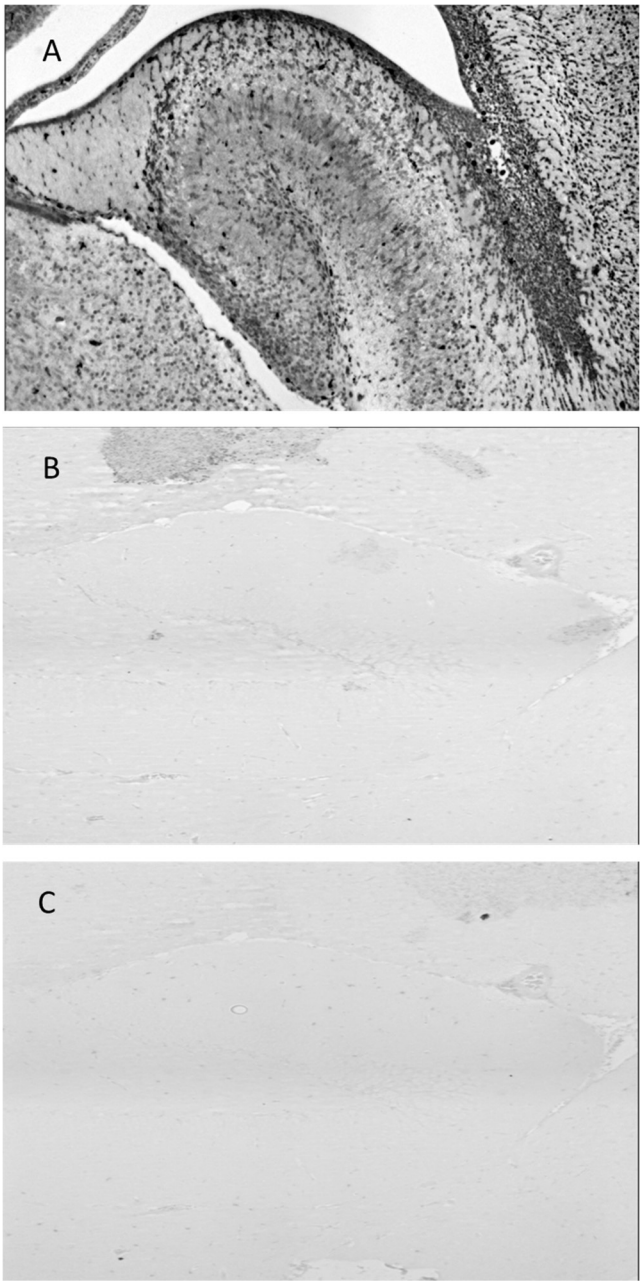


Figure 5: Immunohistochemistry staining results with Iba-1. To demonstrate immunohistochemistry staining results under optimized conditions, we show staining of the hippocampus with a primary and secondary antibody (A), staining without a primary antibody (B), and staining completed without use of the secondary antibody (C).

4.5 Microglial Cell Density and Morphology Analysis

Images of the brain samples were taken using a Nikon Eclipse Ti-E (Nikon) microscope at 20x magnification and automated image stitching. Five regions of the brain previously implicated in ASD were imaged using light microscopy, the cerebellum, hippocampus, olfactory bulb, prefrontal cortex and thalamus (Figure 6-7). The estimated density of each region was obtained by calculating the total number of cells and dividing it by the area of the given region. Cells were classified morphologically into one of two categories: active or ramified. Active microglia have enlarged cell bodies that are typically amoeboid and rounded in shape, while ramified microglia have smaller cell bodies with long thin branches (Figure 8). The percentage of active or ramified microglia was determined by taking the number of active/ramified cells per region and dividing it by that region's total density. The activation state, represented by an amoeboid or ramified cell shape, the longest length of processes, the number of primary branches along with the total number of branches per microglia cell was quantified using NIS-elements software (Nikon). Primary branches were defined as branches that extended directly from the soma, while total branches included all branches from the cell (secondary, tertiary, etc). The length of processes was measured from the surface of the soma to the tip of the longest process per cell (Figure 8). All analyses were completed blind to the condition of the samples

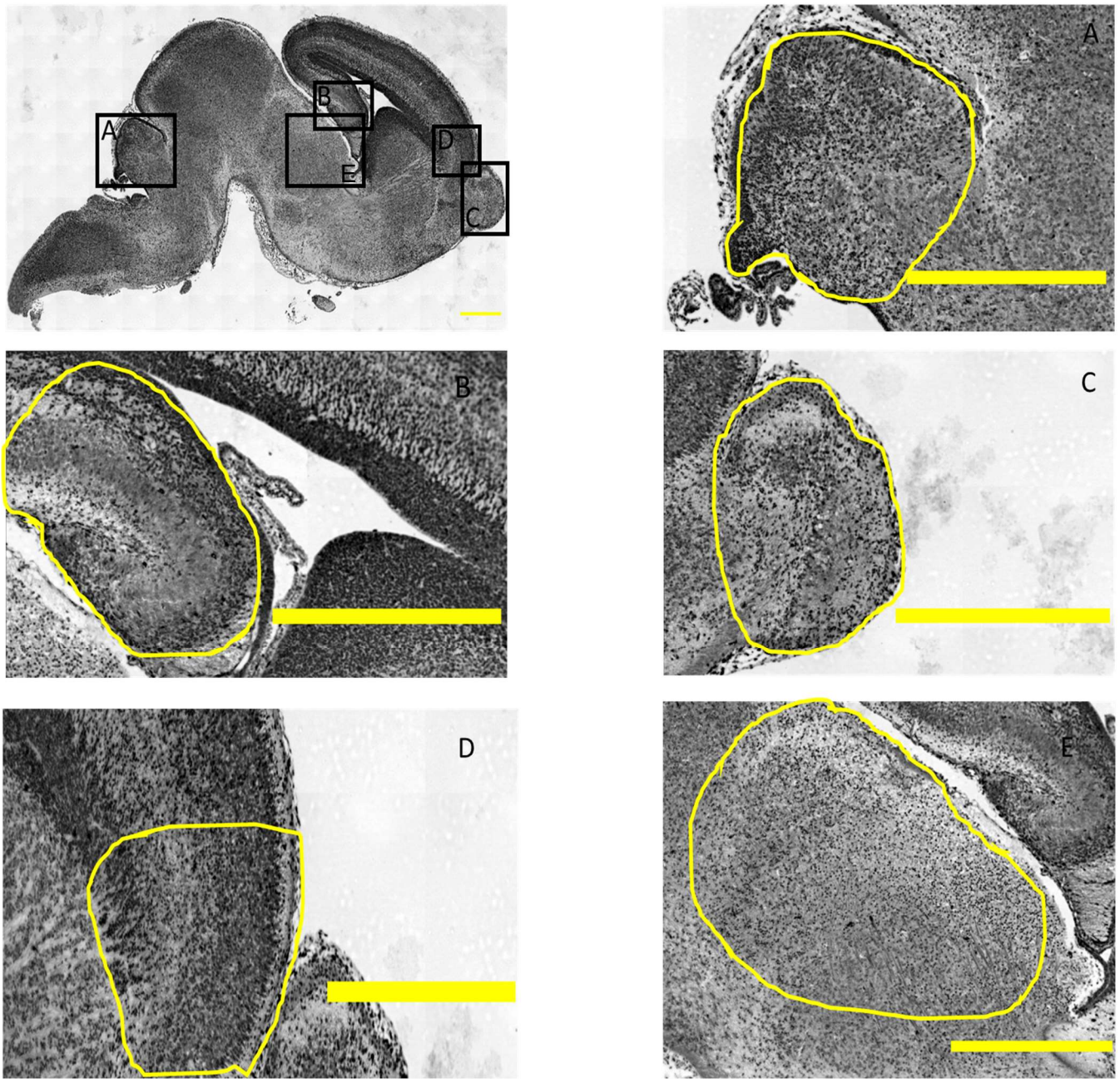


Figure 6. Iba1 immunohistochemistry staining of E19 brain regions. Sagittal slices of the cerebellum (A), hippocampus (B), olfactory bulb (C), thalamus (D) and prefrontal cortex (E) were analyzed (outlined in yellow). Scale bar represents 500 μm.

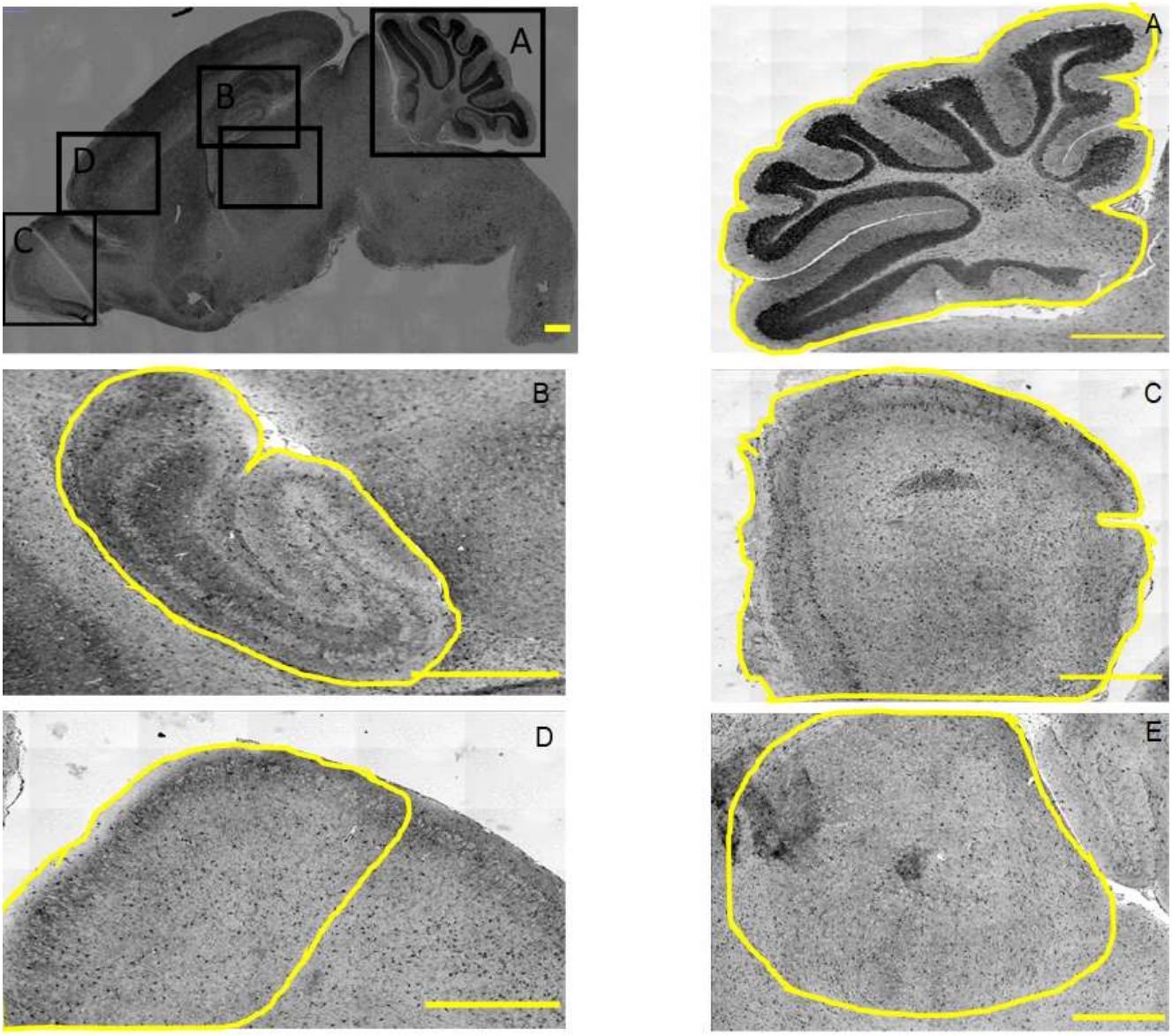


Figure 7: Iba1 immunohistochemistry staining of P25 brain regions. Sagittal slices of the cerebellum (A), hippocampus (B), olfactory bulb (C), thalamus (D) and prefrontal cortex (E) were analyzed (outlined in yellow). Scale bar represents 500 μm.

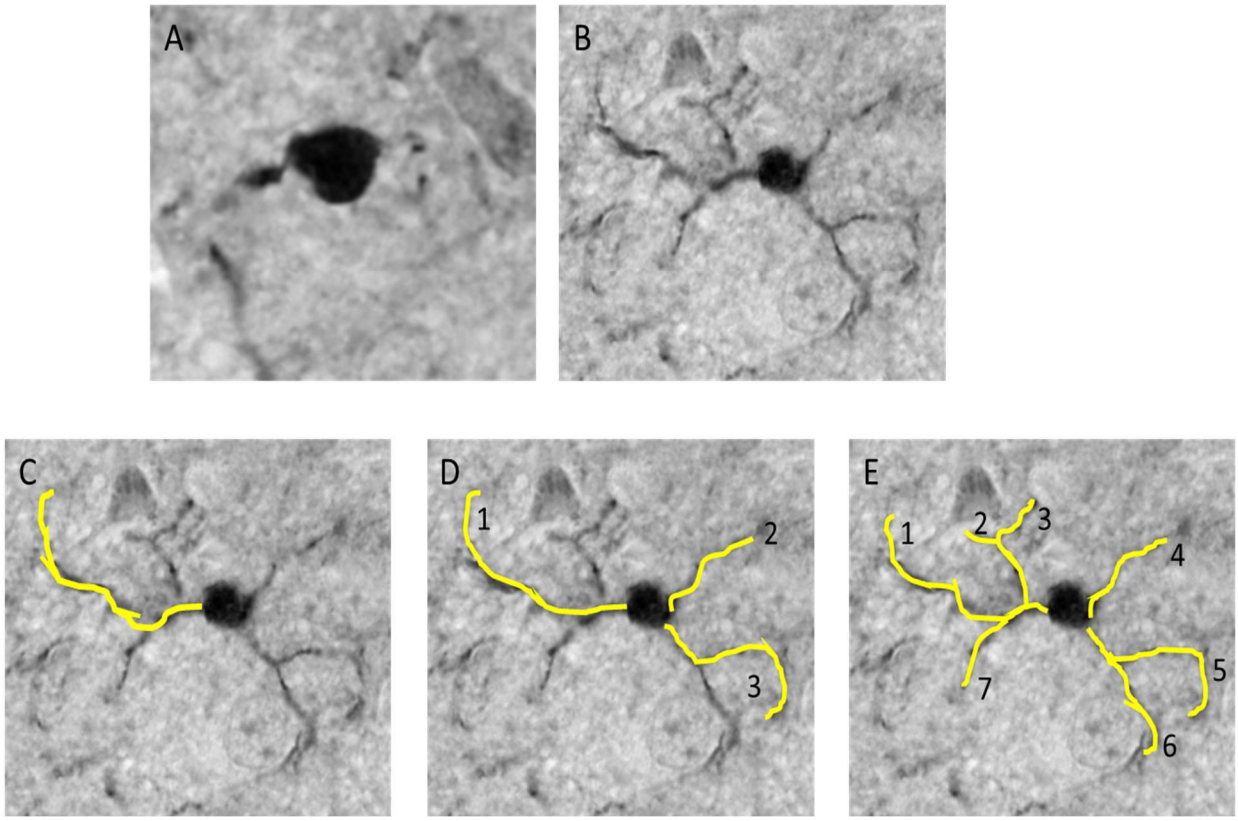


Figure 8: Microglia morphology examples and methods of branch measurements. Active cells were identified based on a large cell body with a rounded or amoeboid form (A). Ramified microglia are known to have a small cell body and large extending branches(B). To examine differences in branching, the length of the largest process was measured from the cell soma, thus only one branch per cell was measured for the given measurement (C). As well primary branches, noted as branches extending directly from the cell soma (D), while total branches included all branches regardless if they were primary or not (E).

4.6 Statistical Analysis

For Immunohistochemistry (IHC) studies a two-way analysis of variance (ANOVA) was completed, with our factors being sex and treatment, followed by post-hoc analysis of a Bonferroni test. We ensured all assumptions for our data were met in order to run the two-way ANOVA. Post-hoc analysis was used to determine differences between experimental groups to the wildtype, as well as sex-based differences. All numerical data is presented as mean ± standard error of the mean (SEM), which represents quantification from at least three separate individuals, with significance for all experiments was determined at $p<0.05$. Within our results, we first present the F-value for the interaction between sex and treatment, along with its *p*-value. This is followed by the comparison of WT to COX animals, showing their means for the given measurement and the *p*-value indicating if there is a statistically relevant difference between the two, to give deeper insight to the effect of the COX-2 disruption. Finally, in order to investigate the effect of sex, we show the means of the WT male, COX-2 male, WT female, and the COX-2 female for the given measurement, followed by any significant differences found through post-hoc comparisons.

5 Results

5.1 Embryonic E19 studies

As previously mentioned E19 is the last possible embryonic day that can be collected before birth. This stage allows us to examine the brain during a period of intense growth and development. To our knowledge, this is the first study within the mouse brain at E19 which studies microglial density and morphology with a focus on sex. Results from the WT and COX-2$^-$KI mice are discussed below.

5.1.1 Altered Microglia Density in Prenatal day 19 Mouse

Microglial precursors begin to invade the mouse brain at approximately E9.5 where they will mature into adult microglia within the first four weeks of the mouse life cycle (Matovitch-Natan et al, 2016). During this time frame the small population of precursors that emerged from the embryonic yolk sac will begin to rapidly undergo division in order to build the self-sustaining microglial population seen within the adult brain. In order to determine differences in microglial density based on the disruption of COX-2 activity within the E19 mouse brain we quantified the total number of microglia within the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus (Figure 9). A two-way ANOVA was used to analyze microglial density (cells/mm^2) for each mentioned region. To examine for sex differences males and females were examined separately.

The cerebellum (Figure 9, $F(3,8)=1.619$, $p=0.204$) had no difference in the combined male and female cerebellar microglial density of WT and COX-2$^-$ KI mice ($p=0.567$, WT=20.50±1.9, COX-2$^-$ KI=22.22±2.4). When sexes were examined separately, we found that WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females had respective microglial densities of 16.71±1.9, 24.56±4.6, 24.29±2.9, 19.87±1.2. Post hoc comparisons found no significance ($p>0.05$).

Within the hippocampus (Figure 9, F(3,8)=2.761, p=0.058) no differences in microglial density between WT and COX-2$^-$ KI mice were found (p=0.325, WT=22.25±2.3, COX-2$^-$ KI=25.78±2.9). The WT male, COX-2$^-$ KI male, WT female, and COX-2$^-$ KI female microglial densities of the hippocampus was respectively 17.77±1.4, 20.74±3.6, 26.73±3.8 and 30.83±4.5. Post hoc comparisons found no significance (p>0.05).

The olfactory bulb (Figure 9, F(3,8)=5.808, p=0.003) had significant differences in microglial density between COX-2$^-$ KI and WT mice (p<0.001, WT=28.96±1.9, COX-2$^-$ KI=43.67±3.3). The olfactory bulb of WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females had respective microglial densities of 31.55±2.1, 40.56±5.4, 26.36±3.1, 46.79±3.9. Post hoc comparisons revealed higher microglial densities in COX-2$^-$KI females compared to WT mice, while no differences were seen in males (p=0.01; p>0.05). No sex differences were seen within the olfactory bulb.

The prefrontal cortex (Figure 9, F(3,8)=0.873, p=0.465) had no statistical differences in microglial density in COX-2$^-$ KI mice compared to WT mice (p=0.492, WT=20.44±1.85, COX-2$^-$ KI=22.59±2.5). WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females had respective microglial densities of 22.44±3.1, 20.10±4.0, 18.43±1.9, and 25.07±2.9. Post hoc comparisons found no differences (p>0.05).

For the thalamus (Figure 9, F(3,8)=2.436, p=0.083), there was no significant differences in microglial density between WT and COX-2$^-$ KI mice (p=0.113, WT=25.05±2.0, COX-2$^-$ KI=21.05±1.6). No significant differences were found between the thalamus of WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females with respective microglial densities of 22.22±2.9, 17.13±1.3, 27.88±1.3, and 24.98±2.4 (p>0.05).

Overall, we found at E19 that within the olfactory bulb that the COX-2$^-$ KI animals were found to have greater microglial density compared to the WT mice, when sex was combined. The cerebellum, hippocampus, prefrontal cortex and thalamus showed no significant changes in density based on the COX-2 mutation. When sexes were examined independently, we found that interestingly found that no regions other than the olfactory bulb had significantly different findings, which were only within females. We saw that COX-2$^-$ KI females had a higher density than WT females, while strikingly no differences were found within the males (p=0.01; p>0.05).

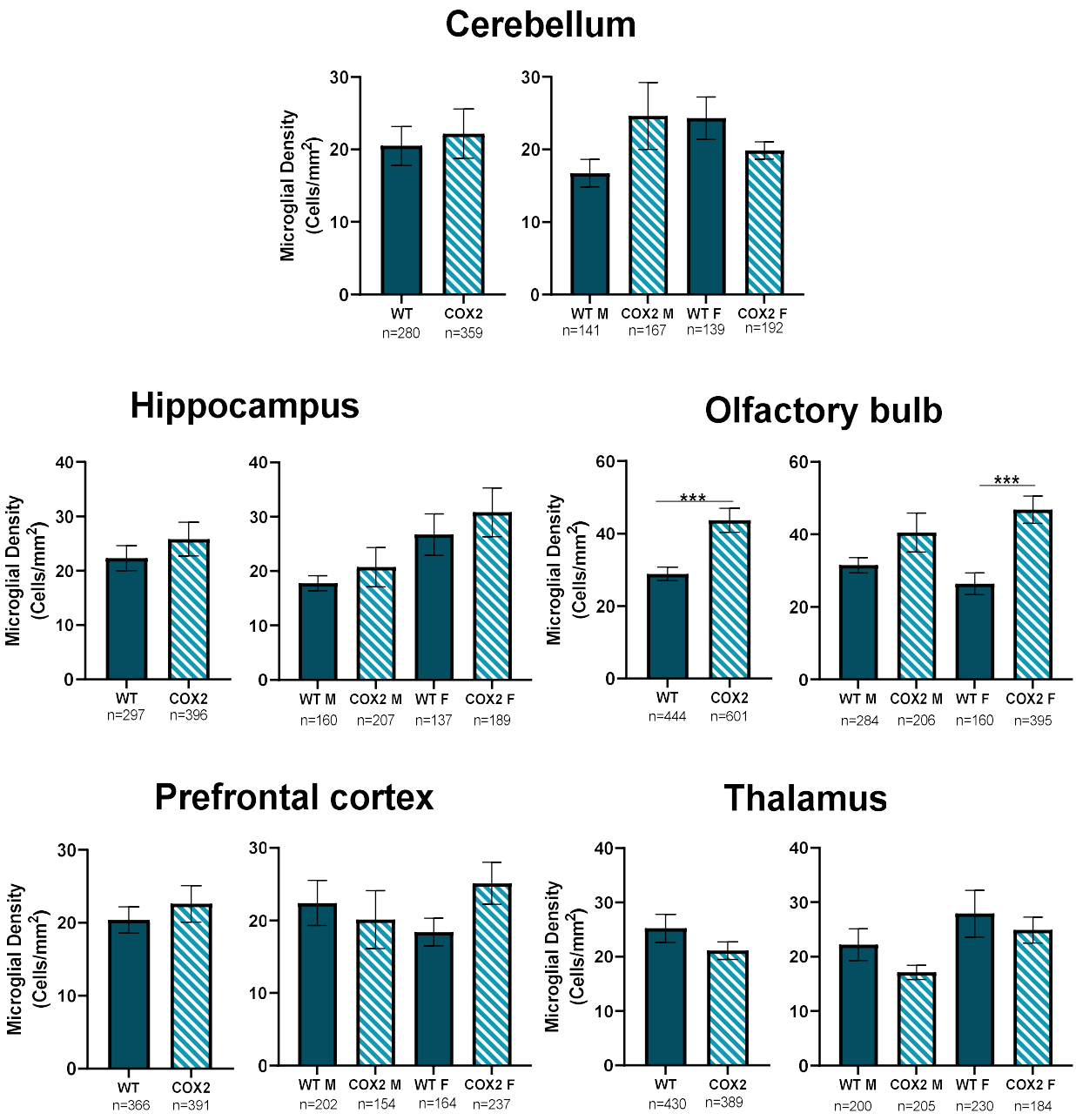


Figure 9. Microglial density changes in embryonic brain. Microglial densities were analyzed within the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus. Each bar represents the average density for at least three animals per experimental condition. Data is presented as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.

5.1.2 Altered Active and Ramified Morphology in Prenatal Day 19 Mouse

Microglia were categorized as either an active or ramified cell, based on their morphological phenotypes. As previously mentioned, active microglia are characterized by having a large round cell body, with little to no branches extending from the soma. Ramified cells on the other hand represent themselves by a small soma and multiple thin branches. Microglia were examined in the cerebellum, hippocampus, olfactory bulb, prefrontal cortex and thalamus of E19 brain samples of COX-2$^-$ KI and WT mice.

Active Morphology Percentages

During embryonic time points microglia are typically seen in an amoeboid morphology, indicating an active state (Ling and Wong, 1993). Recently a growing interest has emerged concerning microglia within the prenatal and developing brain, with many studies suggesting that they engage in phagocytosis of degenerating cells or pathogens and secrete a variety of soluble factors important for healthy development. To determine differences in the percentage of active microglia at E19 counted in our regions of interest, percentages of amoeboid microglia to total microglia were calculated, and a two-way ANOVA was performed.

For the cerebellum (Figure 10, $F(3,8)=2.211$, $p=0.106$) no significance was found in amoeboid microglia morphology percentages when male and female data were combined in WT and COX-2$^-$ KI mice ($p=0.257$, WT=62.39±2.04, COX-2$^-$ KI=66.11±2.4). Within the cerebellum WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females had respective active amoeboid morphology percentages of 62.90±.3.4, 60.88 ±4.1, 61.88 ±2.5, 71.33±2.6. COX-2$^-$ KI females had increased percentages of amoeboid microglia compared to WT females ($p=0.046$), while no differences were seen in males ($p>0.05$). Interestingly when examining for sex

differences only the COX-2$^-$ KI animals showed significant differences with females having greater numbers of amoeboid microglia compared to males (p=0.029).

In the hippocampus (Figure 10, F(3,8)=0.503, p=0.683) amoeboid microglia percentages were not significantly different between WT and COX-2$^-$ KI mice (p=0.627, WT=64.22±2.3, COX-2- KI=66.06±2.9). No statistical differences were observed in the hippocampus of WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females with respective amoeboid microglia percentages of 65.11±4.1, 63.22±3.7, 63.33±2.4, 68.89±4.3 (p>0.05).

For the olfactory bulb (Figure 10, F(3,8)=0.641, p=0.594) no differences in the active amoeboid microglia percentages were seen between COX-2$^-$ KI mice compared to the WT (p=0.877, WT=71.82±2.3, COX-2$^-$ KI=72.36±2.6). Within the olfactory bulb WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females had respective amoeboid microglia percentages of 74.85±3.2, 73.93±3.4, 68.77±3.5, 73.93±3.39. Post hoc comparisons showed no significance (p>0.05).

The prefrontal cortex (Figure 10, F(3,8)=1.792, p=0.168) showed no differences in the quantities of active microglia percentages between WT and COX-2$^-$ KI mice (p=0.752, WT=73.06±2.3, COX-2$^-$ KI=66.67±2.2). For the prefrontal cortex of the WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females had respective amoeboid microglia percentages of 71.56±3.3, 69.00±4.2, 74.56±3.0, 64.34±3.3. While no differences were found between male mice, it was seen that COX-2$^-$KI females had decreased active percentages compared to WT females (p=0.032). No significance was determined through post hoc comparisons between sexes (p>0.05).

For the thalamus (Figure 10, F(3,8)=1.318, p=0.286) no differences were seen in active microglial counts between COX-2$^-$ KI and WT mice (p=0.752, WT=65.44±2.1, COX-2$^-$

KI=64.33±2.8). Within the thalamus WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females had respective amoeboid microglia percentages of 64.66±3.2, 59.55±4.2, 66.22±3.1, 69.11±3.3. Post hoc comparisons found no differences in the thalamus (p>0.05)

Overall, we saw at E19 that there were no differences between COX-2$^-$ KI and WT mice when sexes were combined in terms of active morphology percentages within any of our regions of interest. When sexes were examined independently, we found that within the cerebellum that COX-2$^-$ KI females had greater counts of active microglia compared to WT females. Interestingly within the prefrontal cortex we observed that COX-2$^-$ KI females had a decreased active microglia percentage compared to the WT female. When examining for sex differences only the cerebellum showed significant findings. Interestingly COX-2$^-$ KI females were increased compared to males, while no difference was seen within the wildtypes.

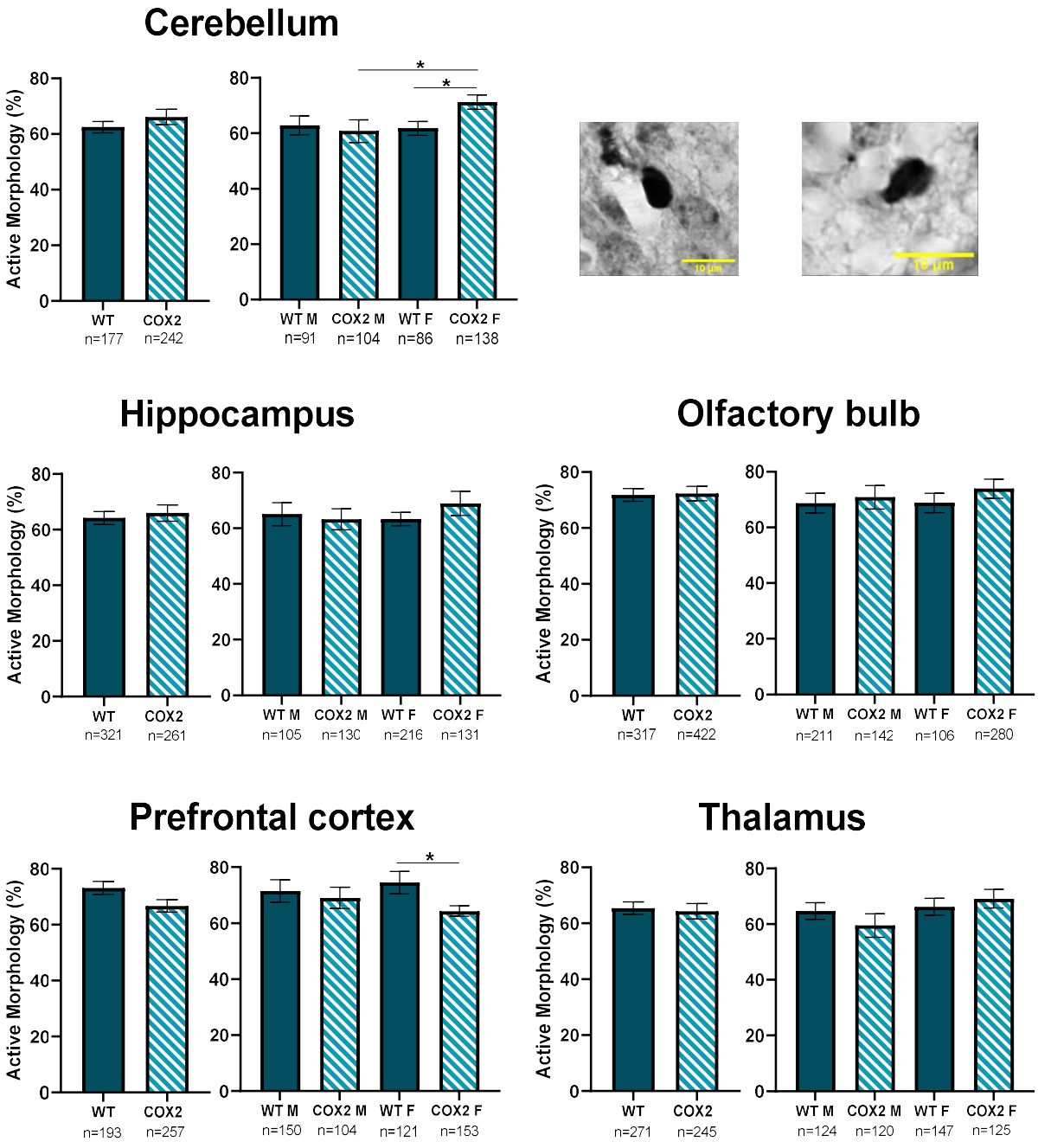


Figure 10. **Active morphology percentage changes in the embryonic brain.** Active microglia percentage were measured within the cerebellum, hippocampus, olfactory bulb, prefrontal cortex and thalamus. Each bar represents the active percentage for at least three animals per experimental condition. In the example of amoeboid microglia scale bars represent 10 µm. Data is presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

Ramified Morphology percentages

Microglia are found to mature as development of the brain progresses and will change their morphology from the rounded large soma to a smaller cell body with long thin processes categorized as ramified cells; as such ramified cells are typically more predominately seen within older stages of the developing healthy brain (Nayak et al, 2014). Ramified microglia are known to influence synaptic activity both through direct contact as well as indirectly through secretions of growth factors such as IGF-1 and TGF-B (Tremblay et al, 2010; Parkhurst et al, 2013; Schafer et al, 2012; Sipe et al, 2016). To determine differences in the percentage of ramified microglia counted in our regions of interest, percentages of ramified microglia to total microglia were calculated, and a two-way ANOVA was performed.

For the cerebellum (Figure 11, $F(3,8)=2.211$, $p=0.106$) no differences in the amount of ramified microglia percentages were found between WT and COX-2$^-$ KI mice when male and female were examined together ($p=0.257$ WT=37.60 ±2.0, COX-2$^-$ KI=33.88±2.7). Within the cerebellum WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females had respective numbers of ramified microglia of 37.1±3.4, 39.1±4.1, 38.1±2.5, 28.6±2.6. We found that there were only differences based on the COX-2 disruption within females, with the COX-2$^-$KI mice having decreased ramified percentages than their sex-matched WT ($p=0.046$). We found that the COX-2$^-$KI males had increased active microglial percentages compared to females ($p=0.029$). Interestingly no sex differences were found between WT mice ($p>0.05$).

For the hippocampus (Figure 11, $F(3,8)=0.503$, $p=0.683$) no difference in the amount of ramified microglia between WT and COX-2$^-$ KI mice was determined ($p=0.387$, WT=35.77±2.3, COX-2$^-$ KI=33.94±2.8). As well, no differences were seen in the hippocampus of WT males,

COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females with respective percentages of ramified microglia of 34.88±4.1, 36.77±3.7, 36.66±2.4, 31.1±4.3 (p>0.05).

The olfactory bulb (Figure 11, F(3,8)=0.641, p=0.594) percentages of ramified microglia were not significantly different between COX-2$^-$KI and WT mice (p=0.877, WT=28.18±2.3, COX-2$^-$KI=27.3±2.6). Within the olfactory bulb WT males, COX-2$^-$KI males, WT females, and COX-2$^-$KI females had respective numbers of ramified microglia of 25.14±2.8, 29.20±4.2, 31.23±3.5, 26.08±3.4. Post hoc comparisons found no differences in the olfactory bulb (p>0.05*)*.

For the prefrontal cortex (Figure 11, F(3,8)=1.792, p=0.1.68) no differences were found in the percentages of ramified microglial in the COX-2$^-$KI mice compared to WT mice (p=0.057, WT=26.94±3.3, COX-2$^-$KI=33.33±3.1). Within the prefrontal cortex WT males, COX-2$^-$KI males, WT females, and COX-2$^-$KI females had respective numbers of ramified microglia of 28.44±4.0, 31.0±3.9, 25.44±2.6, 35.66±1.9. We found that COX-2$^-$KI females had increased numbers of ramified microglia compared to WT females (p=0.032), while no differences were observed in males. No sex differences were seen within the prefrontal cortex (p>0.05).

For the thalamus (Figure 11, F(3,8)=1.318, p=0.286) the percentages of ramified microglia were larger in COX-2$^-$KI compared to WT mice (p=0.752, WT=34.55±2.2, COX-2$^-$ KI=35.66±2.8). Within the thalamus WT males, COX-2$^-$KI males, WT females, and COX-2$^-$ KI females had respective percentages of ramified microglia of 35.33±3.3, 40.4±4.2, 33.77±3.0, 30.88±3.3. Post hoc revealed no significant differences (p>0.05).

When sexes were combined and differences were examined for between ramified morphology percentages between COX-2$^-$KI and WT mice no significance was found within any of our regions of interest at E19. When sexes were examined independently COX-2$^-$KI females had decreased counts of ramified microglia compared to WT females within the cerebellum.

Interestingly, the prefrontal cortex showed that COX-2^{-}KI females had increased ramified microglia percentage compared to the WT females. Sex differences were only found within the cerebellum where COX-2^{-}KI females were decreased in ramified counts compared to males.

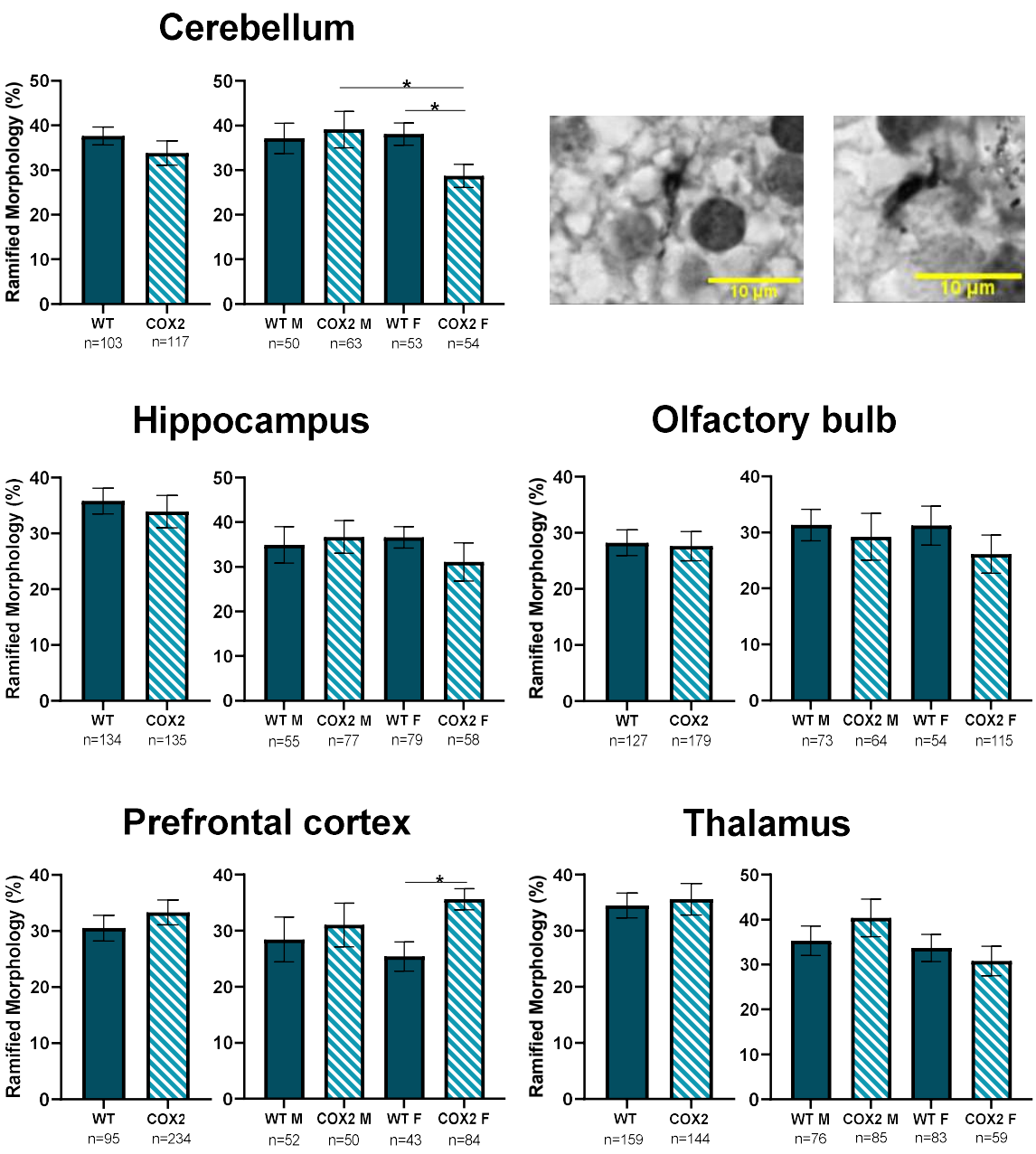


Figure 11. Ramified morphology percentage changes in the embryonic brain. Ramified morphology percentages were measured within the cerebellum, hippocampus, olfactory bulb, prefrontal cortex and thalamus. Each bar represents the ramified percentage for at least three animals per experimental condition. In the example of ramified microglia scale bars represent 10 µm. Data is presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

5.1.3 Altered Microglial Branch Length in Prenatal Day 19 Mouse

Microglia show how highly dependent they on their environment through changes in their morphology, one such indicator of environmental impact is microglial branch length. Differences in microglia branch length are considered to be objective indicators of microglial activation, with shorter branches indicating a state closer to microglial activation than ramified (Morrison and Filosa, 2013; Stence et al, 2001). To determine differences in branch length based on the disruption of COX-2 activity at E19 the longest branch (μm) per microglia was measured within the cerebellum hippocampus, olfactory bulb, prefrontal cortex and thalamus. The values reported represent the average branch length per microglia. A two-way ANOVA was used to analyze average branch length for each mentioned region Males and females were examined separately to examine for sex differences.

For the cerebellum (Figure 12, $F(3,266)=10.236$, $p<0.001$) no statistical differences were seen between WT and COX-2$^-$ KI mice when male and female data were examined together ($p=0.130$, WT=8.69±0.62, COX-2- KI=7.37±0.45). In the cerebellums of WT males, COX-2$^-$KI males, WT females, and COX-2$^-$KI females had respective branch lengths of 9.98±1.06, 9.80±0.85, 7.57±0.69, 5.55±0.4. Post hoc comparisons found COX-2$^-$ KI females had decreased branch lengths compared to WT females ($p=0.039$), while no differences were seen in males ($p>0.05$). Sex differences were only seen in the COX-2$^-$ KI animals with males having larger branch lengths than females ($p<0.001$).

The hippocampus (Figure 12, $F(3,863)=14.29$, $p<0.001$) showed no difference between COX-2$^-$ KI mice compared to WT mice ($p=0.616$, WT=4.06±0.22, COX-2$^-$ KI=3.82±0.2). WT males, COX-2$^-$KI males, WT females, and COX-2$^-$KI females hippocampal analysis revealed respective branch lengths of 4.01±0.3, 4.46±0.3, 4.09±0.3, 3.36±0.2. When sexes were analyzed we found that COX-2$^-$ KI females had decreased branching lengths compared to WT females

(p=0.047), though no differences were seen within males (p>0.05). Interestingly while COX-2$^-$ KI males had greater branching lengths COX-2$^-$ KI females (p=0.003), no differences were seen in WT animals (p>0.05).

For the olfactory bulb (Figure 12, F(3,1030)=0.720, p=0.540) indicated COX-2$^-$ KI no significant differences to the branch lengths of WT mice (p=0.610, WT=4.07±0.2, COX-2$^-$ KI=3.98±0.2). Within the olfactory bulb WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females had respective average branch lengths of 3.81±0.3, 3.83±0.2, 4.35±0.4, 4.05±0.2. Post hoc comparisons found that no differences within the olfactory bulb (p>0.05).

For the prefrontal cortex (Figure 12, F(3,757=3.255, p=0.021) COX-2$^-$ KI mice were seen to have longer average branch lengths compared to WT mice (p=0.03, WT=3.09±0.2, COX-2$^-$ KI=3.87±.0.2). Within the prefrontal cortex WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females had respective branch lengths of 3.17±0.2, 3.75±0.3, 2.99±0.2, 3.95±0.2. Post hoc comparisons showed that COX-2$^-$ KI females had increased branch lengths compared to WT females (p=0.008), while males interestingly showed no differences (p>0.05). No sex differences were observed within the prefrontal cortex (p>0.05).

For the thalamus (Figure 12, F(3,822)=3.815, p<0.010) COX-2$^-$ KI mice were found to have no difference in branch length compared to WT mice (p=0.442, WT=5.05±0.2, COX-2$^-$ KI=5.42±0.3). Within the thalamus WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females had respective branch lengths of 5.41±0.4, 6.17±0.4, 4.73±0.4, 4.55±0.3. Post hoc comparisons found that COX-2$^-$ KI males had greater microglial branch lengths than COX-2$^-$ KI females (p=0.003), while interestingly no sex differences were observed within the WT mice (p>0.05).

Overall, at E19 we found that COX-2$^-$ KI mice had significant increases in average branch lengths in the prefrontal cortex to WT mice. Interestingly within the cerebellum we observed that branch lengths were decreased in the COX-2$^-$ KI animals compared to WT mice. The hippocampus, olfactory bulb and thalamus were not to have any statistically relevant differences despite the disruption of COX-2 activity. When sexes were examined independently between WT and COX-2$^-$ KI mice significant differences were seen in various regions. COX-2$^-$ KI females were seen to have decreased branch lengths compared to WT females within the cerebellum and the hippocampus. Interestingly COX-2$^-$ KI females were seen to be increased in branch length compared to WT females within the prefrontal cortex. Sex based differences were seen only in COX-2$^-$ KI animals where males had greater branch lengths compared to females within the cerebellum, hippocampus, and thalamus.

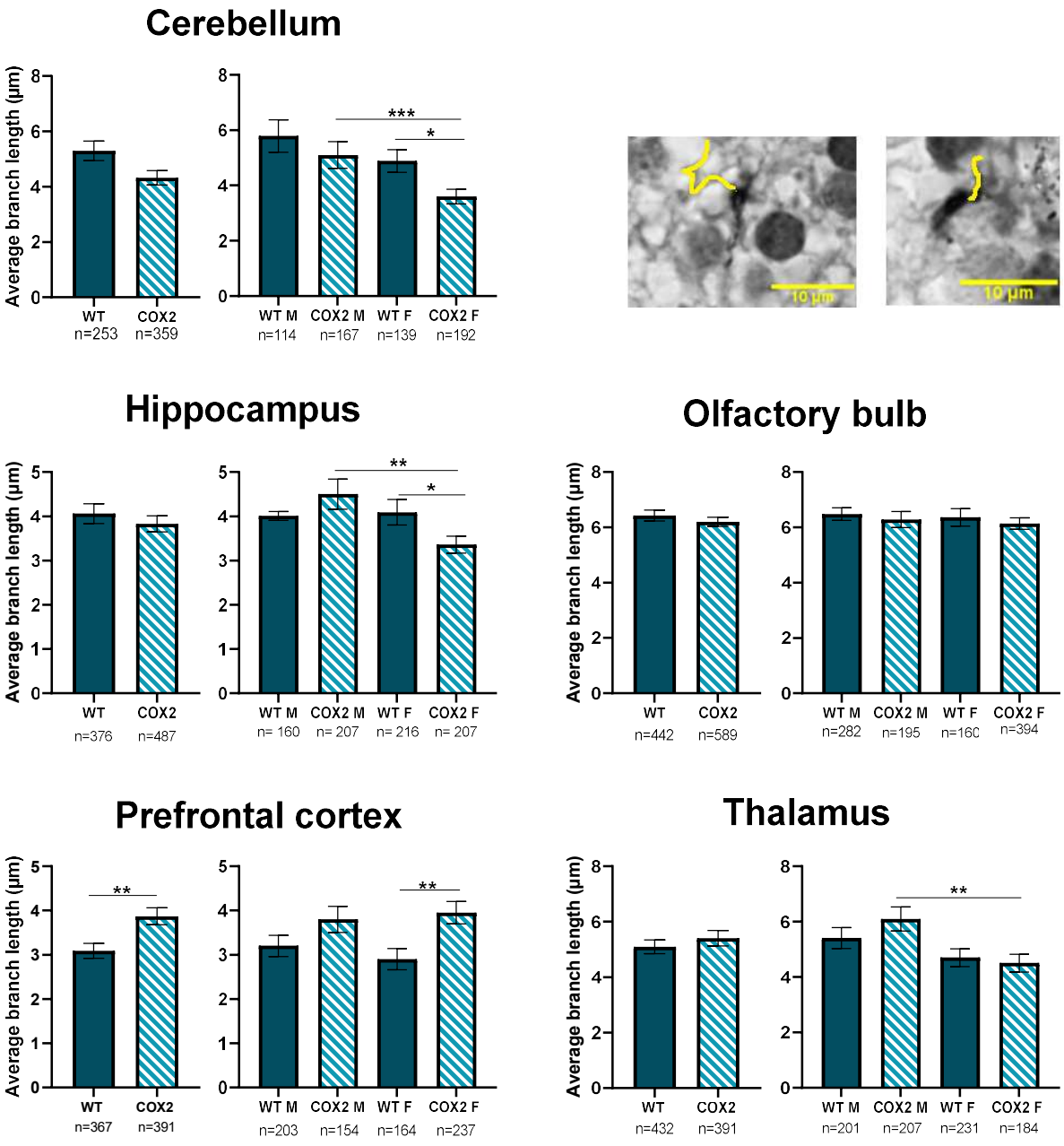


Figure 12. Branch length changes in embryonic brain. The average branch length per cell were analyzed within the cerebellum, hippocampus, olfactory bulb, prefrontal cortex and thalamus. Each bar represents the average branch length for at least three animals per experimental condition. The yellow lines on the representative images of ramified microglia branch length quantification signifies the longest branch measured; scale bar represents 10µm. Data is presented as mean ±SEM, **p*<0.05, ***p*<0.01, ****p*<0.001.

5.1.4 Altered Microglial Primary Branching in Prenatal Day 19 Mouse

Microglial branches have been shown through two-photon microscopy to be highly active, constantly scanning the brain for insult or injury (Nimmerjahn et al, 2005). As time passes and microglia continue to mature an increasingly complex branching network has been demonstrated through a variety studies completed in several brain regions (Schwarz et al, 2012; Wu et al ,1992). To determine differences at E19 based on the disruption of COX-2 activity we quantified primary microglial branches within the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus. Primary branches were identified as branches extending directly from the microglial soma; all values reported are the average number of primary branches viewed per microglia. A two-way ANOVA was used to analyze primary branch density for each mentioned region. To examine for sex differences males and females were examined separately.

For the cerebellum (Figure 13, $F(3,611)=2.647$, $p=0.048$) COX-2$^-$ KI mice had a decreased number of primary branches density compared to WT mice when male and female data was combined ($p=0.017$, WT=1.08±0.07, COX-2$^-$ KI=0.87±0.05). Within the cerebellum of WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females we found primary branches counts to respectively be 1.13±0.1, 0.95±0.05, 1.04±0.09, 0.81±0.07. When sexes were examined separately, we saw that COX-2$^-$ KI females had a decreased number of primary branches compared to the WT females ($p=0.045$), while no differences were seen in males ($p>0.05$). No sex differences were seen within the cerebellum ($p>0.05$).

In the hippocampus (Figure 13, $F(3,862)=2.216$, $p=0.085$) COX-2$^-$ KI mice had no significant difference in primary branches when compared to WT mice ($p=0.079$, WT=1.01±0.05, COX-2$^-$ KI=0.88±0.04). Within the hippocampus WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females had respective average primary branches per cell of

0.99±0.07, 0.96±0.07, 1.03±0.07, 0.82±0.05. Post hoc comparisons determined COX-2$^-$ KI females to have decreased primary branching than their WT counterparts (p=0.017). No differences were noted within the males, nor were any sex differences within this region (p<0.05; p<0.05).

For the olfactory bulb (Figure 13, F(3,1030)=0.622, p=0.601) no difference was seen between the COX-2$^-$ KI and WT mice primary branch counts (p=0.867, WT=0.78±0.04, COX-2$^-$ KI=0.81±0.04). The olfactory bulbs of WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females had respective average primary branches per cell of 0.74±0.05, 0.78±0.06, 0.84±0.07, 0.82±0.04. When sexes were examined separately no differences were seen (p>0.05).

In the prefrontal cortex (Figure 13, F(3,757)=1.768, p=0.152) COX-2$^-$ KI mice had greater quantities of primary branches than WT mice (p=0.047, WT=0.82±0.07, COX-2$^-$ KI=0.97±0.05). Within the prefrontal cortex of WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females respective primary branches values of 0.78±0.07, 0.97±0.07, 0.88±0.08, 0.97±0.06 were seen. Through post-hoc comparisons we found no statistically relevant differences (p>0.05).

For the thalamus (Figure 13, F(3,822)=0.241, p=0.243) no differences were seen in the amount of primary branches between COX-2$^-$ KI and WT mice (p=0.834, WT=1.03±0.05, COX-2$^-$ KI=1.05±0.05). Within the thalamus WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females had respective primary branches per cell of 1.07±0.07, 1.04±0.07, 1.00±0.06, 1.07±0.07. Post-hoc differences determined no differences within the thalamus (p>0.05).

When examining for differences of primary microglial branches between WT and COX-2$^-$ KI animals significant differences were seen within the cerebellum and the prefrontal cortex. Interestingly while the cerebellum showed COX-2$^-$ KI mice to have a decreased number of

primary branches compared to the WT, the prefrontal cortex showed the opposite. The hippocampus, olfactory bulb, and thalamus showed no significant differences in total branching despite the COX-2 disruption. Sexes were examined independently we saw that COX-2$^-$ KI females more primary branches than WTs in the cerebellum and hippocampus. No sex differences were noted in any of the regions of interest.

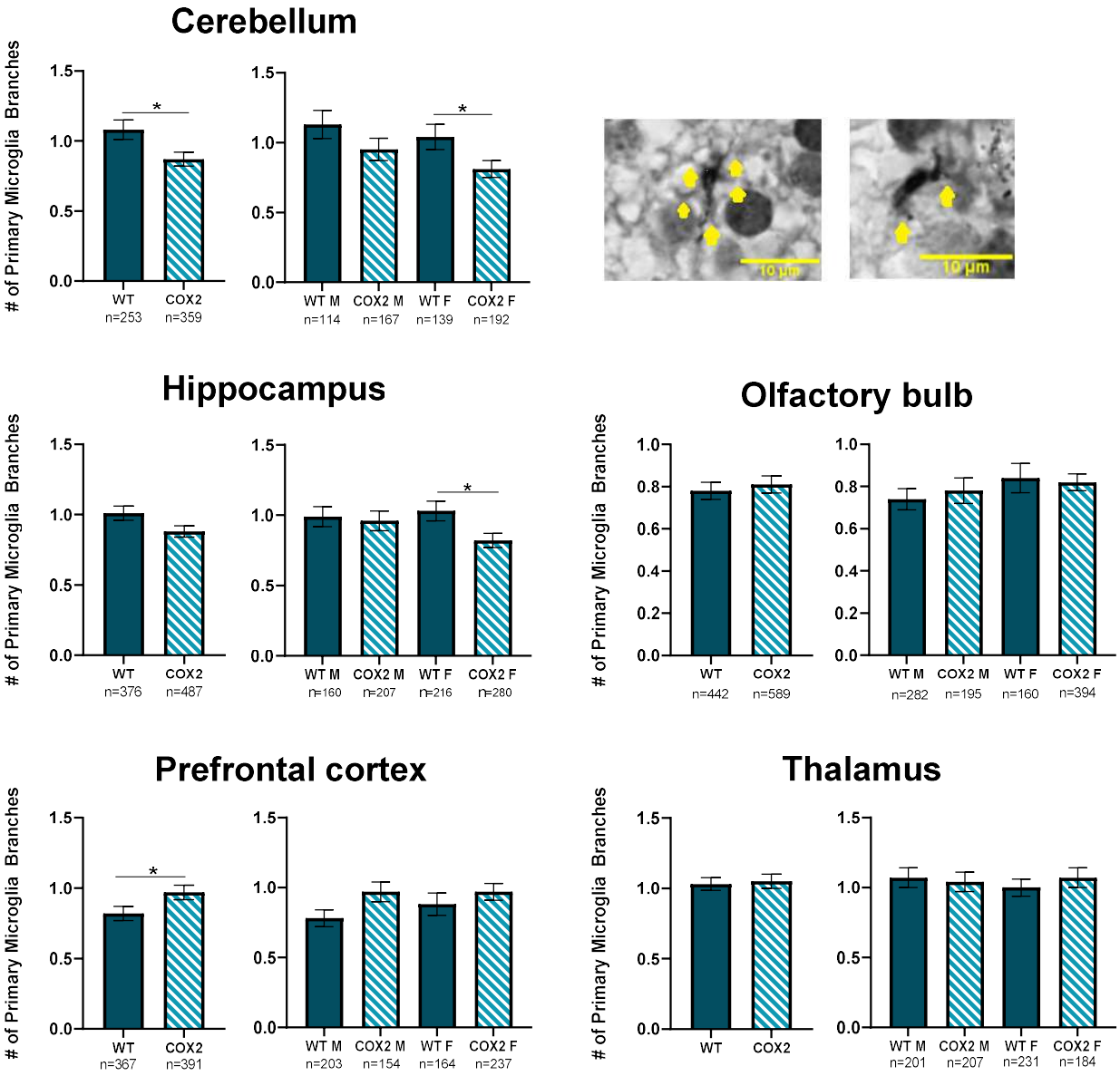


Figure 13. Primary branch count changes in embryonic brain. The average number of primary branches per cell were analyzed within the cerebellum, hippocampus, olfactory bulb, prefrontal cortex and thalamus. Each bar represents the average primary branch count for at least three animals per experimental condition. Yellow arrows represent primary branches counted in ramified microglia; scale bats represent 10 µm. Data is presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

5.1.5 Altered Microglial Branch Total in Prenatal Day 19 Mouse

Ramified cells were previously thought to be simply lying in wait for a pathogen or insult to arise, at which point the cell may transform into an active morphology, permitting it to elicit an effect on overall brain health. This is now known ramified cells actually play a very active role in overall brain health, with a variety of studies showing that branches make direct interactions with synaptic spines and axon terminals (Tremblay et al, 2010). These direct contacts were seen to have large effects on dendritic spines, with only 6% of spines contacted remaining stable in size of mice aged to P28-P39 (Tremblay et al, 2010). To determine differences in total branch quantities per microglia at E19 based on the disruption of COX-2 activity we quantified total microglial branches per ramified microglia within the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus. We examined the branches of only ramified microglia to gain further insight on the amount of possible connections microglia may be making to neurons and thus potentially regulating activity in its ramified state. The values reported are average values for total branches, including secondary and tertiary branches, per cell. Males and females were examined separately to examine for sex differences.

For the cerebellum total branches (Figure 14, $F(3, 214)=2.969$, $p=0.033$) showed COX-2$^-$ KI mice had a decreased amount of total microglia branches compared to WT mice when examining male and female data concurrently ($p=0.040$, WT=2.38±0.09, COX-2- KI=2.14±0.09). When examining sexes independently the cerebellum of WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females had respective total branches per microglia of 2.53±0.13, 2.29±0.1, 2.25±0.14, 1.96±0.14. Post hoc comparisons found no significant differences when sexes were examined independently ($p>0.05$).

For the hippocampus (Figure 14, $F(3, 289)=11.915$, $p<0.001$) no statistical difference was found between WT and COX-2$^-$ KI mice ($p=0.086$, WT=2.22±0.08, COX-2- KI=1.96±0.08).

Within the hippocampus WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females had respective total branches per microglia of 2.05±0.1, 2.38±0.1, 2.34±0.1, 1.66 ±0.07. Post hoc comparisons found in females that COX-2$^-$ KI mice have a decreased total branching count than WT mice (p<0.001), while interestingly COX-2$^-$ KI males were seen to have increased numbers of total branches compared to WT males (p=0.044). When examining for sex differences we saw within the COX-2$^-$ KI mice that males had more total branches compared to females (p<0.001), while no differences were seen within WT mice (p>0.05).

In the olfactory bulb (Figure 14, F(3, 376)=3.171, p=0.024) COX-2$^-$ KI animals were not seen to have greater total branching than in WT mice (p=0.698, WT=1.92±0.08, COX-2$^-$ KI=1.94±0.06). Within the olfactory bulb WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females had respective total branches per microglia of 2.08±0.1, 1.83±0.09, 1.76±0.1, 1.83±0.07. Through post hoc comparisons we found no differences between the WT and COX-2$^-$ KI mice (p>0.05). When examining for sex differences we found that in males for both COX-2$^-$ KI and WT mice males had greater numbers of total branching compared to females (p=0.028, p=0.032).

The prefrontal cortex total branches (Figure 14, F(3, 226)=0.085, p=0.968) had no statistical difference between COX-2$^-$ KI and WT mice (p=0.924, WT=2.00±0.09, COX-2$^-$ KI=1.99±0.07). Within the prefrontal cortex WT males, COX-2$^-$ KI males, WT females and COX-2$^-$ KI females had respective total branches per microglia of 1.96±0.1, 2.00±0.1, 2.05±0.1, 1.99±0.08. Post hoc comparisons determined that there were no differences when sexes were examined separately within the prefrontal cortex (p>0.05).

The thalamus (Figure 14, F(3, 302)=0.103, p=0.958) also showed no statistically different values in total branches between COX-2$^-$ KI and WT mice (p=0.827, WT=2.08±0.07, COX-2$^-$

KI=2.10±0.08). Within the thalamus WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females had respective total branches per microglia of 2.12±0.1, 2.09±0.1, 2.05±0.1, 2.12±0.1. No differences were seen within the thalamus (p>0.05).

Overall, we found that COX-2$^-$ KI mice had statically decreased total branching compared to WT mice in the cerebellum. Within the hippocampus, olfactory bulb, prefrontal cortex, and thalamus the disruption of COX-2 activity was not found to have a statistically relevant effect on total branching values. When sexes were examined independently between WT and COX-2$^-$ KI significant differences were seen in various regions of interest. COX-2$^-$ KI females were seen to have decreased numbers of total branches compared to WTs in the hippocampus. Within WT mice sex-based differences were seen in the hippocampus and the olfactory bulb, where COX-2$^-$KI males had greater branches than females. Interestingly only the olfactory bulb was found to be the only region which maintained the difference of males having greater total branch numbers than females within WT animals.

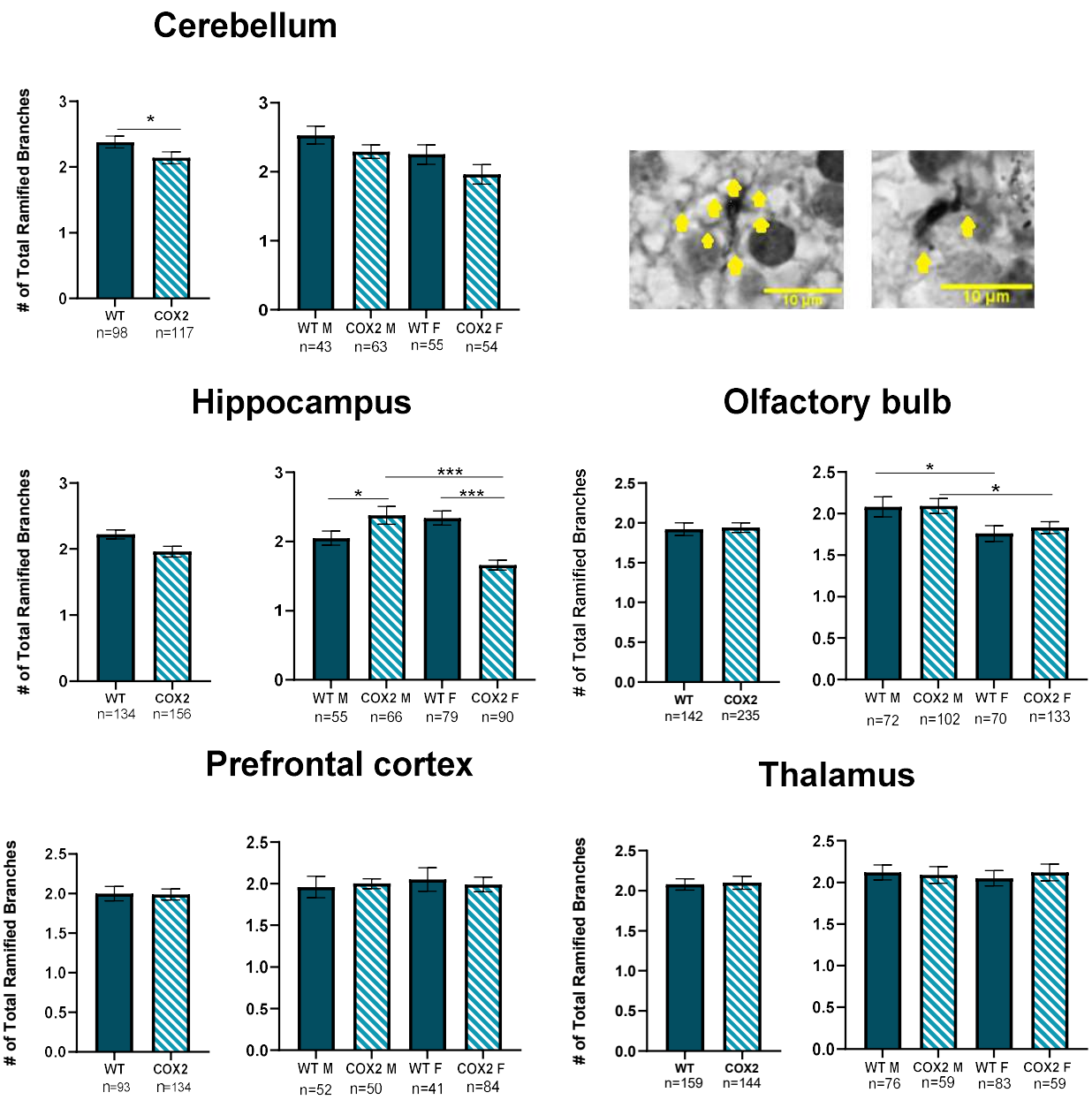


Figure 14. Total branch count changes in embryonic brain. The average number of total branches per cell were analyzed within the E19 cerebellum, hippocampus, olfactory bulb, prefrontal cortex and thalamus. Each bar represents the total branches for at least three animals per experimental condition. Data is presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

5.2 Postnatal Day P25

By postnatal day 25 the mouse brain has fully formed regions of the brain, including our five regions of interest, as previously noted. To our knowledge this is the first study which examines the mouse brain at this stage for microglial density and morphology. The focus we place on sex-differences and how the COX-2 disruption affects the microglia within the P25 brain makes this study even more novel.

5.2.1 Altered Microglia Density in Postnatal day 25 Mouse

Microglial density has been previously found to be altered in individuals with ASD (Morgan et al, 2010; Vargas et al, 2005; Tetreault et al, 2012). Differences in microglial density have been shown to affect several key processes in the brain including neurogenesis, synaptic pruning, and the regulation of neuronal progenitor cells (Bilimoria and Stevens 2015; Frost and Schafer, 2016). To determine differences in microglial density based on the disruption of COX-2 activity within the P25 mouse brain we quantified the total number of microglia within the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus. A two-way ANOVA was used to analyze microglial density (cells/mm^2) for each mentioned region. To examine for sex differences males and females were examined separately.

Findings from the cerebellum (Figure 15, $F(3,8)=2.889$, $p=0.51$) indicated no difference in the combined male and female cerebellar microglial density of WT and COX-2$^-$ KI mice ($p=0.350$, WT=178.70±6.56, COX-2$^-$ KI=187.36±7.37). When sexes were examined separately, we found that WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females had respective microglial densities of 174.11±8.02, 204.75±9.61, 183.30±10.66, 169.96±7.92. Post hoc comparisons found COX-2$^-$ KI males had greater density than WT males ($p=0.024$) as well as COX-2$^-$ KI females ($p=0.011$).

Within the hippocampus (Figure 15, $F(3,8)=5.874$, $p=0.003$) no differences in microglial density between WT and COX-2$^-$ KI mice were found ($p=0.123$, WT=111.88±6.26, COX-2$^-$ KI=122.74±5.07). WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females had microglial densities in the hippocampus respectively of 130.66±9.18, 121.43±8.56, 93.10±4.73 and 124.04±5.96. Post hoc comparisons found that COX-2$^-$KI females had an increased microglial density compared to WT females ($p=0.003$), while interestingly no differences were seen for COX-2$^-$ KI males ($p>0.05$). As well WT males had higher microglial density compared to WT females ($p<0.001$), but no sex-differences were seen in COX-2$^-$ KI animals ($p>0.05$).

The olfactory bulb (Figure 15, $F(3,8)=1.450$, $p=0.247$) showed no differences in microglial density between WT and COX-2$^-$ KI mice ($p=0.665$, WT=119.08±18.72, COX-2$^-$ KI=109.90±6.38). The olfactory bulb of WT males, COX-2$^-$KI males, WT females, and COX-2$^-$ KI females had respective microglial densities of 145.55±33.44, 112.69±9.1, 92.60±14.00, 107.10±9.39. Post hoc comparisons revealed higher microglial densities in WT males compared to WT females ($p=0.05$), while interestingly the sex difference was not found between male and female COX-2$^-$KI animals ($p>0.05$).

In the prefrontal cortex (Figure 15, $F(3,8)=1.742$, $p=0.178$) we found no statistical difference in microglial density in COX-2$^-$KI mice compared to WT mice ($p=0.053$, WT=149.66±4.51, COX-2$^-$ KI=165.17±6.46).Within the prefrontal cortex it was found that WT males, COX-2$^-$KI males, WT females, and COX-2$^-$KI females had respective microglial densities of 143.66±2.86, 167.99±11.59, 154.61±8.42, and 162.34±6.42. Post hoc comparisons found that COX-2$^-$ KI males had greater microglial density to WT males ($p=0.039$). No sex differences were seen within the prefrontal cortex for either mouse model ($p>0.05$).

For the thalamus (Figure 15, $F(3,8)=1.425$, $p=0.254$), there was no significant differences in microglial density between WT and COX-2$^-$KI mice ($p=0.113$, WT=188.05±20.45, COX-2-KI=174.94±25.49). No significant differences were found between the thalamus of WT males, COX-2$^-$KI males, WT females, and COX-2$^-$KI females with respective microglial densities of 155.88±2.86, 146.30±4.43, 165.72±6.16, and 153.37±8.04 ($p>0.05$).

Overall it was found that COX-2$^-$KI mice, with male and female data combined, had no significant effect on microglial density when compared to WT mice. However, when sexes were examined independently, we found region specific differences between the COX-2$^-$ KI and the WT animals. Our results show that COX-2$^-$ KI males to be greater density in the cerebellum and prefrontal cortex compared to the corresponding WT males. The COX-2$^-$ KI females showed increased density only within the olfactory bulb. Within WT mice sex-based differences were seen in the hippocampus where males had greater densities than females, while no significance was found in other regions. Interestingly in COX-2$^-$ KI mice were found to show sex differences in the cerebellum with males having greater densities than females.

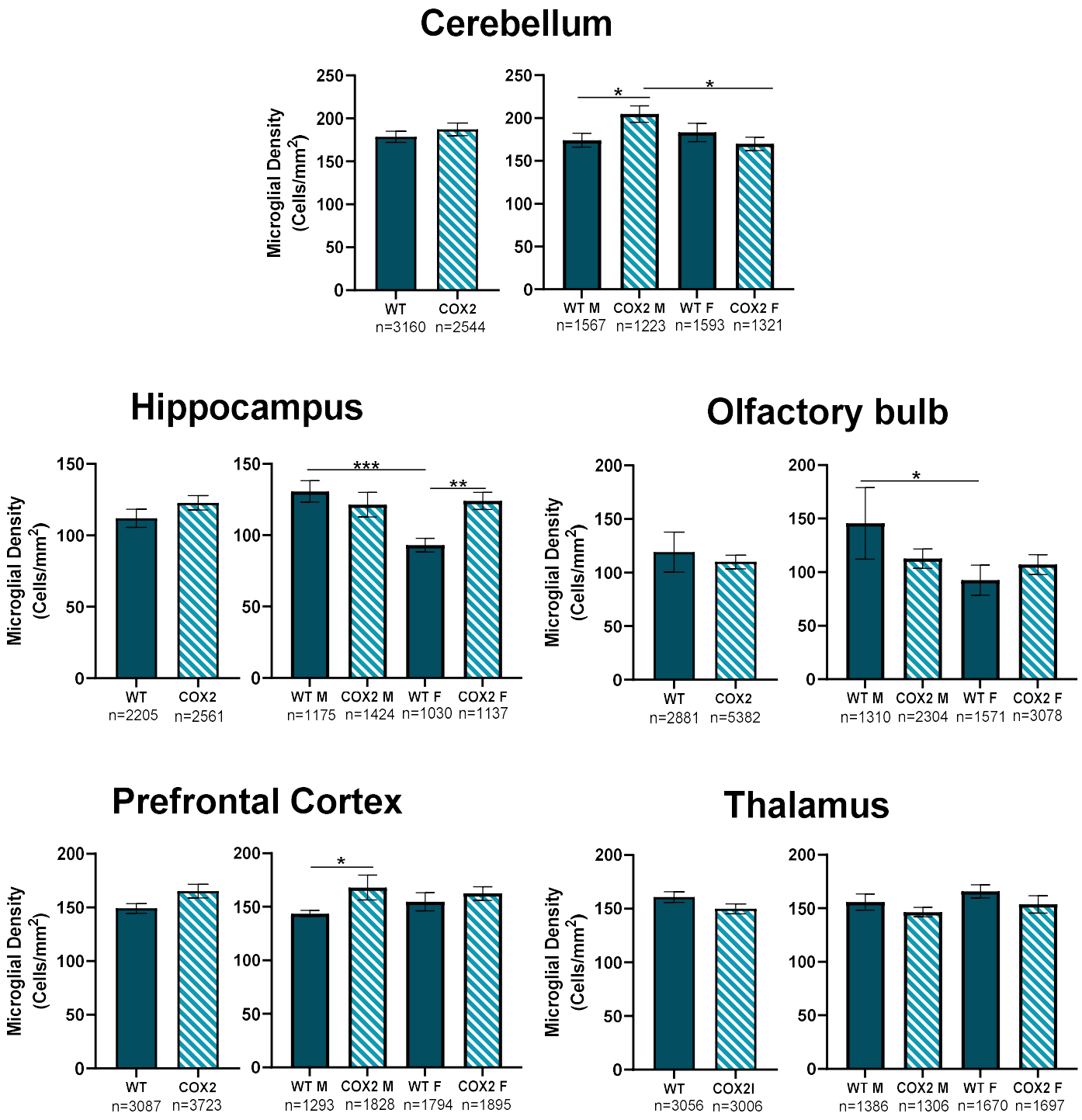


Figure 15: Microglial density changes in postnatal brain. Microglial densities were analyzed within the P25 cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus. Each bar represents the average density for at least three animals per experimental condition. Data is presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

5.2.2. Altered Active and Ramified Morphology in Postnatal Day 25 Mouse

Microglia were categorized as either an active cell, or a ramified cell, based on their morphological phenotypes. Active morphologies are characterized by a large rounded cell soma with little to no branches, while ramified microglia have smaller somas and multiple long and thin branches (Young et al, 2018). In this section, microglia were examined in the cerebellum, hippocampus, olfactory bulb, prefrontal cortex and thalamus in brain samples of COX-2$^-$ KI and WT mice at P25.

Active Morphology Percentages

Active microglia are known to play a variety of roles in the brain that include phagocytosis of dead cells and debris, assisting in tissue repair, and in the regulation of the inflammatory pathway (Harry et Kraft, 2012). To determine differences in the percentage of active microglia counted in our regions of interest, counts of active microglia to total microglia were calculated (see methods), and a two-way ANOVA was performed.

For the cerebellum (Figure 16, $F(3,8)=1.865$, $p=0.155$) we found no significance differences in active microglia morphology percentages when male and female data were combined to look at differences between WT and COX-2$^-$KI mice ($p=0.076$, WT=37.63±1.6, COX-2$^-$KI=32.37±2.4). Within the cerebellum it was determined that WT males, COX-2$^-$KI males, WT females, and COX-2$^-$KI females had respective active morphology percentages of 40.66±.2.1, 32.37 ±3.5, 34.59 ±2.10, 32.37±3.5. COX-2$^-$KI males had decreased percentages of active amoeboid microglia compared to WT males ($p=0.049$). No differences were seen between COX-2$^-$KI females to their respective wild type or any sex differences within this region ($p>0.05$).

In the hippocampus (Figure 16, $F(3,8)=0.846$, $p=0.479$) active microglia percentages were not significantly different between WT and COX-2$^-$ KI mice ($p=0.387$, WT=26.12±1.3, COX-2$^-$ KI=27.11±1.6). No statistical differences were found in the hippocampus of WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females with respective active microglia percentages of 27.58±1.8, 25.52±2.0, 24.67±1.7, 28.71±2.4 ($p>0.05$).

For the olfactory bulb (Figure 16, $F(3,8)=3.692$, $p=0.022$) the active microglia percentages were significantly decreased in COX-2$^-$KI mice compared to the WT when male and female data was combined ($p=0.003$, WT=34.23±2.1, COX-2$^-$KI=26.40±1.2). Within the olfactory bulb WT males, COX-2$^-$KI males, WT females, and COX-2$^-$KI females had respective active microglia percentages of 34.89±1.7, 24.91±1.9, 33.56±4.0, 27.88±1.2. Post hoc comparisons found that COX-2$^-$ KI males had decreased numbers of active microglia cells compared to WT males ($p=0.007$), while there no differences seen within females. No sex differences were found within the olfactory bulb ($p>0.05$).

The prefrontal cortex (Figure 16, $F(3,8)=2.875$, $p=0.051$) showed no differences in the quantities of active microglia percentages between WT and COX-2$^-$KI mice ($p=0.099$, WT=21.72±1.3, COX-2$^-$ KI=18.86±1.2). For the prefrontal cortex of the WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$KI females respective active microglia percentages of 24.46±0.8, 18.06±1.2, 18.98±2.3, 19.67±2.1 were determined. We observed that only COX-2$^-$KI males had a decreased active microglia percentage compared to WT males ($p=0.011$). Sex differences were only seen in WT animals were males had a higher active microglia percentage compared to females ($p=0.028$).

For the thalamus (Fig. 16, $F(3,8)=5.160$, $p=0.005$) the main findings showed that COX-2$^-$ KI mice had a significant decrease in the percentage of active microglia when compared to the WT ($p=0.001$, WT=32.16±1.7, COX-2- KI=24.61±1.1). Within the thalamus it was seen that WT males, COX-2$^-$KI males, WT females, and COX-2$^-$KI females had respective active microglia percentages of 34.01±2.5, 25.11±1.9, 30.30±2.3, 24.12±1.1. Post hoc comparisons found COX-2$^-$ KI males and females, to their respective WT animals, had decreased percentages of active microglia ($p=0.004$; $p=0.039$). No sex differences were reported ($p>0.05$).

Overall it was found that COX-2$^-$KI mice, when male and female data were combined, had decreased active microglia morphology in the olfactory bulb and the thalamus compared to WT mice. Within the cerebellum, hippocampus, and prefrontal cortex the disruption of COX-2 activity was not found to have a statistically relevant effect on active microglia counts. When sexes were examined independently between WT and COX-2$^-$ KI mice significant differences were observed in the cerebellum, olfactory bulb, prefrontal cortex, and thalamus where COX-2$^-$ KI males had decreased active morphology than WT males. As well it was found that WT females had greater percentages than COX-2$^-$KI females in the thalamus. Sex differences were seen the prefrontal cortex of WT mice, where males had greater active morphologies than females. No sex differences were noted in the COX-2$^-$KI mice.

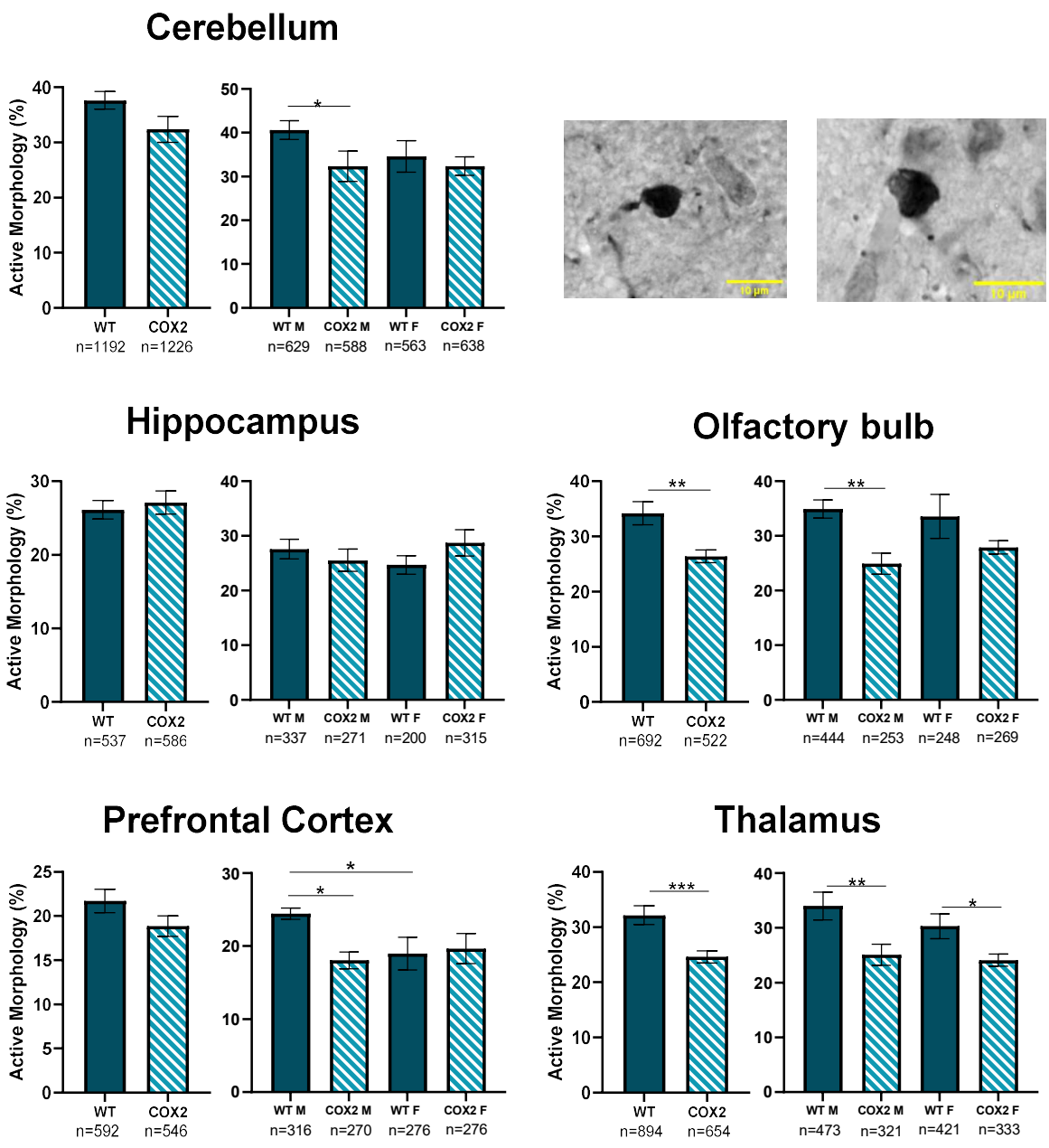


Figure 16. Active morphology percentage changes in postnatal brain. Active microglia percentage were measured within the cerebellum, hippocampus, olfactory bulb, prefrontal cortex and thalamus. Each bar represents the active morphology percentage for at least three animals per experimental condition. In the example of amoeboid microglia scale bars represent 10 µm. Data is presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

Ramified Morphology percentages

Ramified microglia play an active role in surveying the brain via its extensions, assisting in synaptic pruning as well as synaptic maturation (Eyo et al, 2013; Salter and Beggs, 2014). In order to determine differences in the percentage of ramified microglia counted in our regions of interest, counts of ramified microglia to total microglia were calculated (see methods), and a two-way ANOVA was performed.

For the cerebellum (Figure 17, $F(3,8)=1.865$, $p=0.155$) we found no differences in the amount of ramified microglia percentages between WT and COX-2$^-$KI mice when males and females were examined together ($p=0.076$ WT=62.36±1.6, COX-2- KI=67.62±2.4). Within the cerebellum it was found that WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females had respective numbers of ramified microglia of 59.33±2.1, 67.62±3.5, 65.40±2.1, 67.62±3.5. Post hoc comparisons found that COX-2$^-$ KI males had an increased number of ramified microglia compared to WT mice ($p=0.049$). No sex differences were seen within the cerebellum ($p>0.05$).

For the hippocampus (Figure 17, $F(3,8)=0.846$, $p=0.479$) no difference in the amount of ramified microglia between WT and COX-2$^-$KI mice was determined ($p=0.387$, WT=62.36±1.6, COX-2$^-$ KI=67.62±2.4). No differences were seen in the hippocampus of WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$ KI females with respective percentages of ramified microglia of 72.41±1.8, 74.47±2.0, 75.32±1.7, 71.29±2.4 ($p>0.05$).

The olfactory bulb (Figure 17, $F(3,8)=3.692$, $p=0.022$) had larger percentages of ramified microglia in COX-2$^-$ KI mice compared to WTs ($p=0.003$, WT=65.76±2.1, COX-2$^-$ KI=73.59±1.2). Within the olfactory bulb it was found that WT males, COX-2$^-$ KI males, WT females, and COX-2$^-$KI females had respective numbers of ramified microglia of 65.10±1.7,

75.08±1.9, 66.43±4.0, 72.11±1.2. Post hoc comparisons found that COX-2$^-$ KI had greater numbers of ramified microglia compared to WT males (p=0.007). No sex differences were seen within the olfactory bulb (p>0.05).

For the prefrontal cortex (Figure 17, F(3,8)=2.875, p=0.051) no differences were found in the percentages of ramified microglial in the COX-2$^-$ KI mice compared to WT mice (p=0.099, WT=78.27±1.3, COX-2$^-$KI=81.13±1.2). Within the prefrontal cortex it was seen that WT males, COX-2$^-$KI males, WT females, and COX-2$^-$ KI females had respective numbers of ramified microglia of 75.55±0.8, 81.93±1.2, 81.01±2.3, 80.32±2.1. We were able to determine that COX-2$^-$ KI males had increased ramified microglia percentages compared to WT males (p=0.011), while there were no differences between females (p>0.05). In terms of sex differences WT males had lower ramified counts compared to WT females (p=*0*.028), while interestingly no differences were seen between the males and females of the COX-2$^-$ KI mice (p>0.05).

For the thalamus (Figure 17, F(3,8)=5.160, p=0.005) the percentages of ramified were larger in COX-2$^-$KI compared to WT mice (p=0.001, WT=67.83±1.7, COX-2- KI=75.38±1.1). Within the thalamus it was found that WT males, COX-2$^-$KI males, WT females, and COX-2$^-$KI females had respective percentages of ramified microglia of 65.98±2.5, 74.88±1.9, 69.69±2.3, 75.87±1.1. Post hoc comparisons found COX-2$^-$KI females and males to have greater percentages of ramified microglia compared to respective WT animals (p=0.039; p=0.004). No sex differences were found between the WT or COX-2$^-$KI mice (p>0.05).

Overall it was found that COX-2$^-$ KI mice had increased ramified microglia morphology in the olfactory bulb and the thalamus compared to WT mice when male and female data was combined. This is consistent with the results for the amoeboid microglia presented above. Within the cerebellum, hippocampus, and prefrontal cortex the disruption of COX-2 activity was not

found to have a statistically relevant effect on ramified microglia counts. When sexes were examined independently between WT and COX-2$^-$ KI mice significant differences were observed in the cerebellum, olfactory bulb, prefrontal cortex, and thalamus, in that COX-2$^-$ KI males had higher active amoeboid morphology than WT males. As well it was found that COX-2$^-$ KI females had greater counts than WT females in the thalamus. Sex differences were seen in the prefrontal cortex of WT mice, where females had greater ramified morphologies than males. No sex differences were noted in the COX-2$^-$ KI mice.

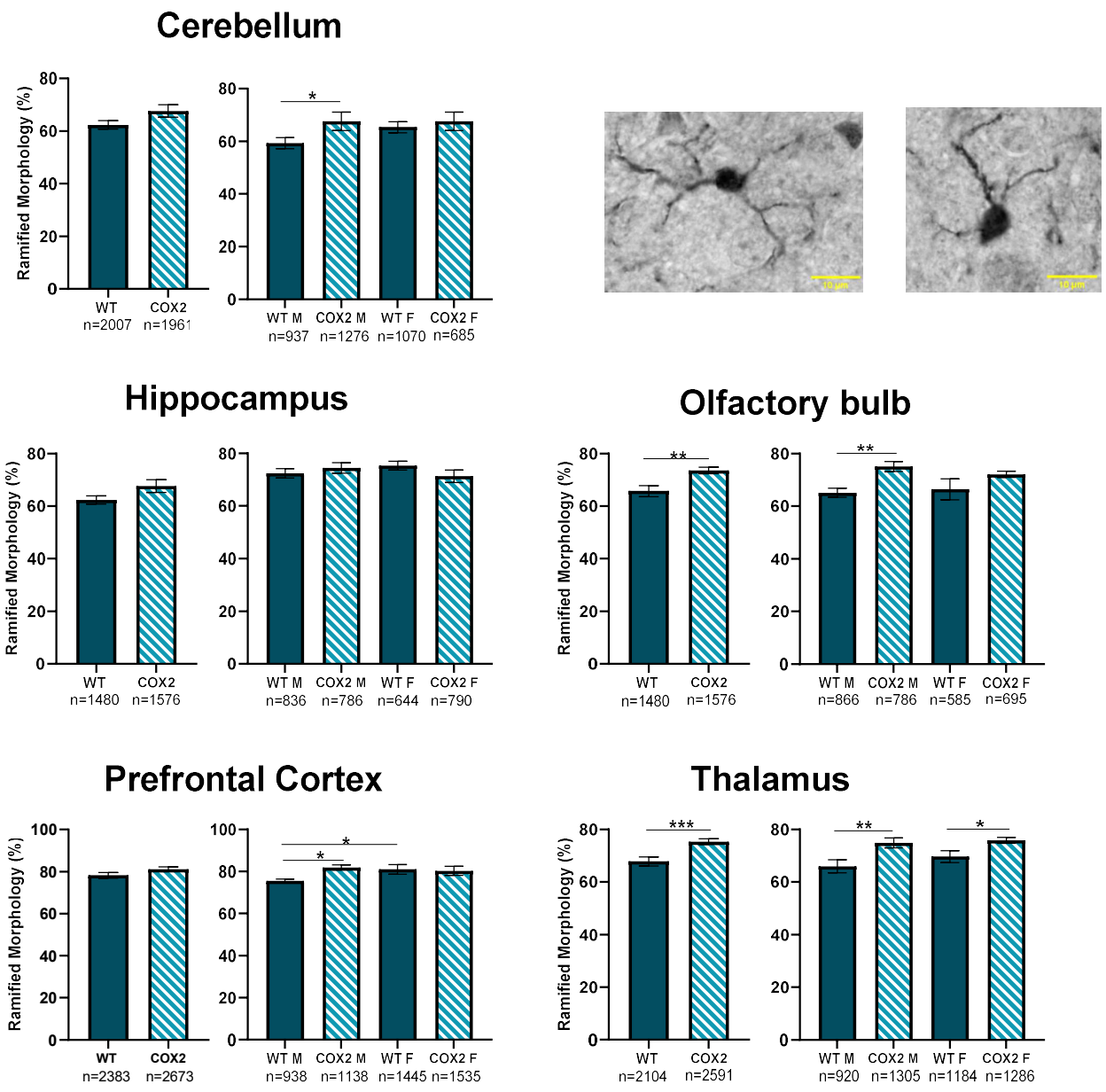


Figure 17. Ramified morphology percentage changes in postnatal brain. Ramified morphology percentages were measured within the cerebellum, hippocampus, olfactory bulb, prefrontal cortex and thalamus. Each bar represents the average ramified percentage for at least three animals per experimental condition. In the example of ramified microglia scale bars represent 10 µm. Data is presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

5.2.3 Altered Microglial Branch Length in Postnatal Day 25 Mouse

Average microglial branch length is an indicator of how ramified a cell is and has been implicated in a cell's ability to survey the brain (Madry et al, 2018; Morrison et al, 2017). As well, changes in branch length have been suggested to be an indicator of the environmental effect on the ramification of a cell as previously stated (Young et al, 2018; Kongsui et al, 2014). In order to determine differences in branch length based on the disruption of COX-2 activity at P25 the longest branch (μm) per microglia was measured within the cerebellum hippocampus, olfactory bulb, prefrontal cortex and thalamus. The values reported represent the average branch length per microglia. A two-way ANOVA was used to analyze average branch length for each mentioned region. Males and females were examined separately to examine for sex differences.

For the cerebellum (Figure 18, $F(3,6242)=5.869$, $p=0.001$) no statistical differences were seen between WT and COX-2- KI mice when male and female data were examined together ($p=0.052$, WT=10.30±0.5, COX-2- KI=10.76±0.2). In the cerebellums of WT males, COX-2$^-$KI males, WT females, and COX-2$^-$ KI females respective branch lengths of 9.68±0.2, 10.87±0.3, 10.89±0.2, 10.66±0.3 were seen. Through post hoc comparisons we show that COX-2$^-$KI males had increased branch lengths compared to WT males ($p<0.001$), while no differences were seen in females ($p>0.05$). Sex differences were only seen in the WT animals with females having larger branch lengths than males ($p=0.001$).

The hippocampus (Figure 18, $F(3,5152)=14.29$, $p<0.001$) of COX-2$^-$KI mice had a significantly increased branch length compared to WT mice ($p=0.001$, WT=15.21±0.2, COX-2-KI=16.27±0.2). WT males, COX-2$^-$KI males, WT females, and COX-2$^-$KI females hippocampal analysis revealed respective branch lengths of 14.79±0.3, 17.18±0.3, 15.69±0.3, 15.23±0.3. When sexes were analyzed separately it was seen that COX-2$^-$KI males had greater branching lengths compared to WT males ($p<0.001$), though no differences were seen within females

(p>0.05). Interestingly while COX-2$^-$KI males had greater branching lengths COX-2$^-$KI females (p<0.001), WT females had greater branch lengths compared WT males (p=0.028).

The olfactory bulb (Figure 18, F(3,7720)=29.61, p<0.001) indicated that COX-2$^-$KI mice had increased branch lengths compared to the WT (p<0.001, WT=8.22±0.1, COX-2$^-$KI=9.67±0.1). Within the olfactory bulb it was found that WT males, COX-2$^-$KI males, WT females, and COX-2$^-$KI females had respective average branch lengths of 8.05±0.2, 10.28±0.2, 8.37±0.2, 9.21±0.1. We found that COX-2$^-$KI males and females had increased average branching length compared to their respective wildtypes through post-hoc comparisons (p<0.001; p<0.001). It was also found that COX-2$^-$ KI males had longer branches than COX-2$^-$KI females (p<0.001), while interestingly no sex differences were seen within the wildtypes (p>0.05).

For the prefrontal cortex (Figure 18, F(3,6917)=24.11, p<0.001) no statistical difference between WT and COX-2$^-$KI mice was determined (p=0.840, WT=13.16±0.2, COX-2$^-$KI=12.93±.0.2). Within the prefrontal cortex we found that WT males, COX-2$^-$KI males, WT females, and COX-2$^-$KI females had respective branch lengths of 11.42±0.3, 12.89±0.2, 14.34±0.2 12.96±0.2. Post hoc comparisons showed that COX-2$^-$KI males had increased branch lengths compared to WT males (p<0.001), while COX-2$^-$KI females interestingly had decreased branching lengths compared to their respective WT (p<0.001). Sex differences were only noted in WT mice where females had greater branch lengths compared to WT males (p<0.001).

For the thalamus (Figure 18, F(3,6795)=30.056, p<0.001) we found COX-2$^-$KI mice to have statistically longer than branches than WT mice (p<0.001, WT=13.01±0.2, COX-2$^-$KI=14.41±0.2). Within the thalamus we saw that WT males, COX-2$^-$KI males, WT females, and COX-2$^-$KI females had respective branch lengths of 11.62±0.3, 15.12±0.3, 14.30±0.2, 13.80±0.2.

Through post-hoc comparisons we found that COX-2^{-}KI males had increased microglial branch lengths compared to WT males (p<0.001), while COX-2^{-}KI females had decreased branch lengths compared to their sex matched WT (p<0.001). Sex differences were found in WT animals with females having a greater branch length to males (p<0.001), while interestingly the inverse was seen in the COX-2^{-}KI animals (p<0.001).

Overall it was found that COX-2^{-}KI mice had significant increases in average branch lengths in the hippocampus, olfactory bulb, and thalamus compared to WT mice. Within the cerebellum and prefrontal cortex, the disruption of COX-2 activity was not found to have a statistically relevant effect on average branch lengths. When sexes were examined independently between WT and COX-2^{-}KI mice significant differences were seen in various regions. COX-2^{-}KI males were seen to have increased branch lengths compared to WT males within all regions of interest. Interestingly COX-2^{-}KI females were seen to be increased compared to WT females within the olfactory bulb but were found to be decreased compared to the WT within the prefrontal cortex and thalamus. Sex based differences were seen in the WT animals where females had greater branch lengths compared to males within the cerebellum, hippocampus, prefrontal cortex and thalamus. Interestingly in COX-2^{-}KI mice males were seen to have greater branch lengths compared to females within the hippocampus, olfactory bulb and thalamus.

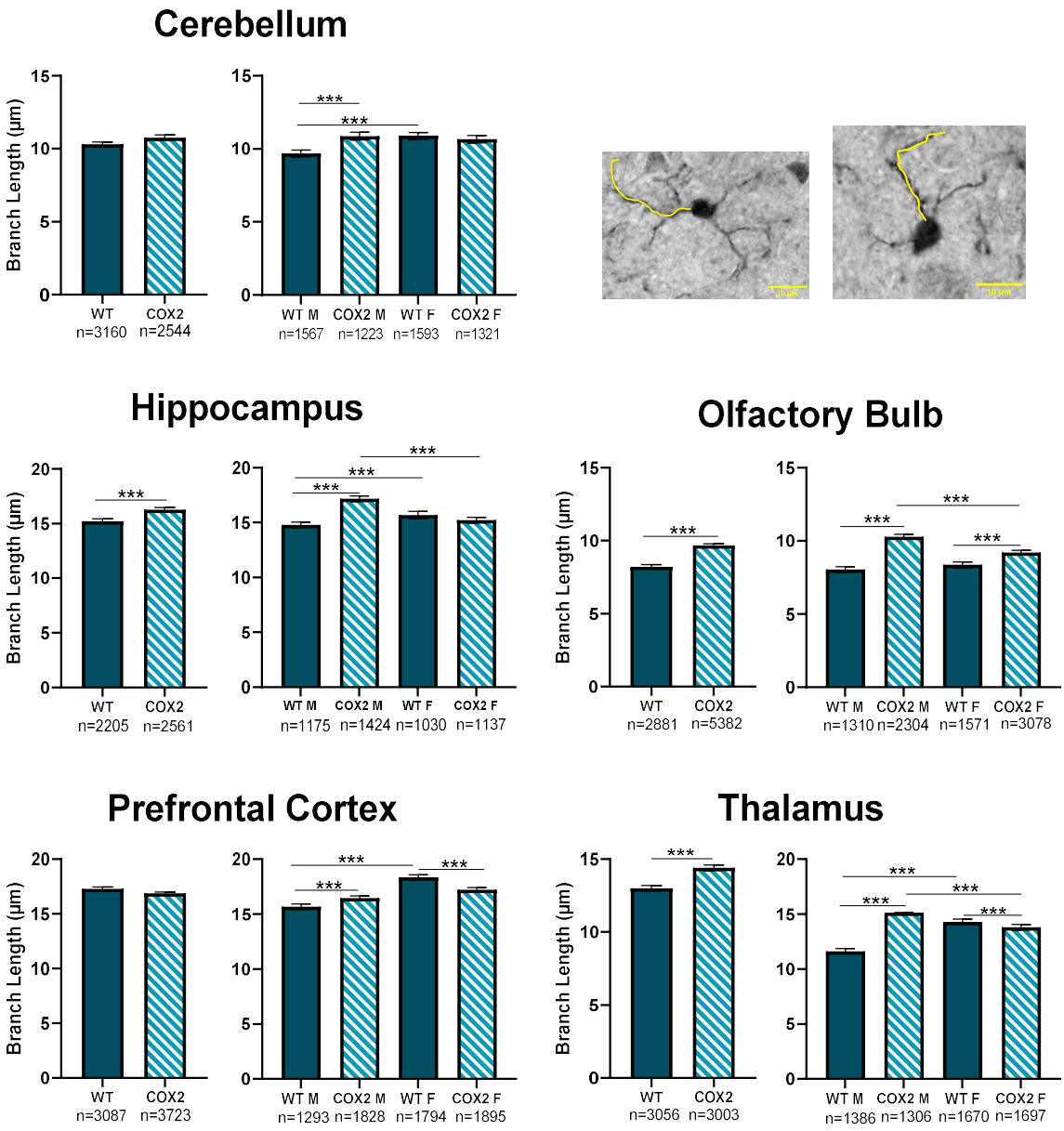


Figure 18. Branch length changes in postnatal brain. The average branch length per cell were analyzed within the cerebellum, hippocampus, olfactory bulb, prefrontal cortex and thalamus. Each bar represents the branch length for at least three animals per experimental condition. The yellow lines on the representative images of ramified microglia branch length quantification signifies the longest branch measured; scale bar represents 10µm. Data is presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

5.2.4 Altered Microglial Primary Branching in Postnatal Day 25 Mouse

Microglia are known during development to become highly branched to create relationships with neurons (Eyo et Dailey, 2013). These contacts with neurons can last up to a few minutes and have been found to regulate neuronal activity within the healthy brain (Li et al, 2012; Wake et al, 2013). To determine differences at P25 based on the disruption of COX-2 activity we quantified primary microglial branches within the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus. Primary branches were identified as branches extending directly from the microglial soma; all values reported are the average number of primary branches viewed per microglia. A two-way ANOVA was used to analyze primary branch density for each mentioned region. To examine for sex differences males and females were examined separately.

For the cerebellum (Figure 19, $F(3,6242)=25.35$, $p<0.001$) it was found that COX-2$^-$KI mice had greater quantities of primary branches density than WT mice when male and female data was combined ($p<0.001$, WT=1.14±0.02, COX-2$^-$KI=1.22±0.02). Within the cerebellum we found that WT males, COX-2$^-$KI males, WT females, and COX-2$^-$KI females had primary branches per cell of 1.02±0.02, 1.30±0.03, 1.24±0.02, 1.14±0.02. When sexes were examined separately, we saw that COX-2$^-$KI males had an increase in primary branches compared to WT males ($p<0.001$). Interestingly, COX-2$^-$ KI females have a decreased number of primary branches compared to the WT females ($p=0.003$). As well, COX-2$^-$KI males were found to have greater primary branch counts compared to COX-2$^-$KI females ($p<0.001$) while WT females were found to be greater compared to WT males ($p<0.001$).

In the hippocampus (Figure 19, $F(3,5152)=28.997$, $p<0.001$) COX-2$^-$ KI mice had increased average quantities of primary branches than WT mice ($p<0.001$, WT=1.81±0.02,

COX-2$^-$ KI=2.09±0.02). Within the hippocampus it was found that WT males, COX-2$^-$KI males, WT females, and COX-2$^-$KI females had respective average primary branches per cell of 1.80±0.03, 2.12±0.03, 1.81±0.04, 2.05±0.03. Post hoc comparisons determined that COX-2$^-$KI males, as well as COX-2$^-$KI females, have increased primary branching than their WT counterparts, (p<0.001, p<0.001). No sex differences were seen within the hippocampus (p<0.05).

For the olfactory bulb (Figure 19, F(3,7720)=16.26, p<0.001) we determined that the average primary branches per cell increased in COX-2$^-$KI mice compared to WT mice (p<0.001, WT=1.39±0.02, COX-2$^-$ KI=1.56±0.02). The olfactory bulbs of WT males, COX-2$^-$KI males, WT females, and COX-2$^-$KI females had respective average primary branches per cell of 1.43±0.03, 1.61±0.02, 1.36±0.03,1.52±0.02. When sexes were examined separately COX-2$^-$KI males and COX-2$^-$KI females were determined to have greater numbers of primary branching compared to their WT counterparts (p<0.001, p<0.001). We also saw that COX-2$^-$KI males had a greater primary branch count compared to COX-2$^-$KI females (p=0.007), interestingly no sex differences were seen in WT animals (p>0.05).

In the prefrontal cortex (Figure 19, F(3,6917)=48.31, p<0.001) COX-2$^-$KI mice had greater quantities of primary branches than WT mice (p<0.001, WT=1.58±0.02, COX-2$^-$ KI=1.78±0.02). Within the prefrontal cortex of WT males, COX-2$^-$KI males, WT females, and COX-2$^-$KI females respective primary branches values of 1.33±0.03,1.77±0.03, 1.75±0.03, 1.79±0.03 were seen. Post hoc comparisons found COX-2$^-$KI males had increased average primary branches compared to WT males (p<0.001), though no differences were seen in females. (p>0.05). In terms of sex differences WT males to have fewer average primary branches

compared to WT females ($p<0.001$), while interestingly COX-2$^-$ KI animals showed no differences based on sex ($p>0.05$).

For the thalamus (Figure 19, $F(3,6795)=26.26$, $p<0.001$) COX-2$^-$KI mice had increased quantities of primary branches than WT mice ($p=0.001$, WT=1.28±0.01, COX-2$^-$ KI=1.43±0.01). Within the thalamus it was found that WT males, COX-2$^-$KI males, WT females, and COX-2$^-$KI females had respective primary branches per cell of 1.20±0.02, 1.52±0.02, 1.35±0.02, 1.36±0.02. Post hoc comparisons determined COX-2$^-$KI males had a greater number of average primary branches compared to WT males ($p<0.001$), no differences were seen in females ($p>0.05$). When examining for sex differences it was seen that WT females had a higher amount of average primary branches compared to WT males ($p<0.001$), while interestingly in COX-2$^-$KI animals males had a greater number of primary branches compared to females ($p<0.001$).

Overall it was found that COX-2$^-$KI mice had greater numbers of primary branches compared to WT mice in all regions of interest. When sexes were examined independently between WT and COX-2$^-$KI mice significant differences were found within various regions. COX-2$^-$KI males were seen to have greater numbers of primary branches in all regions of interest compared to WT males. Interestingly, only within the hippocampus and the olfactory bulb were COX-2$^-$ KI females determined to be higher than WT females. Within the cerebellum COX-2$^-$KI females were seen to have fewer primary branches compared to WT females. In terms of sex differences within the cerebellum, prefrontal cortex and thalamus WT females were seen to have higher numbers of primary branches compared to WT males. Interestingly, in COX-2$^-$KI animals males were found to have higher numbers of primary branches compared to within the cerebellum, olfactory bulb and the thalamus.

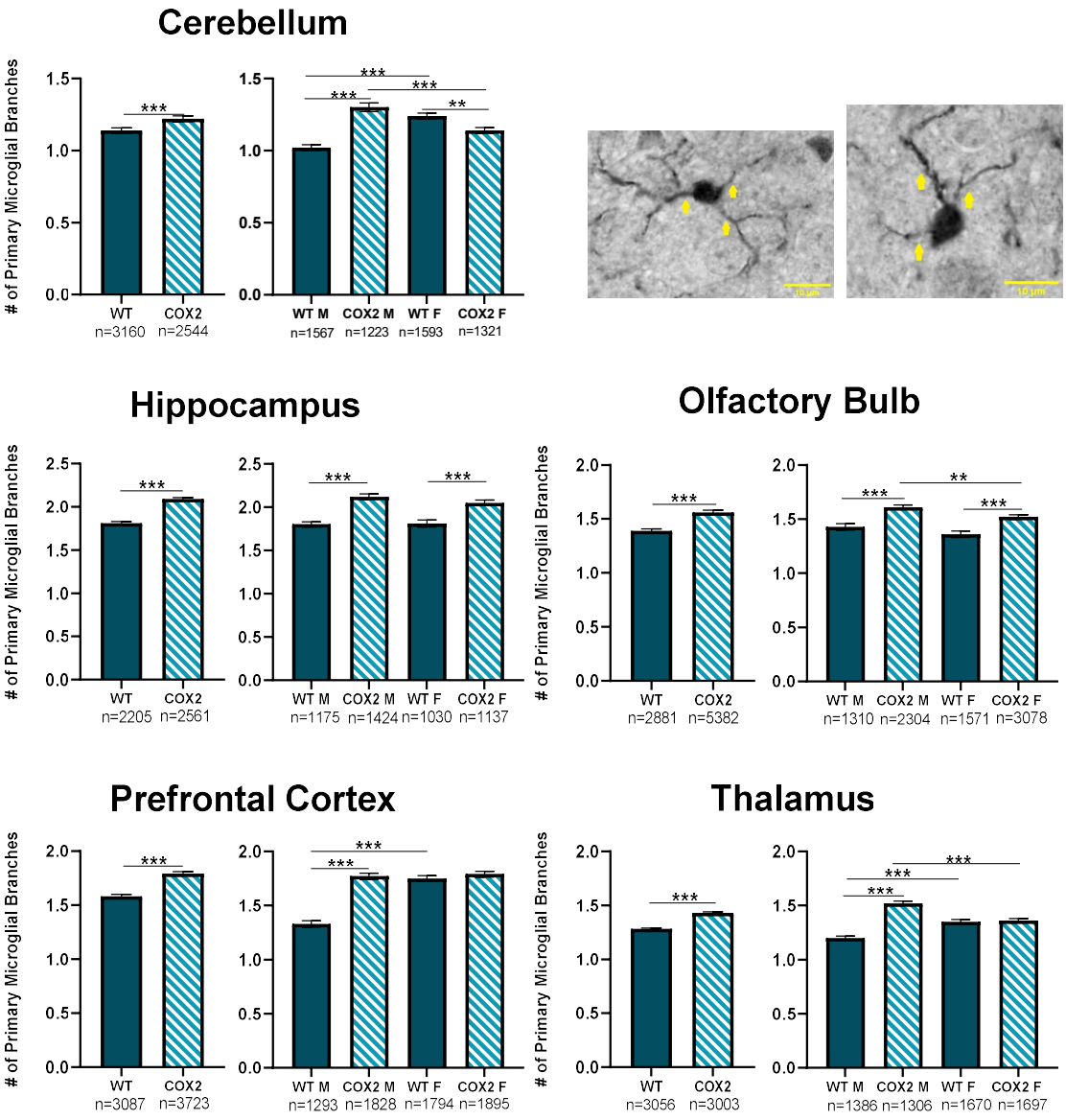


Figure 19. Primary branch count changes in postnatal brain. The number of primary branches per cell were analyzed within the cerebellum, hippocampus, olfactory bulb, prefrontal cortex and thalamus. Each bar represents the primary branch for at least three animals per experimental condition. Yellow arrows represent primary branches counted in ramified microglia; scale bats represent 10 μm. Data is presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

5.2.5 Altered Microglial Branch Total in Postnatal Day 25 Mouse

Ramified microglia processes are known to be constantly scanning the brain, attracted to a variety of factors such as synaptic activity. Processes have been shown to modulate neuronal activity and assist in the pruning, elimination and maturation of synapses (Szepesi et al, 2018; Salter and Beggs, 2014). To determine differences in total branch quantities per microglia at P25 based on the disruption of COX-2 activity we quantified total microglial branches per ramified microglia within the cerebellum, hippocampus, olfactory bulb, prefrontal cortex, and thalamus. Examining only ramified microglia was completed in attempts to gain further insight on the amount of possible connections microglia may be making to neurons and thus potentially regulating activity. The values reported are average values for total branches, including secondary and tertiary branches, per cell. Males and females were examined separately to examine for sex differences. A two-way ANOVA was used to analyze total branch counts for each mentioned region.

For the cerebellum total branch counts (Figure 20, $F(3, 3824)=7.4621$, $p<0.001$) showed no differences between WT and COX-2$^-$ KI mice when examining male and female data concurrently ($p=0.348$, WT=1.97±0.02, COX-2$^-$ KI=2.00±0.03). When examining sexes independently the cerebellum of WT males, COX-2$^-$KI males, WT females, and COX-2$^-$KI females had respective total branches per microglia of 1.87±0.02, 2.02±0.03, 2.07±0.03, 1.98±0.03. Post hoc comparisons found in males that COX-2$^-$KI mice had increased total branching when compared to male WT mice ($p=0.001$), while no differences were found in females ($p>0.05$). Sex differences were only determined in WT mice where females had greater numbers of total branches compared to WT males ($p<0.001$).

For the hippocampus (Figure 20, $F(3, 3493)=47.11$, $p<0.001$) no statistical difference was found between WT and COX-2$^-$KI mice ($p=0.177$, WT=2.88±0.03, COX-2$^-$ KI=2.99±0.03).

Within the hippocampus it was found that WT males, COX-2$^-$KI males, WT females, and COX-2$^-$KI females had respective total branches per microglia of 2.53±0.05, 3.18±0.05,3.25±0.05, 2.74±0.05. Post hoc comparisons found that COX-2$^-$KI males have increased total branching compare to WT male mice (p<0.001). Interestingly COX-2$^-$ KI females show decreased numbers of total branches per cell compared to WT females (p<0.001). As well it was seen within COX-2$^-$ KI males that there were statistically greater numbers of total branches compared to COX-2$^-$KI females (p<0.001). Interestingly, in WT mice females had greater numbers than males (p<0.001).

In the olfactory bulb (Figure 20, F(3, 4861)=19.47, p<0.001) we found COX-2$^-$KI animals to have increased total branching compared to WT mice (p<0.001, WT=2.29±0.02, COX-2$^-$ KI=2.49±0.02). Within the olfactory bulb we determined that WT males, COX-2$^-$KI males, WT females, and COX-2$^-$KI females had respective total branches per microglia of 2.16±0.03, 2.60±0.04, 2.39±0.03, 2.46±0.02. We show through post hoc comparisons that COX-2$^-$ KI males had increased total numbers of branches per cell compared to WT males (p<0.001), but no differences were noted between COX-2$^-$KI and WT females (p>0.05). When examining for sex differences it we found that in WT mice females had greater numbers of total branching compared to males (p<0.001), while interestingly in COX-2$^-$ KI mice males had higher total branching counts compared to females (p=0.008).

For the prefrontal cortex total branches (Figure 20, F(3,5298)=25.26, p<0.001) no statistical difference was seen between COX-2$^-$KI and WT mice (p=0.129, WT=3.04±0.02, COX-2$^-$ KI=3.05±0.02). Within the prefrontal cortex it was found that WT males, COX-2$^-$ KI males, WT females and COX-2$^-$ KI females had respective total branches per microglia of 2.75±0.03, 3.02±0.03, 3.23±0.03,3.07±0.03. Post hoc comparisons found COX-2$^-$KI males had

increased total branch counts compared to WT males (p<0.001), while interestingly COX-2$^-$KI females had decreased total branching counts compared to WT females (p=0.001).When examining for sex differences only WT animals exhibited significant changes, with females having greater total branching than males (p<0.001).

The thalamus (Figure 20, F(3,4694)=25.15, p<0.001) had no statistically different values in total branches between COX-2$^-$KI and WT mice (p=0.069, WT=2.51±0.02, COX-2$^-$KI=2.55±0.02). Within the thalamus it was found that WT males, COX-2$^-$KI males, WT females, and COX-2$^-$KI females had respective total branches per microglia of 2.26±0.03, 2.64±0.03, 2.70±0.03, 2.45±0.03. Post hoc comparisons found that COX-2$^-$ KI males were seen to have increased total numbers of branches per cell compared to WT males (p<0.001), while in females COX-2- KI mice had decreased total branching that WT females (p<0.001). Sex differences were seen in COX-2- KI where males had greater total branches per cell compared to COX-2- KI females (p<0.001). Interestingly in WT mice females were found to have greater numbers of total branches compared to WT males (p<0.001).

Overall it was found that COX-2$^-$ KI mice had statically greater total branching compared to WT mice in the olfactory bulb. Within the cerebellum, hippocampus, prefrontal cortex, and thalamus the disruption of COX-2 activity was not found to have a statistically relevant effect on total branching values. When sexes were examined independently between WT and COX-2$^-$ KI significant differences were seen in various regions of interest. COX-2$^-$ KI males were seen to have increased numbers of total branches compared to WTs in all regions, while interestingly COX-2$^-$ KI females had decreased numbers of total branches compared to WTs in the hippocampus, prefrontal cortex and thalamus. Within WT mice sex-based differences were seen in all regions of interest with females having greater total numbers of branches compared to

males. Interestingly in COX-2^{-} KI mice males were found to show greater numbers of total branches compared to females in the hippocampus, olfactory bulb, and the thalamus.

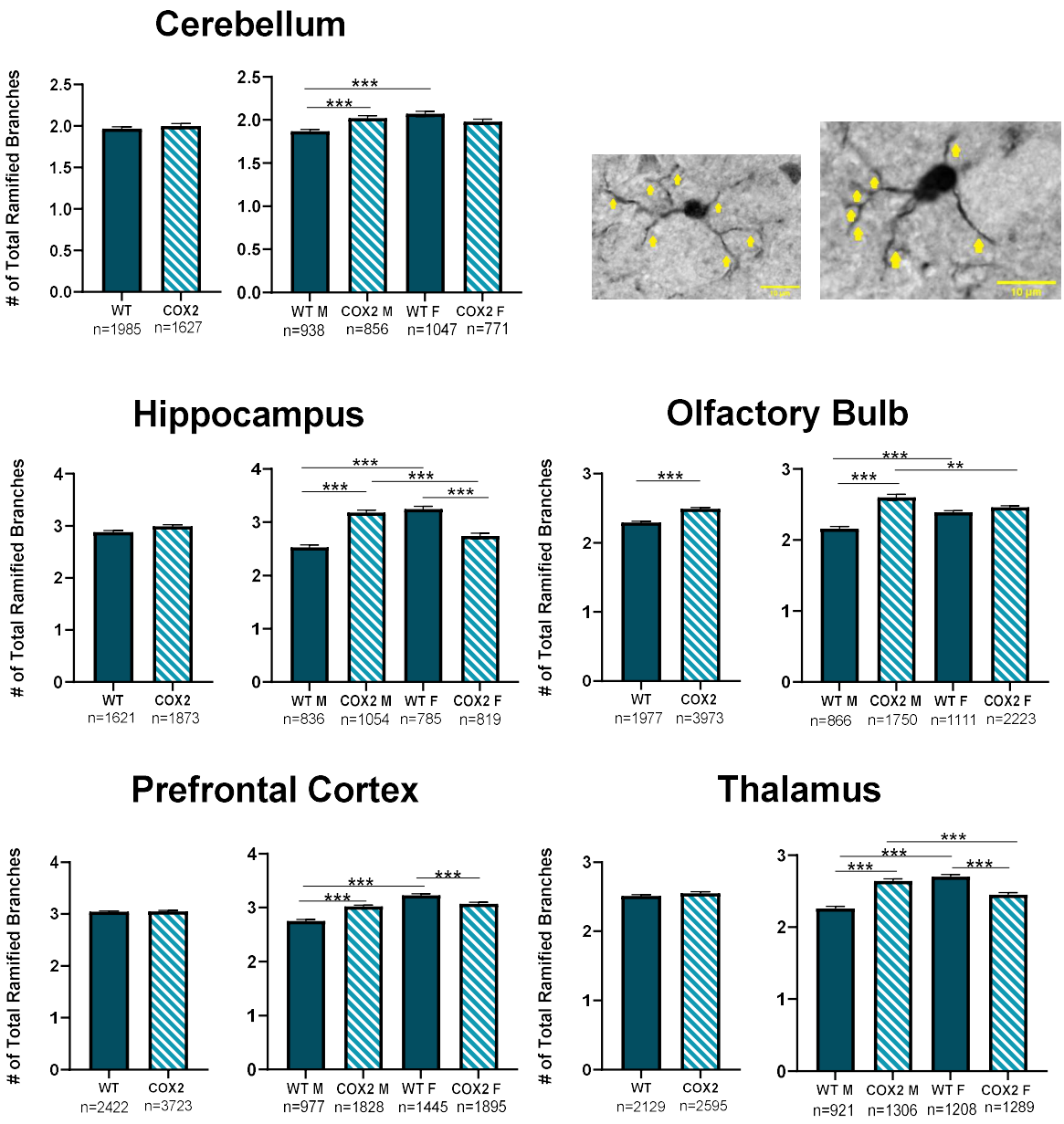


Figure 20. Total branch count changes in postnatal brain. The average number of total branches per cell were analyzed within the cerebellum, hippocampus, olfactory bulb, prefrontal cortex and thalamus. Each bar represents the total branch counts for at least three animals per experimental condition. Yellow arrows represent branches quantified in representative ramified microglia; scale bars represent 10 μm. Data is presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

5.3 Progression trajectories across the developing healthy and COX-2^{-}KI brain

To examine the effect of sex and COX-2 deficiency on microglial density and morphology across development we created graphs compiling the data presented in the E19 and P25 results sections. To emphasize the importance on sex-based studies within the brain, we first aimed to investigate the differences in male and female wildtypes from E19 to P25. We next show the impact of the COX-2 disruption on microglia density and morphology by comparing the progression trends of the COX-2^{-}KI mouse to their sex-matched WT. All methods of measurement and statistical analysis for our respective comparisons were previously stated in the E19 and P25 results.

5.3.1 Sex differences in microglia density in the developing healthy brain

Where sex differences are greatly under studied, we aimed to investigate differences in microglial density between the WT males and females across development. For the first time we show the density trajectories of both sexes within the cerebellum, hippocampus, olfactory bulb, prefrontal cortex and thalamus across the E19 and P25 stages. In general, both males and females were seen to increase exponentially over development across all regions of interest. However, we determined in a region-specific manner that sex plays a role on the microglial density trajectory within the WT mouse brain.

Regions including the cerebellum, prefrontal cortex, and thalamus demonstrated no differences in the trajectory of density over development between sexes, indicated by the similar values in density pre- and postnatally within each region (Figure 21). Interestingly, we found that the hippocampus and olfactory to exhibit sex differences by P25. Postnatally we found males to have greater density values compared to females in the two aforementioned areas (HPC: $p<0.001$, OB: $p=0.05$). Here we clearly show that despite similar density values at E19 the effect

of sex was found to influence the overall microglia counts within the postnatal hippocampus and olfactory bulb.

Overall, we found that sex differences in microglial density were apparent at P25 within our WT model in a region-specific manner. Males within the hippocampus and olfactory bulb demonstrated a greater overall trajectory than females, as shown by greater density values postnatally.

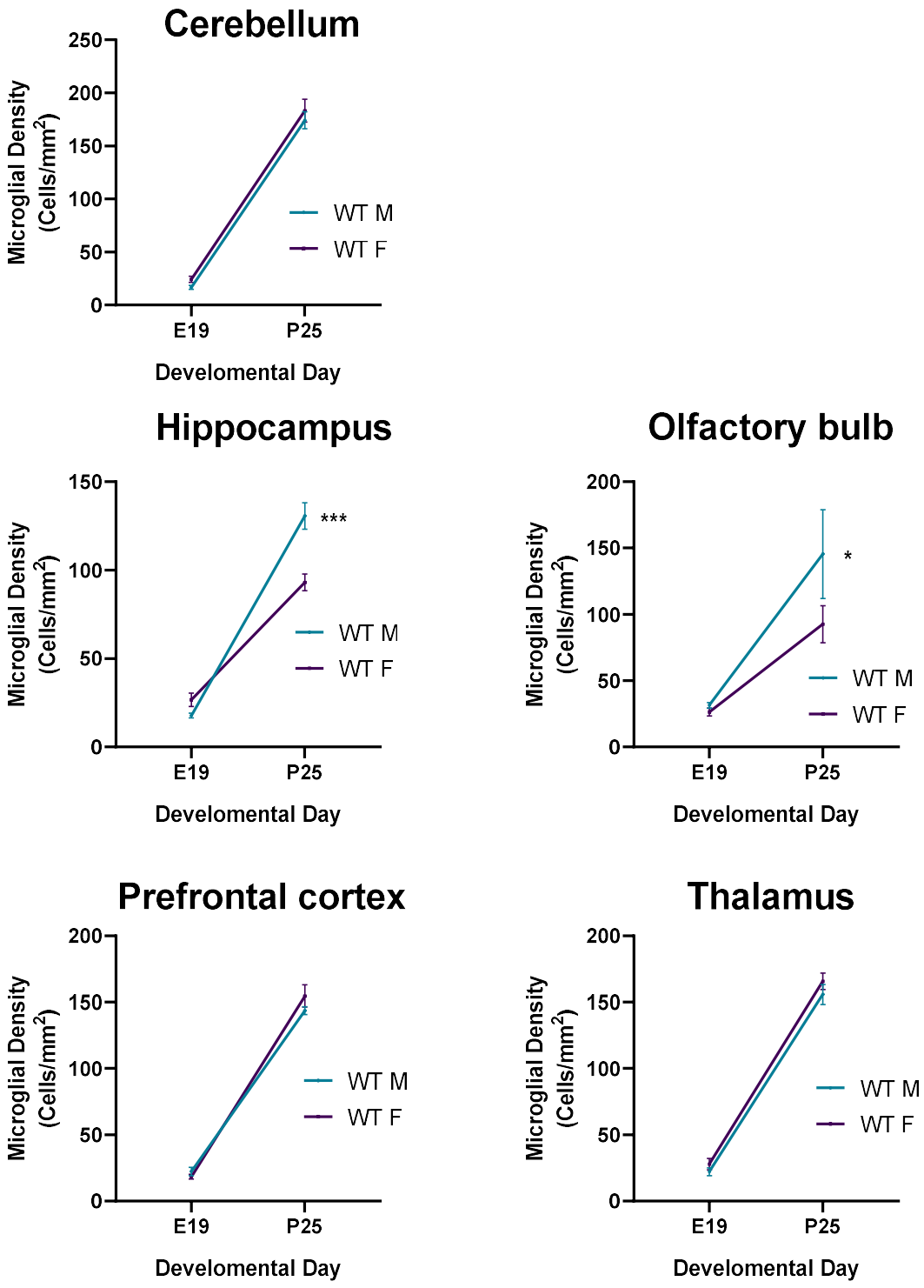


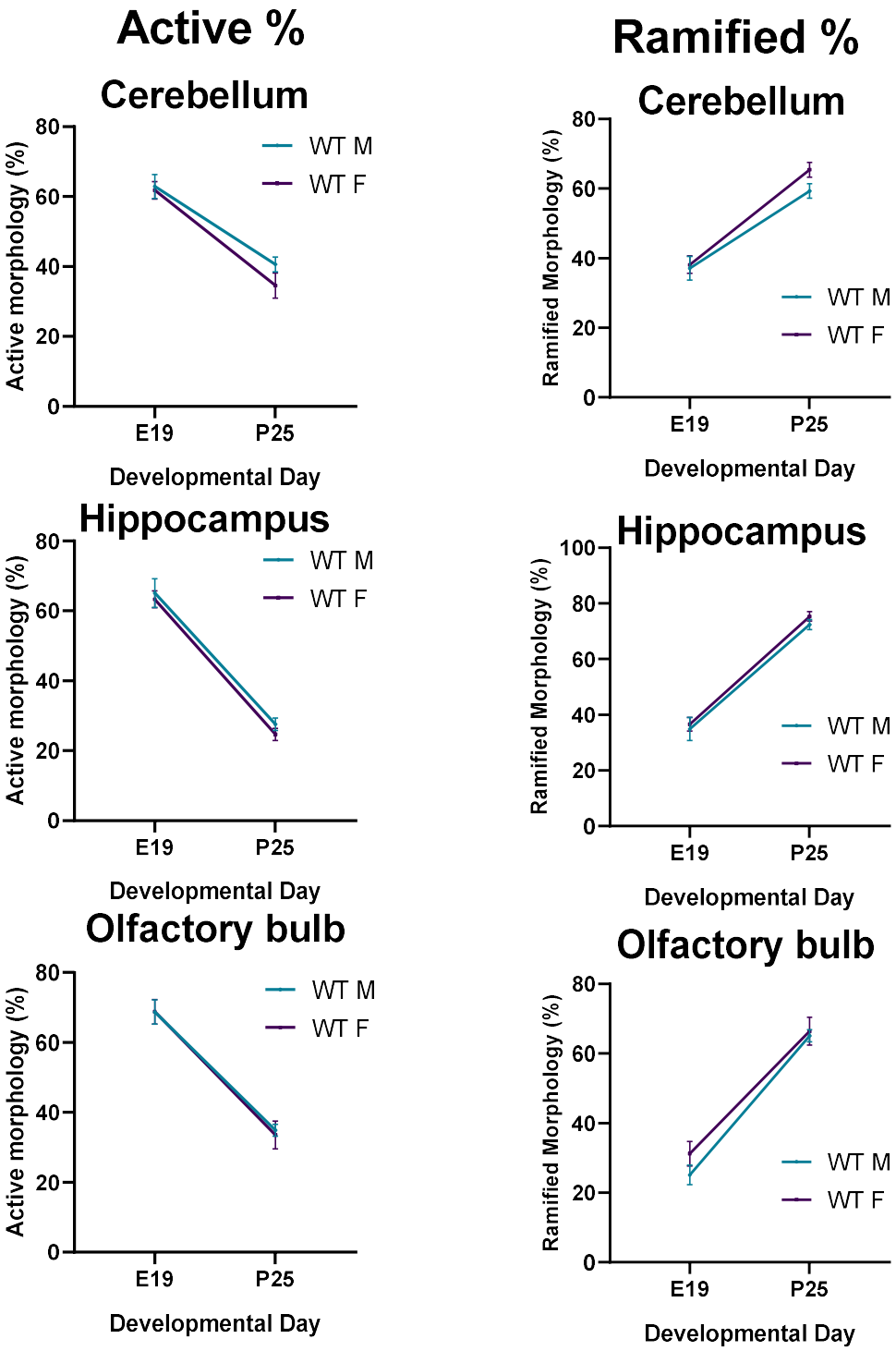
Figure 21: Sex-based microglial density differences of the healthy mouse. Values used in these studies were obtained from the E19 and P25 density studies. Data points are presented as mean±SEM, *p<0.05, **p<0.01, ***p<0.001.

5.3.2 Sex differences in active and ramified microglia percentages in the developing WT brain

Male and female microglia are known to have clear sex differences within the neurotypical brain; literature shows that males typically exhibit greater active microglia percentages, compared to females, although this has not yet been proved at either E19 or P25 (Lenz et al, 2012, Hanamsagar et al, 2017, Schwarz 2012). In general, both males and females were seen to decrease exponentially over development across all regions of interest, with a reciprocal increase in ramified microglia percentage as the brain developed. We will discuss the active and ramified trends over development within the same section as changes we see in active percentages are mirrored for ramified percentages.

Regions including the cerebellum, hippocampus, olfactory bulb and thalamus demonstrated no differences in the trajectory of active or ramified microglia percentages over development between sexes, indicated by the similar values in density pre- and postnatally within each region (Figure 22). Interestingly, we found that the male prefrontal cortex had a greater percentage of active microglia compared to females at P25 (PFC: p=0.028). We accordingly observed an increased percentage of ramified microglia within the female prefrontal cortex compared to the male (PFC: p=0.028). As such, we demonstrate sex differences within this region, despite similar active or ramified percentages values at E19, the effect of sex was found to influence the overall microglia morphology percentages within the postnatal prefrontal cortex.

Overall, we found that sex differences in microglial active and ramified percentage were apparent at P25 within our WT model in a region-specific manner. As demonstrated though the increased values of active and ramified microglia within the prefrontal cortex, for males and females respectively.


Active %
Cerebellum
Active morphology (%)
80
60
40
20
0
WT M
WT F
E19
P25
Developmental Day
Ramified %
Cerebellum
Ramified Morphology (%)
80
60
40
20
0
WT M
WT F
E19
P25
Developmental Day
Hippocampus
Active morphology (%)
80
60
40
20
0
WT M
WT F
E19
P25
Developmental Day
Hippocampus
Ramified Morphology (%)
100
80
60
40
20
0
WT M
WT F
E19
P25
Developmental Day
Olfactory bulb
Active morphology (%)
80
60
40
20
0
WT M
WT F
E19
P25
Developmental Day
Olfactory bulb
Ramified Morphology (%)
80
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20
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WT M
WT F
E19
P25
Developmental Day

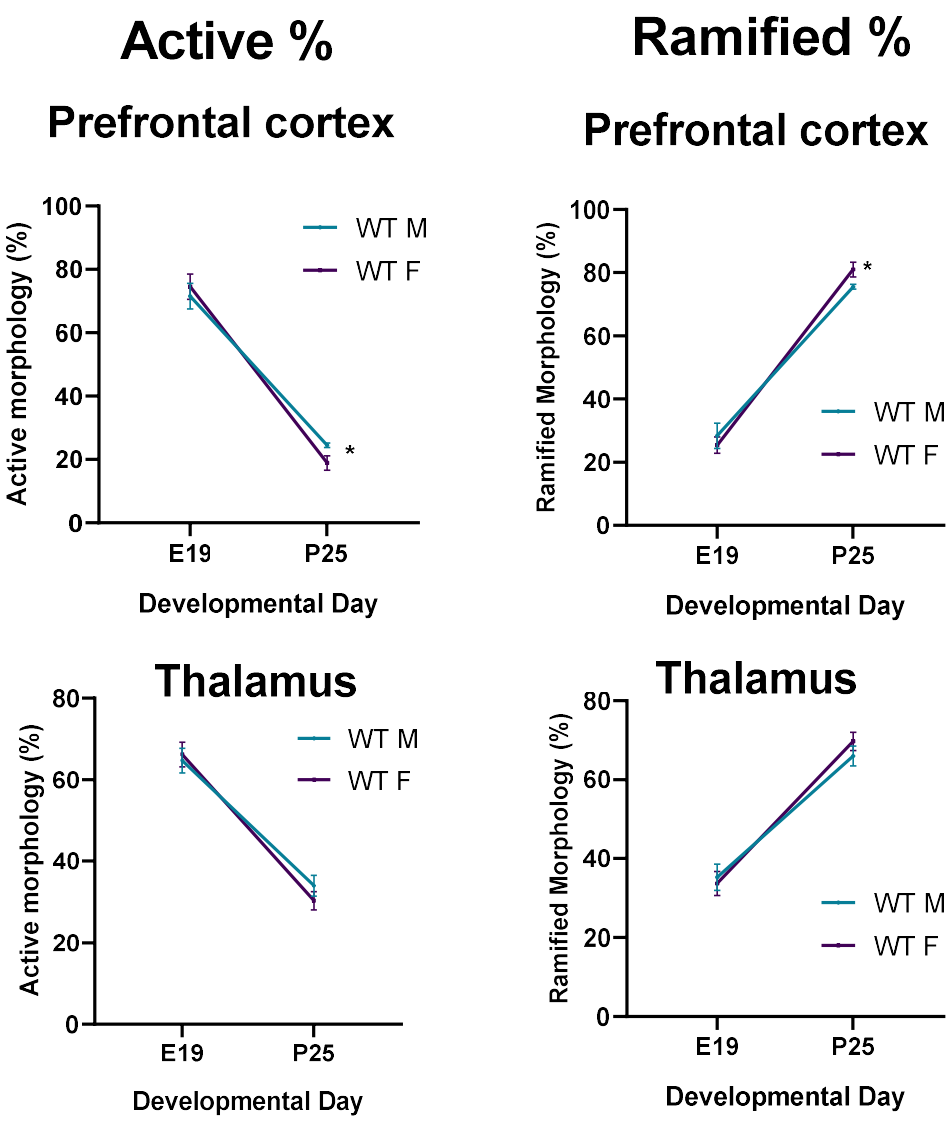


Figure 22: Sex-based differences in active and ramified percentage in the healthy mouse. Values used in these studies were obtained from the E19 and P25 active and ramified morphology studies. Data points are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

5.3.3 Sex differences in microglial branch length in the developing WT brain

As males are known to have greater levels of active microglia, as previously mentioned, they have also been demonstrated to have shorter microglial branches within the rat and mouse brain (Lenz et al, 2012). As no current literature reports whether this trend remains true at E19 and P25 within the mouse, we compared the data collected to examine for sex differences in the WT mouse. In general, both males and females were seen to increase branch length over development across all regions of interest.

The olfactory bulb was the only region where no significant differences in the trajectory of microglial branch length within the developing brain was found between sexes, indicated by the similar values in density pre- and postnatally within each region (Figure 23). Interestingly, we found that the female cerebellum, hippocampus, olfactory bulb and prefrontal cortex all had greater branching lengths compared to males at P25 (CB: p=0.001, HPC: p=0.028, PFC: p<0.001, TH: p<0.001). Here we show within these regions, despite similar values at E19, that sex has a significant effect on microglial branch length within the WT mouse.

Overall, we found that sex differences in the cerebellum, hippocampus, prefrontal cortex, and thalamus were apparent at P25 within our WT model in a region-specific manner. This was demonstrated though greater values observed for female branch length within the aforementioned regions at P25.

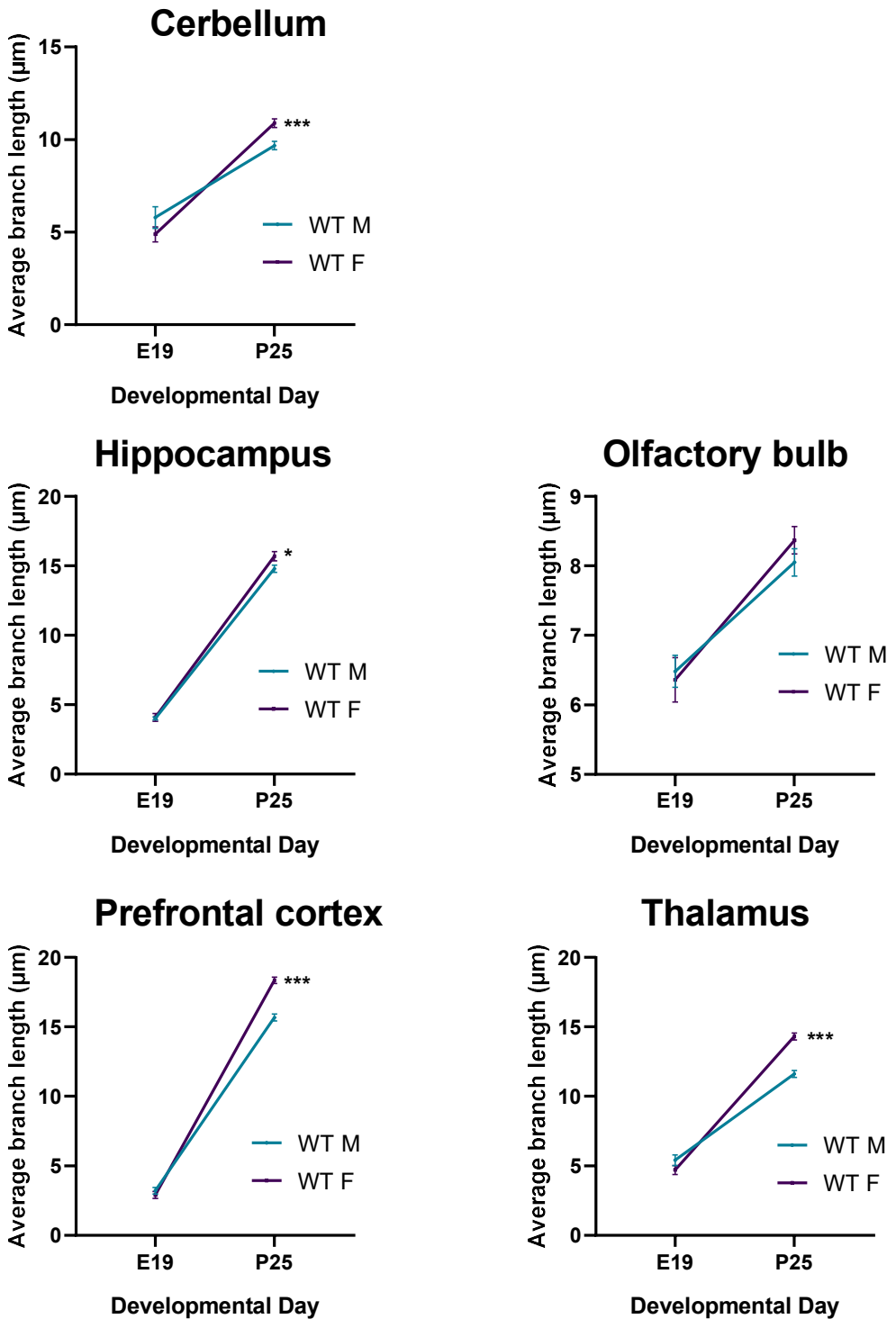


Figure 23: Sex-based differences in average branch length in the healthy mouse. Values used in these studies were obtained from the E19 and P25 branch length studies. Data points are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

5.3.4 Sex differences in primary microglia branches in the developing WT brain

As previously mentioned, male and female microglia have been previously found to have differences in morphology, with females commonly having greater branching compared to males (Lenz et al, 2012, Hanamsagar et al, 2017, Schwarz 2012). We examine for the first time if females maintain greater branching compared to males at the E19 and P25. In general, both males and females were seen to greater primary branch counts over development across our regions of interest. We find that in a region-specific manner that females have greater numbers of primary branches compared to males, consistent with the current literature.

Regions such as the hippocampus and olfactory bulb demonstrated no differences in the trajectory of primary branch counts between males and females, demonstrated through similar values at E19 and P25 (Figure 24). Interestingly, we find drastic differences in primary branch counts within the cerebellum, prefrontal cortex and thalamus, with P25 females having greater primary branch counts compared to the males (CB: $p<0.001$; PFC: $p<0.001$; TH: $p<0.001$). We demonstrate that sex has a clear effect on primary microglial branching in a region-specific manner at P25, despite there being no differences at E19.

Overall, we found that sex differences in microglial active and ramified percentage were apparent at P25 within our WT model in a region-specific manner. As demonstrated though the increased values of active and ramified microglia within the prefrontal cortex, for males and females respectively.

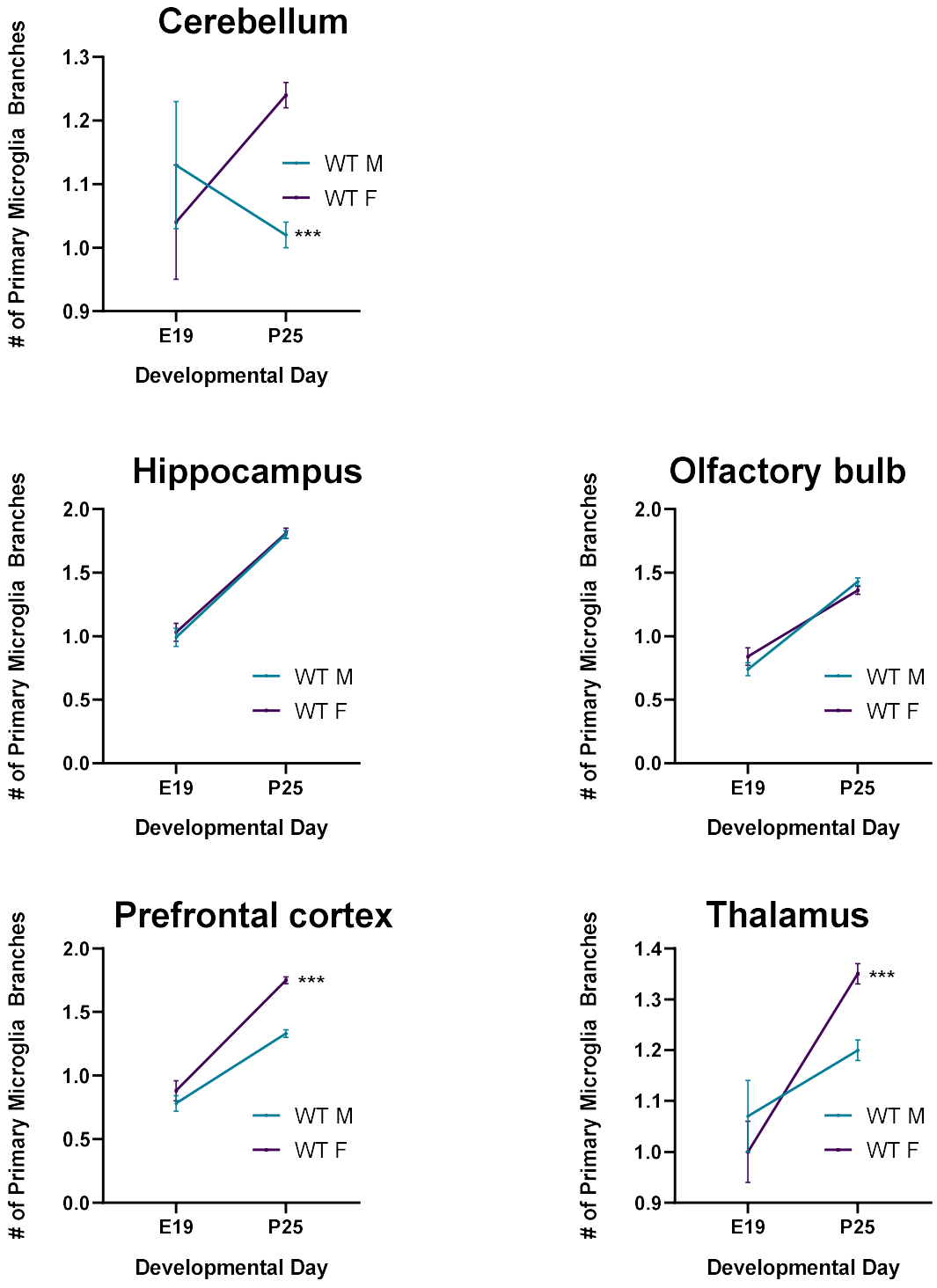


Figure 24: Sex-based differences in primary branches in the healthy mouse. Values used in these studies were obtained from the E19 and P25 primary branch studies. Data points are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

5.3.5 Sex differences in total microglia branching in the developing WT brain

Finally, we examined for sex differences in the total branching counts between male and female wildtypes at E19 and P25. As previously mentioned, females are typically seen to have a more ramified profile compared to males. We have so far shown females to have increased branch length and primary branch counts compared to males in a region-specific manner, which agrees with the literature. In terms of total branching counts in general, while both males and females were seen to greater total branch counts over development across all regions of interest, sex differences were found. We find that in a region-specific manner that females have greater numbers of primary branches compared to males.

For the first time we see differences in the prenatal at E19 within the olfactory bulb, with a surprising finding that males had greater total branch counts at females (OB: p=0.032) (Figure 25). We also found drastic differences in primary branch counts within the cerebellum, hippocampus, prefrontal cortex and thalamus, with P25 females having greater primary branch counts compared to the males (CB: p<0.001; HPC: p<0.001 PFC: p<0.001; TH: p<0.001). Of particular note, we see that the male cerebellum had decreased total branch counts from E19 to P25, a trend we also report in the primary branch trajectories. We demonstrate that sex has a clear effect on primary microglial branching in a region-specific manner at P25, despite there being no differences at E19.

Overall, we found that sex differences in microglial active and ramified percentage were apparent at P25 within our WT model in a region-specific manner. As demonstrated though the increased values of active and ramified microglia within the prefrontal cortex, for males and females respectively.

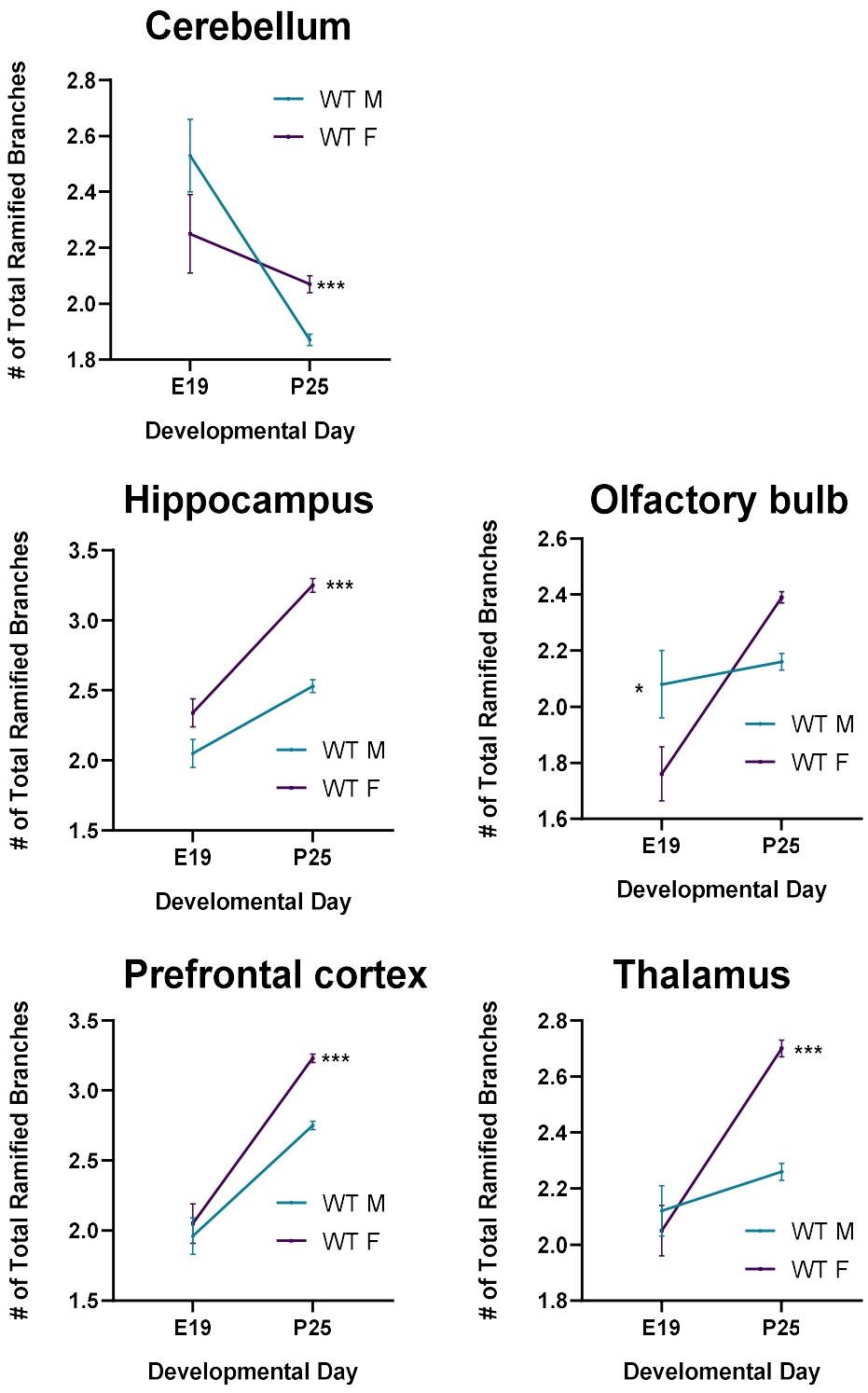


Figure 25: Sex-based differences in total branch counts in the healthy mouse. Values used in these studies were obtained from the E19 and P25 total branch counts studies. Data points are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

5.4 Changes between COX-2^{-}KI mice across development

We next wanted to determine how the COX-2 disruption would influence microglial density and morphology within the developing brain. In order to do so we compiled the data from our E19 and P25 studies and created trajectory charts. We compared COX-2^{-}KI males and females to their respective WTs in charts shown below, which clearly demonstrate sex-based differences from the WT trends within the COX-2^{-}KI mouse.

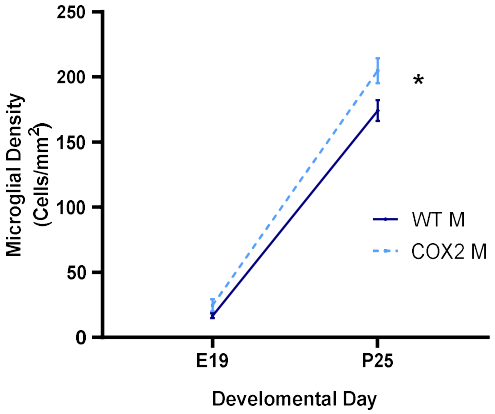
5.4.1 Changes in microglia density in the developing COX-2^{-}KI male and female brain

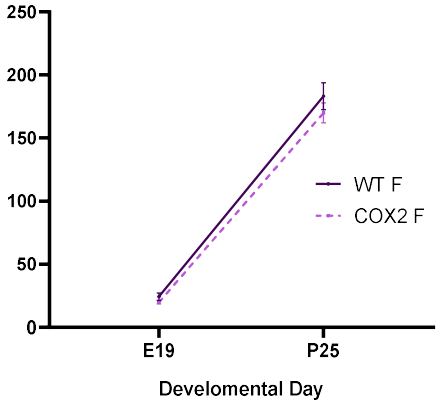
Previous literature shows that as development progresses the microglial density increases at a steadily until approximately P14 within the healthy mouse brain, followed by a decrease in the population until P21 where it stabilizes to the density that will be seen within the adult (Lenz and Nelson, 2018). To investigate the impact of the COX-2 disruption on microglial density in the developing brain, we compared the trajectory of microglial density at E19 and P25 of both COX-2^{-}KI animals to their sex-matched WT. In general, we found that both the WT and COX-2^{-}KI mouse increased in microglial density as the brain developed, however we found differences from the WT trends in a region and sex-specific manner discussed below.

Within the thalamus, we found no differences in the density trajectory from E19 to P25 within either sex of the COX-2^{-}KI mouse compared to their respective WT (Figure 26). However, in a sex and time-specific manner we demonstrate differences in microglial density within the cerebellum, hippocampus, olfactory bulb, and prefrontal cortex. COX-2^{-}KI males within the cerebellum and prefrontal cortex at P25 deviated from the WT trend by having greater density counts (CB: p= 0.024, PFC: p=0.039). Prenatally at E19 we demonstrate that the female COX-2^{-}KI mouse has a greater density compared to its sex matched control in the olfactory bulb, as well, postnatally at P25 in the hippocampus we found increased microglial density of the COX-2^{-}KI mouse compared to the WT (OB: p=0.01, HPC: p=0.003).

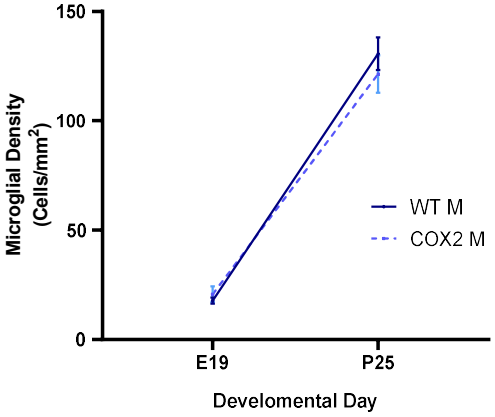
Overall, we show that the COX-2 deficit has a significant impact on the trajectory of microglia density from E19 to P25 for males within the cerebellum and prefrontal cortex and for females within the hippocampus and olfactory bulb. All fore-mentioned differences were found to be increased in microglial density values compared to their sex-matched WT at the respective developmental stage. No differences were seen within the thalamus. These findings were compiled into a summary chart presented below (Table 2). Overall, we provide evidence for the first time that the disruption of COX-2 impacts microglial density in a sex specific manner across development in specific brain regions.

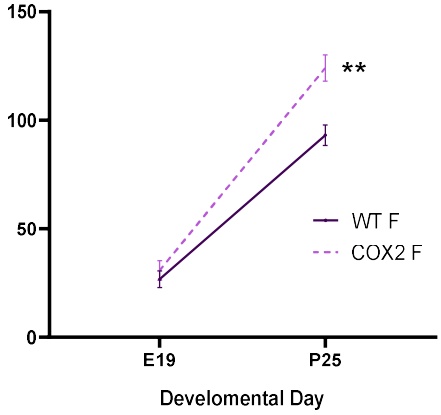
Cerebellum



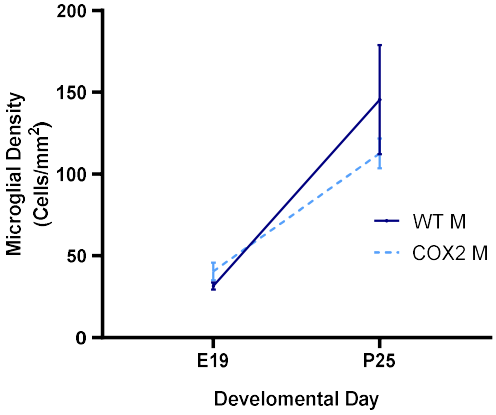


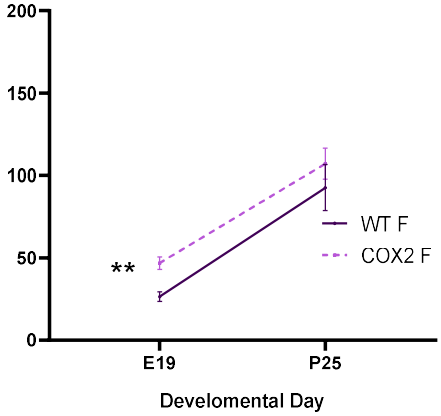
Hippocampus





Olfactory bulb





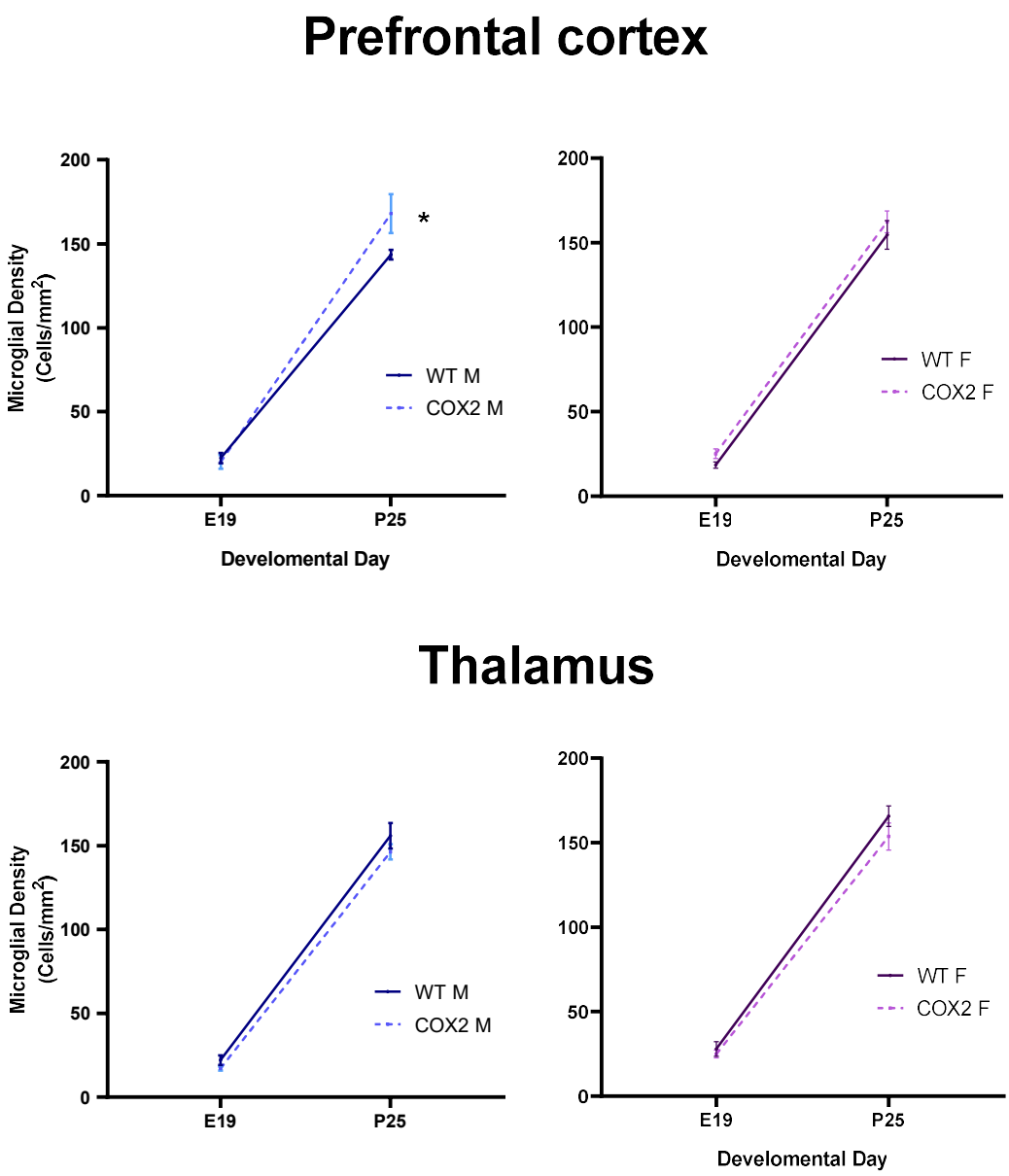


Figure 26: Progression trends for microglial density. Trends for microglial density between WT and COX-2^{-}KI mice were compared over developmental time points embryonic day 19 and postnatal day 25 within our five regions of interest. Values used in these studies were obtained from the E19 and P25 microglial density studies. Data points are presented as mean ±SEM, **p*<0.05, ***p*<0.01, ****p*<0.001.

Table 2: Microglial density in the developing COX-2^{-}KI male and female brain. A summary of density progression results. Arrows represent increased or decreased density values compared to their sex matched wildtype.

DENSITY	COX2 M E19	COX2 F E19	COX2 M P25	COX2 F P25
Cerebellum	-	-	↑	-
Hippocampus	-	-	-	↑
Olfactory	-	↑	-	-
Prefrontal cortex	-	-	↑	-
Thalamus	-	-	-	-

5.4.2 Amoeboid active and ramified morphology changes in the developing brain

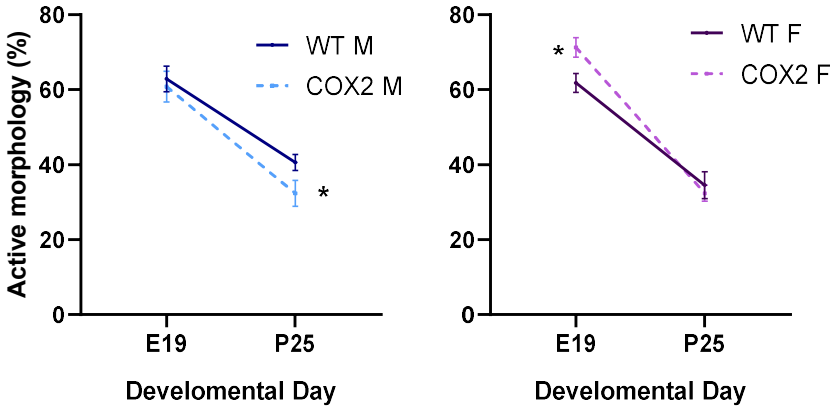
As the brain continues to age and mature as do the microglial cells inhabiting it. As previously mentioned in the E19 active morphology percentage results, the immature microglia have an amoeboid form that over time develops into mature microglia. This transition can be visually noted by morphological change in the microglia from the large round cell body with few extensions to the typical morphology of ramified cells. Using values previously stated within the E19 and P25 active and ramified morphology percentage sections we show the influence of COX-2 disruption across development. In general, we found high levels of active amoeboid microglia at E19, which by P25 had decreased substantially. Accordingly, the decline of active amoeboid microglia we saw was accompanied by an increase in the counts of ramified microglia, as we anticipated.

The hippocampus was the only region we found no differences in active and ramified microglia percentages trajectory from E19 to P25 within either sex of the COX-2^{-}KI mouse compared to their respective WT (Figure 27-28). However, in a sex and time-specific manner we

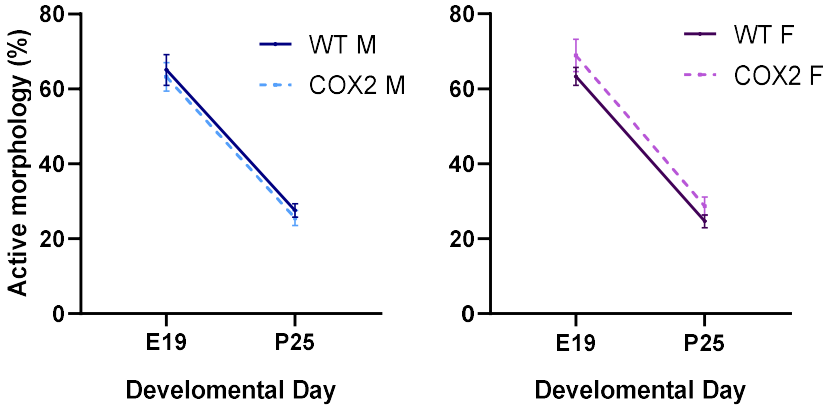
observe that the cerebellum, olfactory bulb, prefrontal cortex and thalamus all show different trajectories within the COX-2^{-}KI mice to the their sex matched WT. COX-2^{-}KI males within the cerebellum, olfactory bulb, prefrontal cortex and thalamus at P25 all deviated from the WT trend by having decreased active microglia percentages and greater ramified microglial percentages (CB: p=0.049 ,OB: p=0.007; PFC: p=0.011, TH: p=0.039). We also show that the female COX-2^{-}KI thalamus deviated from the WT trend, demonstrating a steeper decrease in active microglia morphology over development, represented by a lower active percentage and higher ramified percentage at P25 (TH: p=0.032). Prenatally at E19 we demonstrate that the female COX-2^{-}KI mouse has a greater active percentage and lesser ramified percentages to its sex matched control in the cerebellum, while interestingly a decreased trend of microglial activation and increased ramified percentages were noted within the female prefrontal cortex at E19 (CB: p=0.046; PFC: p=0.032).

Overall, we found that the disruption of COX-2 had an impact on the decreasing trajectory of active amoeboid morphology percentages across E19 to P25 for males in the prefrontal cortex and females in the cerebellum. No differences were observed within the hippocampus. The findings of this section are summarized in table 3. These findings emphasize the known importance of COX-2 function on microglia active amoeboid morphologies in a region-specific manner.

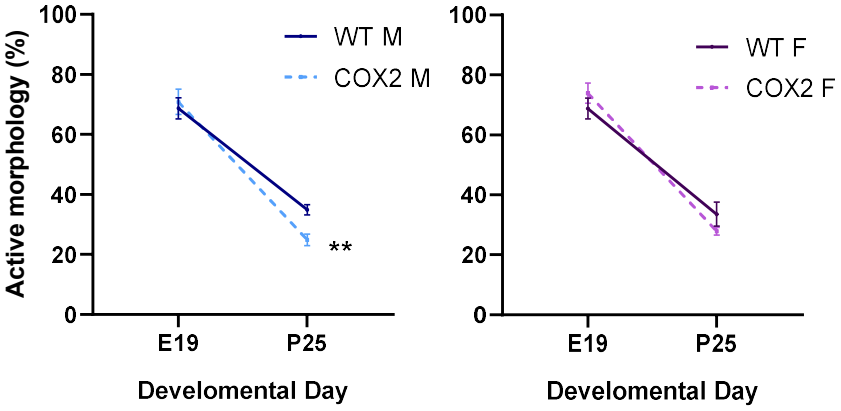
Cerebellum



Hippocampus



Olfactory bulb



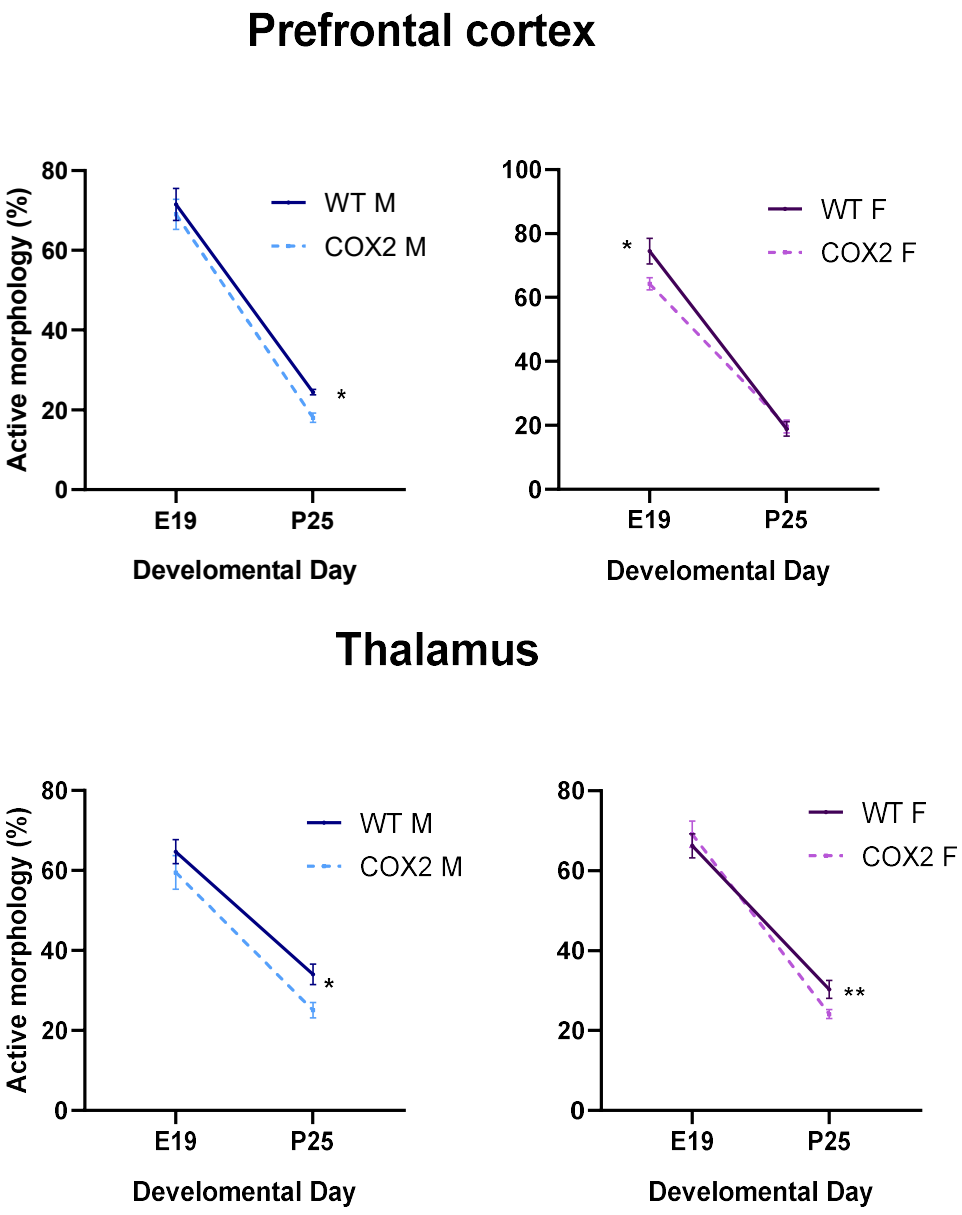
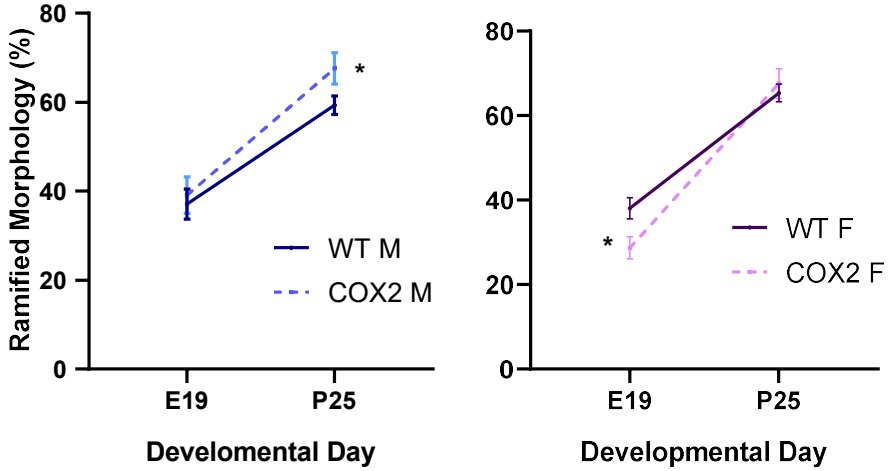
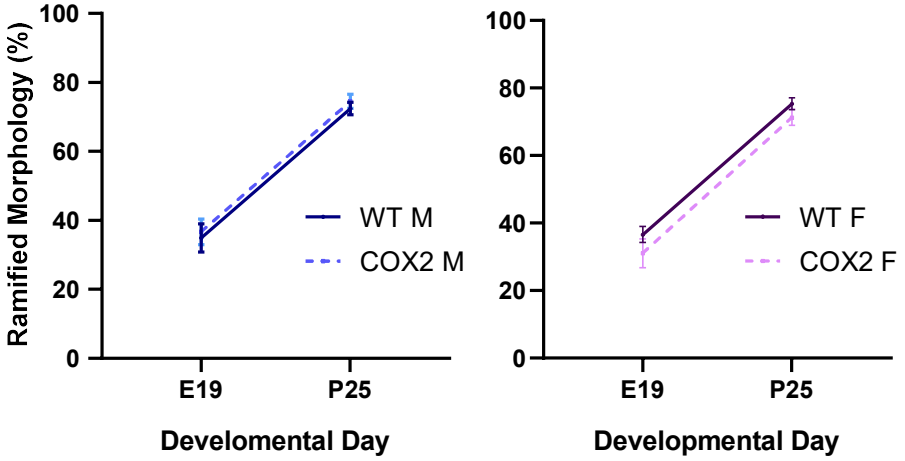


Figure 27: Progression trends for active and ramified percentages. Trends for active microglia percentages between WT and COX-2^{-}KI mice were compared over developmental time points embryonic day 19 and postnatal day 25 within our five regions of interest. Values used in these studies were obtained from the E19 and P25 active morphology percentage studies. Data points are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

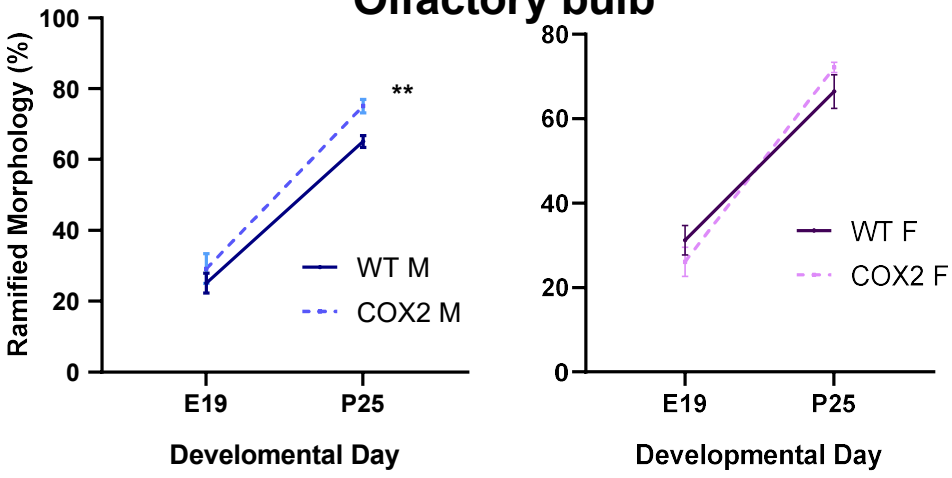
Cerebellum



Hippocampus



Olfactory bulb



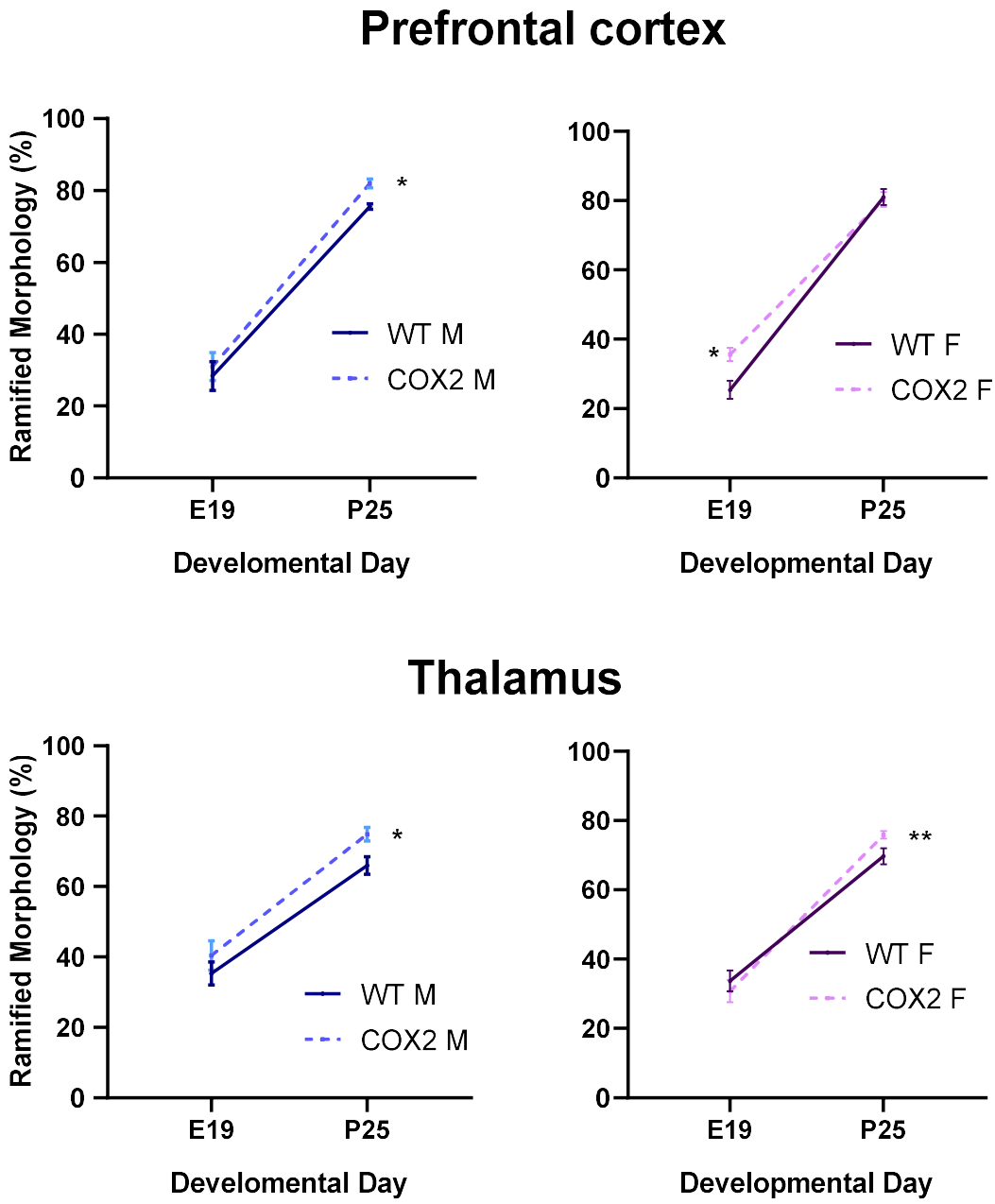


Figure 28: Progression trends for active and ramified percentages. Trends for ramified microglia percentages between WT and COX-2^{-}KI mice were compared over developmental time points embryonic day 19 and postnatal day 25 within our five regions of interest. Values used in these studies were obtained from the E19 and P25 ramified morphology percentage studies. Data points are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

Table 3: Microglial active and ramified percentages in the developing COX-2^{-}KI male and female brain. A summary of active and ramified morphology percentage progression results. Arrows represent increased or decreased values compared to their sex matched wildtype.

ACTIVE AMOEBOID %	COX2 M E19	COX2 F E19	COX2 M P25	COX2 F P25
Cerebellum	-	↑	↓	-
Hippocampus	-	-	-	-
Olfactory	-	-	↓	-
Prefrontal cortex	-	↓	↓	-
Thalamus	-	-	↓	↓

RAMIFIED %	COX2 M E19	COX2 F E19	COX2 M P25	COX2 F P25
Cerebellum	-	↓	↑	-
Hippocampus	-	-	-	-
Olfactory	-	-	↑	-
Prefrontal cortex	-	↑	↑	-
Thalamus	-	-	↑	↑

5.4.3 Change in microglial branch length across development

Branch length has been suggested to be a method of looking at microglial cell activation as the shorter the branch is the closer it is to the active morphology. We analyzed the differences in branch length between our two developmental time points within all five regions of interest. Across development, in general, we see that from E19 to P25 microglial branch lengths increase, with deviations from the WT trends discussed below.

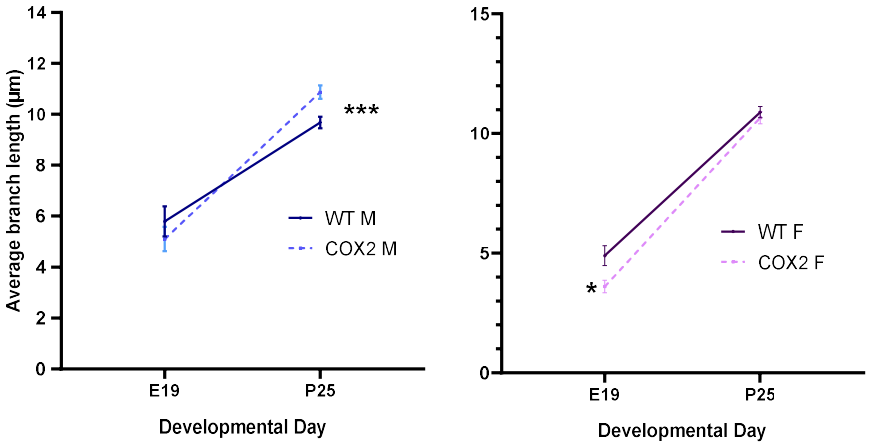
All regions of interested showed sex and developmental time point differences in microglial branch length between WT and COX-2^{-}KI mice (Figure 29). All regions showed that

males of the COX-2^{-}KI mice had greater rates of microglial branch growth over development as indicated by increased branch lengths at the P25 stage (CB: $p<0.001$; HPC: $p<0.001$; OB: $p<0.001$; PFC: $p<0.001$; TH: $p<0.001$). Females were seen to have more complex trends with differences across development found in the cerebellum, hippocampus, olfactory bulb and prefrontal cortex. Both the female COX-2^{-}KI cerebellum and hippocampus were found to deviate from the WT at E19, having lower branch lengths prenatally, but by P25 similar branch length were demonstrated (CB: $p= 0.045$; HPC: $p= 0.047$). This indicates that between these times points the disruption of COX-2 resulted in increased branch growth rates in order for values to reach similar measurement. The olfactory bulb showed that the COX-2^{-}KI females had increased branch growth rates as well, represented by increased branch lengths at P25 (OB: $p<0.001$). The female prefrontal cortex was seen to be heavily impacted by the COX-2 disruption, having increased branch lengths at E19 but decreased lengths by P25 (E19: $p=0.008$; P25: $p<0.001$). Indicating during this period of development branch length had a decrease rate of growth within the female COX-2^{-}KI prefrontal cortex.

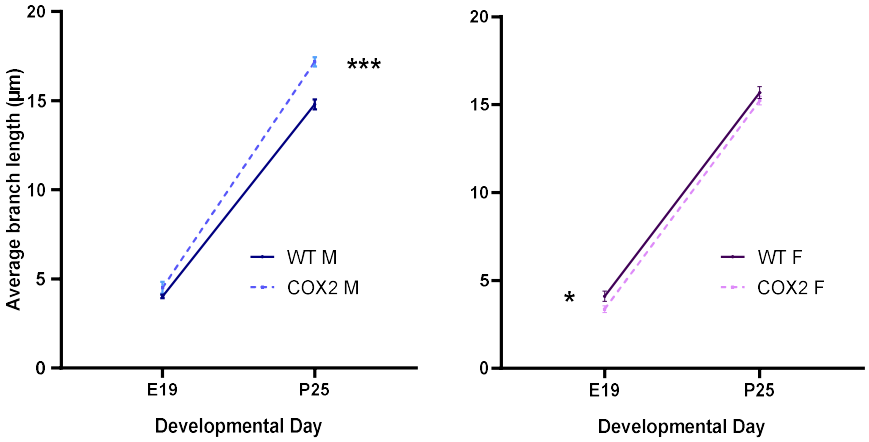
Overall, we show that the deficit of COX-2 had an effect on the trajectory of microglial branch lengths in both males and females. Male COX-2^{-} KI animals were found to have increased branch lengths within the all five regions of interest postnatally compared to WT males. Female COX-2–KI females were seen to have decreased branch lengths compared to their sex-matched WTs prenatally within the cerebellum and hippocampus, and postnatally within the prefrontal cortex. Interestingly prenatally, COX-2^{-}KI females were found to have increased branch length within the prefrontal cortex and olfactory bulb. Table 4 serves as a summary to all findings from this section. We provide evidence here that the COX-2 disruption within our

COX-2^{-}KI model effects microglial branch lengths of males and females in a region and time specific manner.

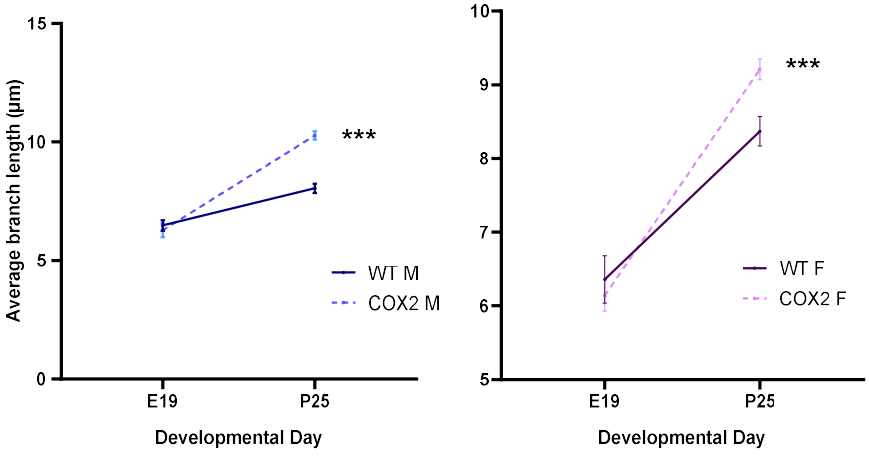
Cerebellum



Hippocampus



Olfactory bulb



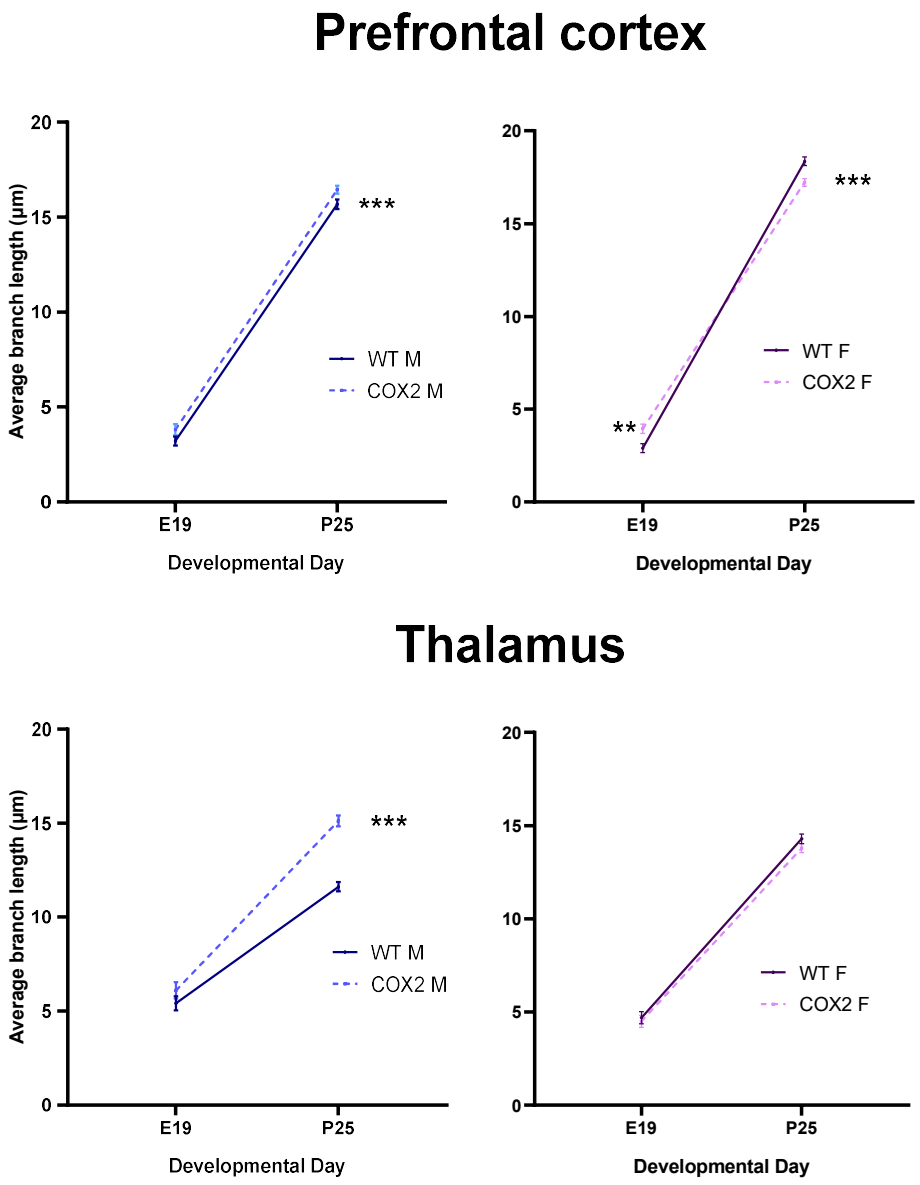


Figure 29: Progression trends for average branch length. Trends for average microglial branch length between WT and COX-2^{-}KI mice were compared over developmental time points embryonic day 19 and postnatal day 25 within our five regions of interest. Values used in these studies were obtained from the E19 and P25 average branch length studies. Data points are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001.

Table 4. Branch length in the developing COX-2^{-}KI male and female brain. A summary of average microglial branch length progression results Arrows represent increased or decreased values compared to their sex matched wildtype.

BRANCH LENGTH	COX2 M E19	COX2 F E19	COX2 M P25	COX2 F P25
Cerebellum	-	↓	↑	-
Hippocampus	-	↓	↑	-
Olfactory	-	-	↑	↑
Prefrontal cortex	-	↑	↑	↓
Thalamus	-	-	↑	-

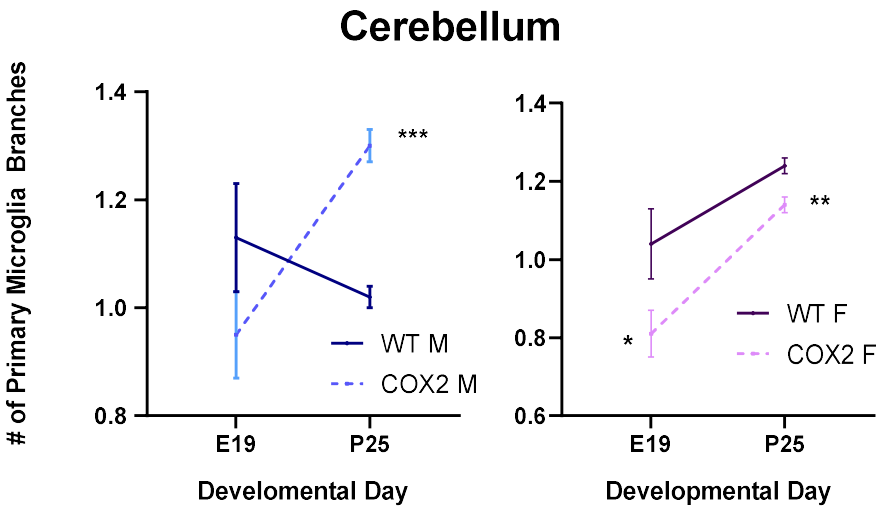
5.4.4 Sex-differences in primary branches across development of healthy and COX-2^{-} KI mice

As previously noted, microglia mature their branching networks will become progressively complex, as such we looked into differences of between E19 to P25 stages to see if the COX-2 disruption affects the progression of primary branching within our ASD experimental model. Primary branches were identified as branches extending directly from the microglial soma; all values reported are the average number of primary branches viewed per microglia.

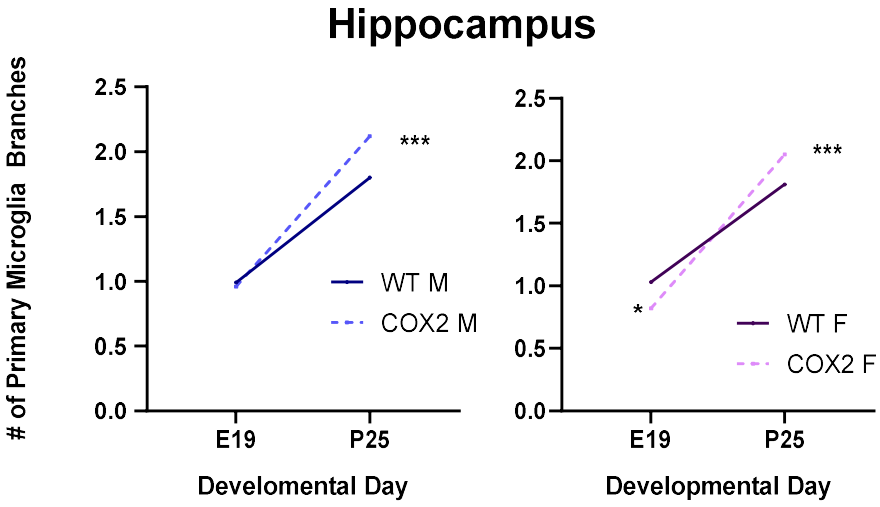
Males all showed similar effects on primary branch counts due to the COX-2 disruption, having greater rates of microglial branch growth over development, indicated by greater branch lengths at the P25 stage (CB: $p<0.001$; HPC: $p<0.001$; OB: $p<0.001$; PFC: $p<0.001$; TH: $p<0.001$) (Figure 30). Females were found to have more complex trends compared to males, with differences seen within the cerebellum, hippocampus and olfactory bulb. Prenatally at E19 we saw that cerebellums of the COX-2^{-}KI females had decreased counts of primary branches compared to the WT, a trend which was maintained at P25 (CB E19: $p=0.039$; CB P25: $p=0.003$). Showing that the COX-2 disruption decreased the amounts of primary branches in the

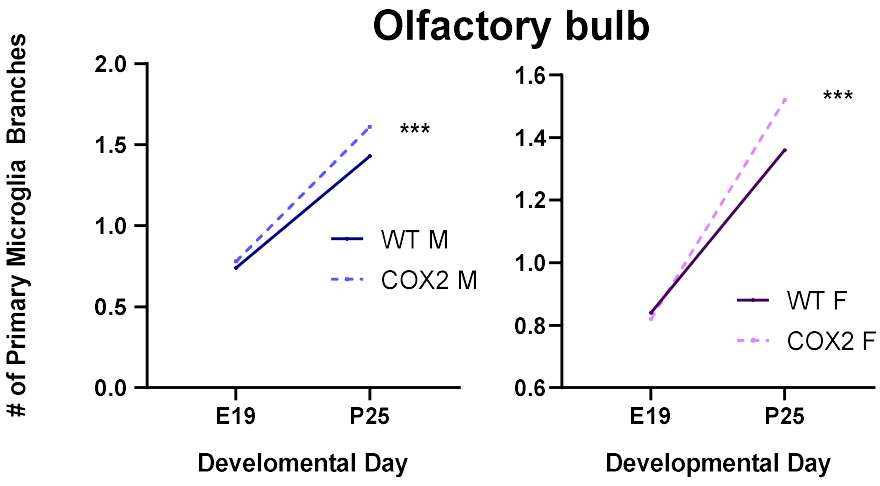
female cerebellum over development. The female COX-2^{-}KI hippocampus was also seen to deviate from WT trends, appearing to have lower primary branching counts at E19, but showing increased values by P25 (HPC E19: p=0.047*;* HPC P25: p<0.001). This suggests that the COX-2^{-}KI mouse had increased rates of primary branch production across development, as despite the deficit early in life, they surpassed the WT counts postnatally. The final change we noted in terms of primary branch counts was seen postnatal olfactory bulb, where the COX-2 disruption resulted in increased primary branch production over development. This is due to the increased primary branch counts seen at within the P25 COX-2^{-}KI females compared to the WT (OB: p<0.001).

Overall it was found that the COX-2 disruption had an impact on the trajectory of microglial primary branch counts in both males and females. Male COX-2^{-}KI animals were found to have increased primary branches within the all five regions of interest postnatally compared to WT males. Female COX-2^{-}KI females were seen to have decreased branch lengths compared to their sex-matched WTs prenatally within the cerebellum and hippocampus, and postnatally within the prefrontal cortex. Interestingly prenatally, COX-2^{-}KI females were found to have increased branch length within the hippocampus and olfactory bulb. A summary of all findings from this section can be found in table 5. We provide evidence for the first time here that the disruption of COX-2 effects microglial primary branch counts of males and females in a region specific, and time specific manner.


Cerebellum
# of Primary Microglia Branches
1.4
1.2
1.0
0.8
***
WT M
COX2 M
E19
P25
Develomental Day
1.4
1.2
1.0
0.8
0.6
**
*
WT F
COX2 F
E19
P25
Developmental Day

*


Hippocampus
# of Primary Microglia Branches
2.5
2.0
1.5
1.0
0.5
0.0
***
WT M
COX2 M
E19
P25
Develomental Day
2.5
2.0
1.5
1.0
0.5
0.0
***
*
WT F
COX2 F
E19
P25
Developmental Day


Olfactory bulb
# of Primary Microglia Branches
2.0
1.5
1.0
0.5
0.0
***
WT M
COX2 M
E19
P25
Develomental Day
1.6
1.4
1.2
1.0
0.8
0.6
***
WT F
COX2 F
E19
P25
Developmental Day

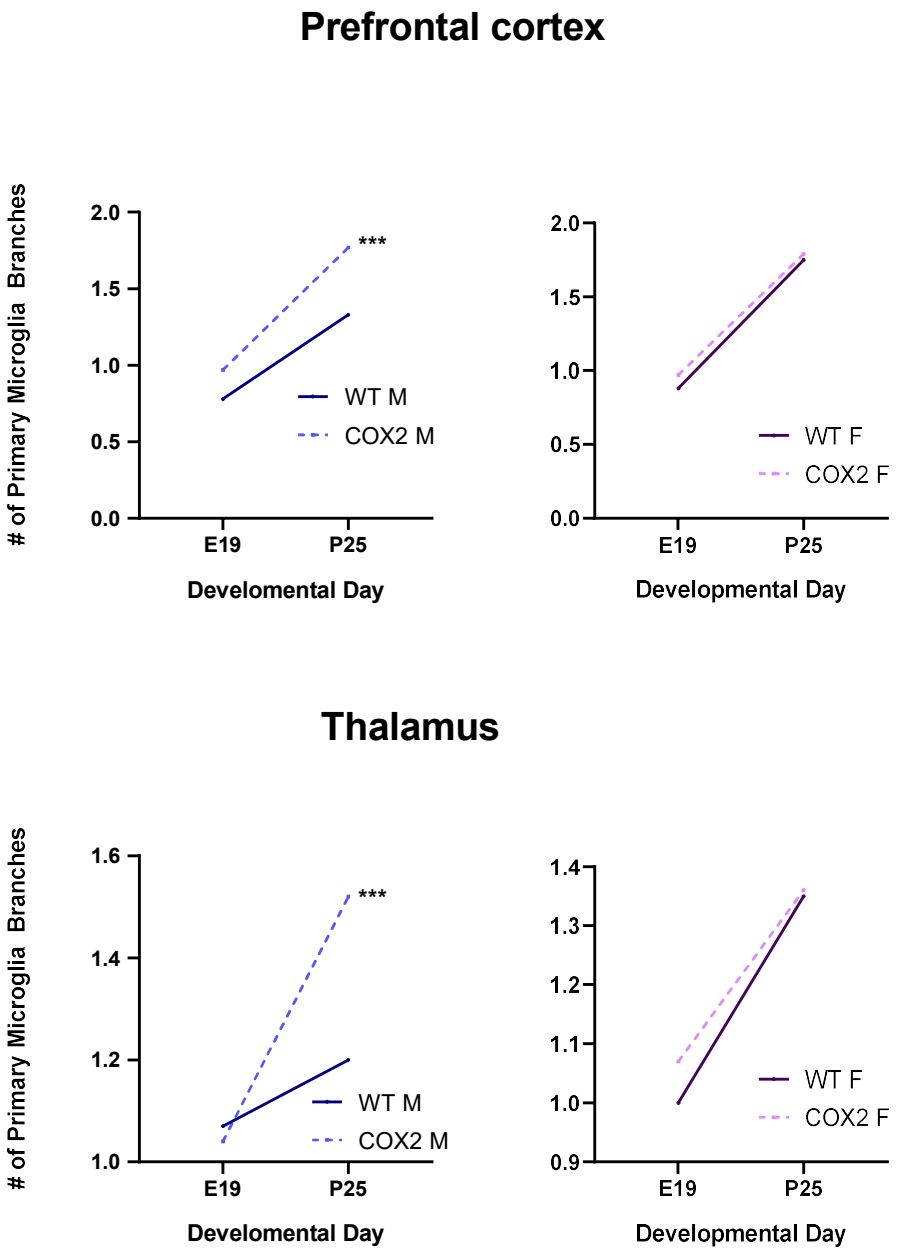


Figure 30. Progression trends for primary branch counts. Trends for primary microglial branch counts between WT and COX-2^{-}KI mice were compared over developmental time points embryonic day 19 and postnatal day 25 within our five regions of interest. Values used in these studies were obtained from the E19 and P25 primary branch count studies. Data points are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001

Table 5: Primary branches in the developing COX-2^{-}KI male and female brain. A summary of primary microglial branch counts percentage progression results. Arrows represent increased or decreased values compared to their sex matched wildtype.

PRIMARY BRANCHES	COX2 M E19	COX2 F E19	COX2 M P25	COX2 F P25
Cerebellum	-	↓	↑	↓
Hippocampus	-	↓	↑	↑
Olfactory	-	-	↑	↑
Prefrontal cortex	-	-	↑	-
Thalamus	-	-	↑	-

5.4.5 Sex-differences in total branches across development of healthy and COX-2^{-} KI mice

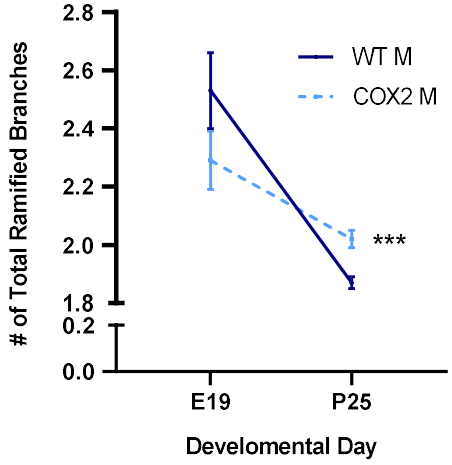
Total branches of ramified cells were examined between our two time points as branches of ramified microglia have been found to be increasingly important to overall brain development and maintenance. As mentioned in our primary branch progression section, as the brain develops microglia are anticipated to obtain a more complex branching pattern, that will be demonstrated through not only primary branches, but also by secondary and tertiary branches.

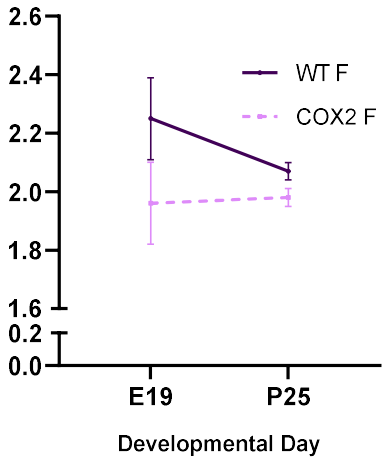
Total branch counts in all regions for the males were seen to be affected by the COX-2 disruption, resulting in increased total branch production indicated by greater total branches at the P25 stage (CB: $p<0.001$; HPC: $p<0.001$; OB: $p<0.001$; PFC: $p<0.001$; TH: $p<0.001$) (Figure 31). Females showed more complex effects to the brain due to the COX-2 mutation, with significant deviations from the WT found in the hippocampus, olfactory bulb, and prefrontal cortex. The female hippocampus was found to be affected by the COX-2 disruption resulting in decreased total branch production within the COX-2^{-}KI mouse, demonstrated by decreased counts at E19 and P25 compared to the WT (E19 HPC: $p<0.001$, HPC P25: $p<0.001$). This

difference in total branches suggests a decreased total branch production within the COX-2^{-}KI females in the developing brain. Postnatally we see that the female olfactory bulb was affected by the COX-2^{-}KI disruption, resulting in an increased rate of total branching production, where counts of total branches were increased in the COX-2^{-}KI mouse compared to the WT at P25 (OB: $p<0.001$). The final difference we saw within the female thalamus, with the COX-2^{-}KI mouse having an apparent decreased rate of total branch production compared to the WT female. While values were similar at our embryonic time point, by P25 the COX-2 disruption resulted in an overall decrease in total branch counts compared to its sex matched control (TH: $p<0.001$)

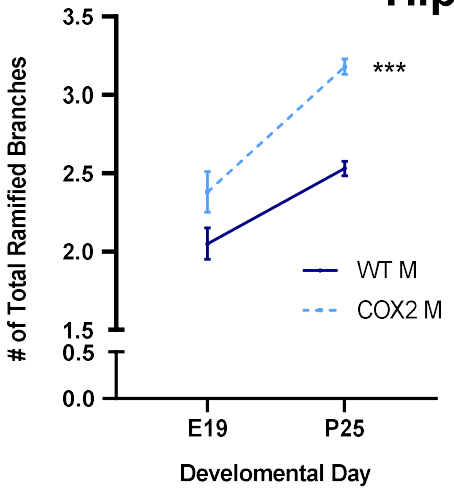
Overall, we demonstrate that the COX-2 disruption had an impact on the developmental trajectory of microglial total branch counts in both males and females within different regions and different developmental periods. Male COX-2^{-} KI animals were found to have increased total branch counts within the all five regions of interest postnatally compared to WT males. COX-2–KI females were seen to have decreased branch lengths compared to their sex-matched WTs prenatally within the hippocampus across development. Interestingly within the olfactory bulb we see an increase in total branch production. A summary of the differences due to the COX-2 disruption reported in this section can be found in table. We provide evidence for the first time that the disruption of COX-2 effects microglial total branch counts of males and females in a region specific, and time specific manner.

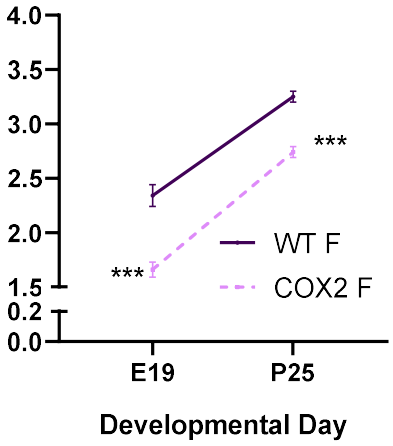
Cerebellum



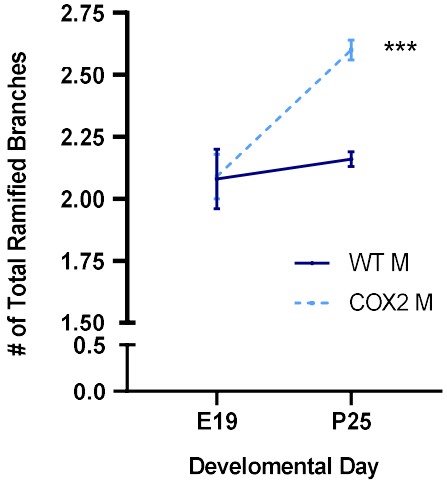


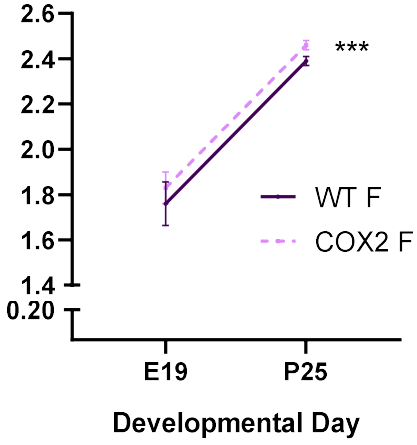
Hippocampus





Olfactory bulb





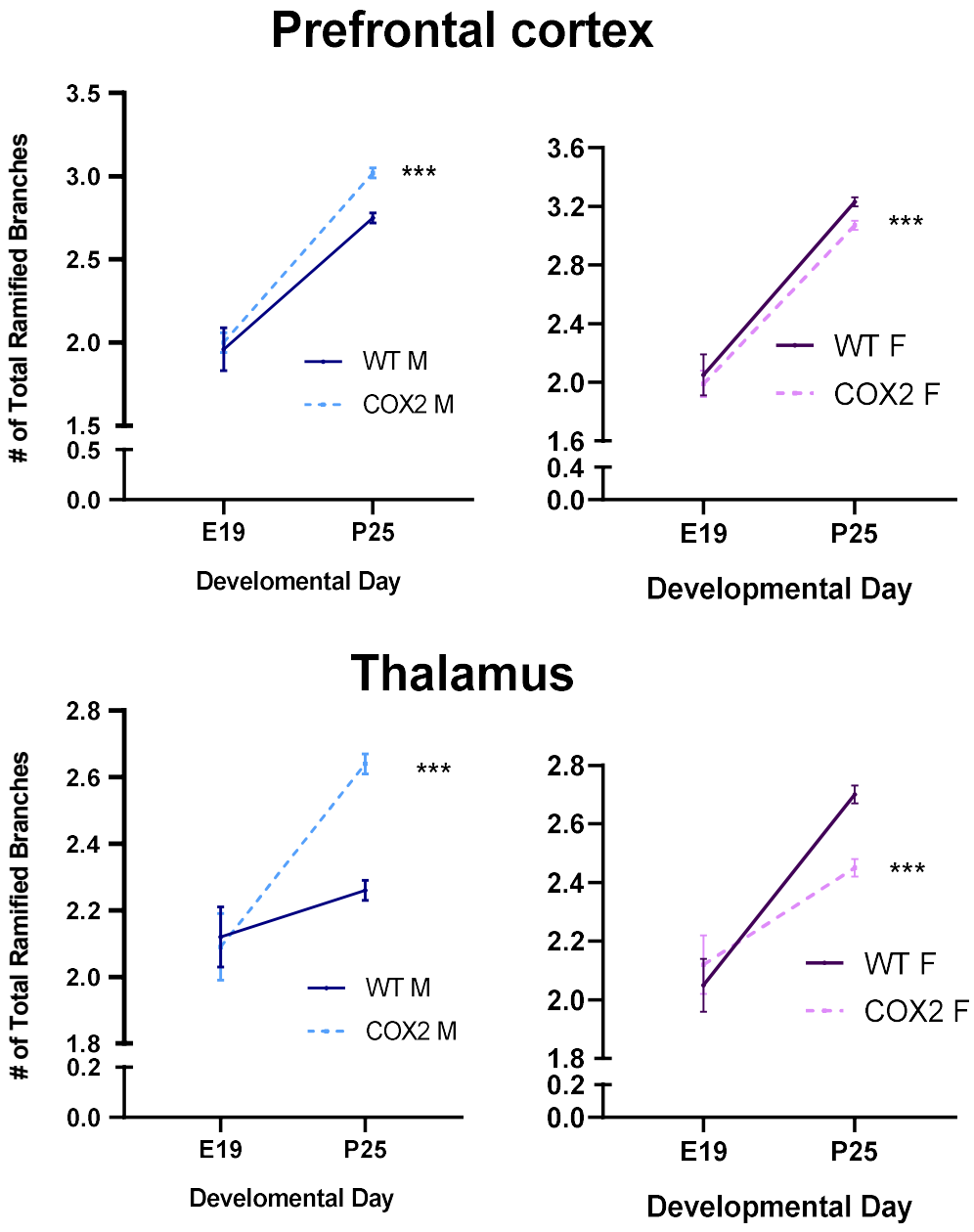


Figure 31: Progression trends for total branch counts. Trends for total microglial branch counts between WT and COX-2^{-}KI mice were compared over developmental time points embryonic day 19 and postnatal day 25 within our five regions of interest. Values used in these studies were obtained from the E19 and P25 total branch count studies. Data points are presented as mean ±SEM, *p<0.05, **p<0.01, ***p<0.001

Table 6: Total branches in the developing COX-2^{-}KI male and female brain. A summary of total microglial branch counts percentage progression results. Arrows represent increased or decreased values compared to their sex matched wildtype.

TOTAL BRANCHES	COX2 M E19	COX2 F E19	COX2 M P25	COX2 F P25
Cerebellum	-	-	↑	-
Hippocampus	-	↓	↑	↓
Olfactory	-	-	↑	↓
Prefrontal cortex	-	-	↑	↑
Thalamus	-	-	↑	↓

6. Discussion

ASD is an increasingly relevant disorder within our society that has still has much left to be answered concerning its etiology. Work completed by our lab looks to answer some of these questions through demonstrating the importance of lipid signaling in the healthy development of the brain, and its connection to pathologies of ASDs (Tamiji and Crawford, 2010b; Wong et al, 2015; Rai-Bhogal et al, 2018). In particular, we focus on PGE2, the most abundant lipid signaling molecule within the brain. Mounting evidence suggests that its abnormal levels is linked to ASD. Levels of PGE2 can be influenced by both genetic and environmental factors such as diesel exhaust, pesticides, and drugs such as aspirin or misoprostol and have been associated with ASD (Wong and Crawford, 2013; Wong et al, 201; Chang et al, 2018; Zablotsky et al, 2015).

Our lab has demonstrated that both the increased and decreased levels of PGE2, resulting from maternal exposure to PGE2 during critical prenatal period (PGE2-injected) or the lack of PGE2 producing enzyme (COX-2^{-}KI model, also used in this study), results in molecular and behavioral changes previously implicated to ASD pathologies. This current study stems from work completed in the P8 stage of PGE2-injected and COX-2^{-}KI offspring (Wong et al, unpublished) that showed significant effects based of the irregular levels of PGE2. PGE2 exposed mice exhibited higher microglial density, greater active microglia and lower ramified microglia, complemented by decreased branching and process length. While the COX-2^{-}KI mice also showed increased density compared to the healthy brain, they showed decreased active microglia and increased percentages of ramified microglia, paired with increase branching lengths and process counts.

In this study, we examined for the first time five different regions of the brain at E19 and P25 previously implicated in ASD in order to assess the effect of the PGE2 pathway on

microglia morphology and whether the effect is sex-dependent. Literature demonstrates that PGE2 can influence microglia morphology between active and ramified (Quan et al, 2013; Chen et al 2018). In this study, we hypothesized that within our regions of interest microglial density, activity and branching behaviors would be dysregulated based in the brain of COX-2^{-}KI offspring as our established ASD model. We show for the first time to our knowledge the effect of abnormal COX-2/PGE2 signaling during a prenatal and postnatal time point on microglial density and morphology at E19 and P25. We observed differences in microglial density, active and ramified morphology percentages, microglial branch length, as well primary and total branch counts within our COX-2^{-}KI model. Below we discuss the effects of the disrupted COX-2/PGE2 pathway on microglia, the potential pathological implications for the development and maintenance of the brain, and the overall implications to ASD.

6.1 COX-2^{-} KI mice demonstrate increased microglial density over development

We began by examining microglial density within the E19 and P25 mouse brain within our regions of interest, selected based on their associations to ASD. To our knowledge this is the first time these time points have been examined within any of the given brain areas, in both the healthy WT and COX-2^{-}KI mouse. We found sex differences in microglial density within the WT mice, demonstrating that postnatally males had greater microglial density compared to females in the hippocampus and olfactory bulb. These sex differences were not maintained for either region of the COX-2^{-}KI mouse. This is likely due to the significant increase in density in the COX-2^{-}KI females compared to the WT control within the hippocampus, and that within the olfactory bulb we found a decrease in COX-2^{-}KI male densities compared to the control. The changes in the olfactory bulb were not significant. These findings suggest that the COX-2/PGE2 pathway may play a role within the sex-differences we found in the postnatal brain in a region-specific manner.

When looking to the effect on microglial density due to the disruption of COX-2 compared to the WT, we found in a region, developmental stage and sex specific manner, that the COX-2^{-}KI mice had increased microglial density. Specifically, we found within the prenatal brain at E19 the female olfactory bulb had greater density values compared to the WT female. At P25 we found that the male COX-2^{-}KI cerebellum and prefrontal cortex, and female COX-2^{-}KI hippocampus all showed significant increases compared to their sex-matched control. In summary, this implicates that the lack of the COX-2 enzyme results in increases in microglial density in a sex, time and region-specific manner.

In the healthy brain microglia are well spread-out throughout all regions, with varying densities from one region to the next. (Lawson et al, 1990; Yang et al, 2012**)**. Within our density study we found for the first time for these developmental time points that each region we studied presented differences in microglial density from one area to the next, following the trends of previously shown in literature (Lawson et al, 1990; Yang et al, 2013; Keller et al, 2018).

Alterations of microglial density from healthy brains have been found in a variety of diseases and disorders and are recognized as a sign of pathology (Furube et al, 2018). Typically, microglial proliferation in both the adult rodent and human brain is slow with a stable density maintained throughout adulthood (Askew et al, 2017). Increased microglial density has been noted in a variety of pathologies in the brain such as traumatic brain injuries, prion diseases, schizophrenia, Alzheimer's disease, and ASD in humans (Uranova et al, 2018; Morgan et al, 2010; Tetreault et al, 2012). Post mortem studies of human ASD patients have shown increased microglial density in a variety of areas including the prefrontal cortex and cerebellum (Vargas et al, 2005; Morgan et al, 2010; Suzuki et al, 2013). The increase in microglial density poses concern to the developing brain as studies have correlated the disruption of neural progenitor

proliferation as well as the inhibition of neuronal differentiation to increased microglial densities (Smith et al, 2014; Appel et al, 2018, Cunningham et al, 2013). The exact mechanisms are not yet known for how the increase in density directly results in such abnormalities in progenitor proliferation and neuronal differentiation, although it has been suggested that the phagocytic activity of microglia and the wide range of soluble factors microglia can secrete may play a role (Appel et al, 2018; VanRyzin et al, 2018).

Interestingly, while we found an increase in density beyond the healthy brain in both sexes of the COX-2^{-}KI animals, we saw greater changes in the females. Within the healthy brain we typically found that males had greater density than females, but in our COX-2^{-}KI model the exaggerated density values beyond the healthy brain were similar between the sexes. This may relate to the Extreme Male Brain (EMB) theory of autism (Ferri et al, 2018). EMB is the theory that the "autistic brain" is identified by having more extreme versions of the typical male brain, exhibited through differences in brain morphology seen in both males and females. This agrees with our findings in the male brains as well, where COX-2^{-}KI mice exhibited increased density compared to the control. A relationship between the masculinization of the brain and the COX-2/PGE2 pathway has been demonstrated in the past (Amateau and McCarthy, 2004; Wright et al, 2008). It has been found that in the healthy male mouse brain that during development there is an upregulation of the COX-2 enzyme, which will ultimately result in high levels of PGE2 (Amateau and McCarthy, 2004). As well, new born female mice when injected with PGE2 were found to display male sexual behaviors, implying the importance of the COX-2/PGE2 pathway to the masculinization of the brain.

One possible explanation for the increases in microglia density we demonstrate across development is the long-term effect of PGE2 on microglia recently discovered. While PGE2 is a

known microglial activator, discussed in further detail in the next section, it has been recently reported that PGE2 also induces apoptosis in microglia after stimulation in cell models (Nagano et al, 2014; Fu et al, 2015; Yang et al, 2006). In particular, the prostaglandin E2 receptor (EP2) was found induce apoptotic effects on the cell once activated, while EP1, EP3 and EP4 had no effect on cell viability (Fu et al, Nagano et al, 2014). The downstream effect of PGE2 activation of EP2 is increased intracellular levels of cyclic monophosphate (cAMP) and is believed to induce caspase-3 activity in microglia resulting in apoptosis (Narumiya et al, 1999, Fu et al 2015). Due to the disruption in the production of PGE2 in our COX-2$^-$KI model we can speculate that decreased EP2 activation potentially results in less activity-driven apoptosis within our ASD mouse model.

6.2 COX-2$^-$ KI mice generally demonstrate decreased active and increased ramified morphologies in the developing brain

We next examined the differences in microglial morphology within the E19 and P25 brains of our COX-2$^-$KI and WT mice. Microglia function is heavily tied to its morphology, with active cells having large round somas and few extensions, and ramified microglia exhibit small somas with large branching networks (Fernandez-Arjona et al, 2017). Microglia transition between the active and ramified morphology, taking on a given form based on the stimulus the cell receives, either influencing into an active or ramified state. As we have classified microglia as either active or ramified, trends seen within one morphology will be reversed for the other. For example, the decrease in active microglia over development we see is accompanied by an equivalent increase in ramified microglia.

The prenatal brain at E19 demonstrated no differences between males and females in our studies on the healthy brain. Interestingly within the COX-2$^-$KI cerebellum studies we demonstrate that females have higher levels of microglial activation compared the males. This

change in sex-differences only appearing in our mouse model is likely attributed to the increase in active morphology percentages we determined in the female COX-2^{-}KI compared to the WT female. Accordingly, we see that males have greater levels of ramified microglia compared to females within the COX-2^{-}KI cerebellum at this time, with differences attributed to lower levels of ramified microglia found within the COX-2^{-}KI females.

Postnatally we see sex differences only within the WT prefrontal cortex, with males having greater levels of active microglia compared to females. This was complemented by increased ramified morphologies within the WT female compared to the male within the region. One of the well-established sexual-dimorphisms between male and female microglia is that males are commonly found to have a more active morphology than females (Villa et al, 2018). These sex-differences concerning percentages of active morphologies further implicate the COX-2/PGE2 pathway in the sexual differentiation of the brain.

In general, we saw that the disruption of COX-2 resulted decreased levels of microglial activation in a sex and region-specific manner across development. In particular, we demonstrate for the first time that within the prenatal brain at E19, the female prefrontal cortex was seen to have decreased counts of active microglia while interestingly the female cerebellum had increased counts of active morphologies compared to their sex-matched controls. Postnatally at P25 we observed that within the male cerebellum, olfactory bulb and prefrontal cortex, as well as the female thalamus, that the disruption of COX-2 resulted in decreased active morphology percentages.

Studies have shown that COX-2 is a known mediator of microglial activation, and when removed or inhibited decreased levels of active microglia have been seen in mouse models, aligning with our findings (Vijitruth et al, 2006). PGE2, the major product of COX-2 activity,

plays a critical role in microglial activation through the activation of the microglial EP2 receptor. The activation of this receptor increases intracellular cAMP and induces migration and phagocytic properties in the microglia, both of which being key functions of microglia (Nagano et al, 2014). Thus, within our ASD model we are reducing the amount of PGE2 within the brain we anticipated finding the decrease in microglial activation as we did.

Surprisingly, we found an increase in active microglia morphologies within the prenatal female cerebellum at E19, but by P25 no differences were found. While highly theoretical, this may be a hint at the female cerebellum trying to compensate for the COX-2 disruption. Haida et al (2019) used a component of the gram-negative bacterial outer membrane, known as a lipopolysaccharide (LPS) to stimulate an infection model within mice. In their study they found that females exhibited less abnormal motor behaviors and cellular effects within the cerebellum, compared to males in this study complementing the theory. Further studies would be needed in order to determine if the female cerebellum did have a protective function or not.

Within the developing brain only recently have microglia come to be recognized as important factors for the its healthy development, with focuses on the diffusible factors and phagocytotic properties characteristic of active microglia (Lenz and Nelson, 2018). As previously mentioned, microglia have been found within the last few years to assist in neurogenesis, myelination, and inducing cell death or survival as well as assisting in synaptic maturation, among other roles (Paolcelli et al, 2011; Lenz and Nelson, 2018). These are processes implicated in developmental dysregulation associated with ASD (Gilbert and Man, 2017; Graciaerna et al, 2019).

As the brain develops it creates approximately 50% more neurons than will be seen in the adult brain, indicating the need for the proper regulation of synaptic pruning. In

neurodevelopmental disorders such as ASD, diagnosed individuals commonly show lower levels of pruning of these synapses compared to controls (Neniskyte and Gross, 2017; Tang et al, 2014). As active microglia are known to be involved in synaptic stripping and influencing cell death of excess neurons, their importance in this process was highly suggested. Several studies have recently proven that microglia are indeed crucial in this pruning in both mouse and human models (Tang et al, 2014; Courchesne et al, 2011, Paolicelli et al, 2011). The lack of pruning during development has been shown in mouse models to affect the ability to learn, defects in social behaviors and increased repetitive behaviors, which are common in ASDs (Kim et al, 2017; Afroz et al, 2016). As well a common trait of ASD individuals is synaptic overconnectivity of the brain, which could relate to the decrease in activity we demonstrate within our COX-2^{-}KI model (Keown et al, 2013).

Other potential implications of the decreased levels of activated microglia in our COX-2^{-} KI are the altered levels variety of pro-inflammatory cytokines to be released (Masi et al, 2017). Studies have shown that humans with ASD had cytokine levels that differed in the blood, brain and CSF samples compared to healthy controls, including decreases levels of IL-1B, IL-8, MIP-1B and VEGF (Masi et al 2017; Xu et al, 2015). These cytokines play important roles in the brain such as cellar defense, tissue repair, and within the inflammatory pathway (Hewett et al, 2012). Interestingly, it has been noted as well that ASDs can result in increased levels of pro-inflammatory cytokines as related to increased levels of activated microglia (Rodriguez and Kern, 2011). These findings fit nicely to the findings that our lab has demonstrates in that both an increase or decrease in PGE2 levels within the brain are related to ASD pathologies.

While a multitude of information can be found concerning activated microglia as it is what is most commonly associated with pathologies in the brain much is left to be desired about

the impact that ramified microglia have on the brain. Within the adult mouse brain, it was shown that branches of ramified microglia are continuously moving around the brain surveying their environment (Nimmerjahn et al, 2005). Considering that this discovery was reported in 2005, where microglia were first discovered in 1932, it indicates that little focus has been given to the ramified morphology over the years (del Rio-Hortega, 1932).

Microglia are known to interact with neurons within the ramified state, as previously mentioned, showing that they influence synaptic activity as well as synaptic pruning. The importance of ramified microglia and their processes interacting with neurons have been found to play a key role in synaptic pruning (Tremblay et al, 2010). Wake et al, (2009) demonstrated that microglia branches do not randomly contact synapses within the healthy mouse brain, which have been demonstrated to be due to neuronal activity (Wake et al, 2009; Tremblay et al, 2010). Microglia can not only phagocytize synapses but are also important in the maturation of synapses and in decreasing neuronal activity (Li et al, 2012; Miyamoto et al, 2013). These similar studies emphasize the new-found importance of ramified microglia cells, but further investigation is still required to demonstrate the underlying mechanisms.

6.3 Increased branch length in COX-2^{-}KI mice over development

Microglial morphology provides clues to the cell's biological function, having drastically altered morphological states between active and ramified classified cells as previously mentioned (York et al, 2018). Shape measurements of microglia, including branch length and number of branches, are commonly used to differentiate healthy microglial trends from those associated with pathologies (York et al, 2018). Here we demonstrate that the disruption of the COX-2 enzyme in the developing mouse brain generally caused an increase in microglial branch length in a sex and region-specific manner, within the E19 and P25 mouse brain.

Within the prenatal brain at E19 we specifically observed differences only within the female brains of the COX-2^{-}KI animals compared to the WTs, with increased branch lengths noted in the prefrontal cortex, but decreased within the cerebellum and hippocampus. Postnatally males were predominately affected, showing increased branch lengths within all five regions of interest when compared to the WT male. We found that P25 COX-2^{-}KI females only showed significant differences from the WT in terms of branch length in the olfactory bulb and prefrontal cortex. The COX-2^{-}KI female olfactory bulb had greater branch lengths, while interestingly the prefrontal cortex lengths were decreased compared to the WT female.

As our COX-2^{-}KI model has the *Ptgs2* mutation, resulting in a disruption of PGE2 production compared to the WT, we initially anticipated that we would find increased branch lengths within the model mouse. This is because PGE2 is a activates microglia, which is typically morphologically coupled with short branch lengths (Morrison and Filosa, 2013; Stence et al, 2001). While this is the trend seen within all males that reported differences, we found two regions at E19 and one region at P25 for females had decreased branch lengths compared to the WT. The decrease in branch lengths seen within the female cerebellum compliments the previously discussed findings of increased microglial activation percentages at E19. Microglia are highly sensitive to their local environment, and individual brain regions have been shown to have differences in gene expression, this may explain why we see differences in branching patterns across the different areas (Grabert et al, 2016). These changes in gene expression may be a potential explanation for these changes we see in brain, although further experiments such as a regional gene expression study, would be needed for further clarification.

Another possible reason that we may see less effects within the E19 stage mouse brain compared to the postnatal is that at this point microglia are still in the process of maturing

(Matcovitch-Natan et al, 2016). The concept of microglia developing in the brain to become "mature microglia" as noted by Matcovitch et al (2016) is quite new, and there have been few follow up studies at this point. It has been demonstrated that there were differences in microglial gene expression at during the different developmental points of microglia and we unfortunately have no current data which can confirm the levels of the e-prostanoid receptors on these immature microglia. It could potentially assist in explaining the fewer differences seen overall within our E19 mice across all microglial studies we completed based on the COX-2 disruption.

We found as well that the postnatal male brain at P25 in all regions of interest that microglial branch length significantly increased in the COX-2^{-}KI mouse. Within the developing mouse the male brain differentiates itself from the female based on the surge of testosterone from the fetal gonads as previously mentioned (McCarthy and Nugent, 2015). This surge in testosterone was found by Lenz et al (2013) to be extremely important for PGE2 production in the male brain, leading to the understanding of the prostaglandin's role in sex differences noted within the brain. It was demonstrated that microglia are also extremely important in the masculinization process, as when microglia are activated by estradiol, the aromatized version of testosterone seen within the mouse brain they will begin PGE2 production themselves. This creates what Lenz et al (2013) call a "positive feedforward" mechanism, where microglia maintain a sustained production of PGE2 within the brain based on their activated state. Microglial inhibition resulted in less masculine traits within the rat brain, which lines up nicely with our findings, as longer microglial branch lengths are more typical of females.

Microglial activation is associated decreased branch lengths, where we demonstrate and discuss earlier that the COX-2 disruption resulted in increased ramified morphologies in our studies, the general increase we observe in branch length completement our previous findings.

We discuss the implications to the brain based on differences observed in branch length, primary branches, and total branches together in later sections.

6.4 Increased primary branches in COX-2^{-}KI mice over development

As mentioned in the previous section, changes in primary branch counts are commonly quantified in order to examine differences in microglia from a heathy control to a model of pathology. In our studies of the mouse brain at E19 we again saw differences only within the female brains of the COX-2^{-}KI animals compared to the WTs as we did in branch length studies. We saw that within the female cerebellum and hippocampus that there were decreased primary branching counts within the COX-2^{-}KI model compared to the WT, complementing the decreases in branch length we saw. Postnatally males were predominately affected, showing increased primary branch counts within all five regions of interest when compared to the WT male. We found that P25 COX-2^{-}KI females showed significant differences from the WT in primary branch counts in the cerebellum, olfactory bulb and prefrontal cortex. The COX-2^{-}KI female hippocampus and olfactory bulb had greater branch lengths, while interestingly the prefrontal cortex lengths were decreased compared to the WT female.

The complexity of the microglial network across development has been demonstrated through our progression studies, as well as through the literature of others, to become more complex as the brain continues to mature. The increase in branch length and primary branch counts that we demonstrate within our COX-2^{-}KI mice, in both males and females in the different regions of the brain, do indicate a more ramified morphology of microglia. Ramified microglia have been shown to be of importance to overall brain health as of recent years, but their exact relevance to pathologies have only recently come into question.

Mouse and rat models of chronic-stress induced through forced swim and restraint test resulted in hyper-ramification of microglia compared to controls, showing that microglia may

have alternative responses to pathology aside from changing to the active morphology (Hellwig et al, 2016). We see within our model increases in the branching network as noted within these models as well. It remains to be elucidated how the increased branch number and branch length resulting in hyper-ramification result in pathologies within the brain (Hellwig et al, 2016).

6.5 Increased total branching in COX-2^{-}KI mice over development

Where branches of ramified microglia have been recently found to influence the synaptic activity of neurons, our final morphological measurement examined the difference in total microglial branches based on the COX-2 disruption within our mouse model. These measurements completement our studies in branch length and primary branch counts, where in general increased counts of total branches were found in the ASD model.

In our studies of the mouse brain at E19 we again saw differences only within the female brains of the COX-2^{-}KI animals compared to the WTs, in our last two microglial measurements. We saw that within the female hippocampus that there were decreased primary branching counts within the COX-2^{-}KI model compared to the WT, complementing the decreases in branch length and primary branch counts. Postnatally males were predominately affected, showing increased primary branch counts within all five regions of interest when compared to the WT male. We found that P25 COX-2^{-}KI females showed significant differences from the WT in total branch counts in the hippocampus and the olfactory bulb. The COX-2^{-}KI female hippocampus maintained a lower count of total microglial branches at the P25 stage, while the olfactory bulb had greater total branch counts.

The importance of the branching network has only come to light in recent years, where ramified microglia were thought to have no overall importance to brain maintenance, as previously mentioned. Most work concerning abnormalities in active to resting microglia percentages within pathology focus only on what the change in active microglia means to the

brain (Tay et al, 2018; Bachiller et al, 2018; Graeber and Streit, 2010; Bechade et al, 2013). This increase in the branching network, demonstrated by the increase in branch length, primary branch count, and total branch count noticed within the COX-2^{-}KI mouse, overall demonstrate a more complex branching network compared to the WT. More work needs to be done to conclusively understand the functional relevance of a more complex branching network on the brain.

It is possible that this branching network may be of importance in terms of ASD pathologies includes learning and memory, which have been found to be altered in the disorder (Boucher, 1981; Mostofsky et al, 2000; Parkhurst et al, 2013; Dawson et al, 2008). Many lines of evidence implicate that experience-dependent synaptic plasticity is important for both learning and memory, and as mentioned previously, microglia interact with synapses in an activity dependent manner (Tremblay et al, 2010; Grutzlendler et al, 2002). The fact that microglial branch dynamics are regulated by neuronal activity and are known to prune synapses, may suggest they play a role in learning (Parkhurst et al, 2013). The abnormal increase in microglial branches may speak to abnormal regulation of synaptic activity and pruning.

6.6 Sex differences in COX-2^{-} KI and healthy mouse model

Male and female microglia are known to have clear sex differences within the neurotypical brain; literature shows that males have greater active microglia percentages, typically paired with decreased branch numbers with shorter branch lengths compared to females (Lenz et al, 2012, Hanamsagar et al, 2017, Schwarz 2012). Our findings show clear sex differences within the WT mice that follow these studies as demonstrated within our progression graph studies.

Within our study of the E19 mouse brain little sex differences were noted for both the healthy and COX-2^{-}KI mouse brain, a finding complemented by the work done by Schwarz et al

(2012) who found a similar lack of sex-based differences when looking at microglia within the heathy male and female mouse brain at E17. As mentioned, microglia are very sensitive to their environment, and the lack of sex-dependent changes may be based on the fact that in mice testosterone is known to spike within the brain at E18, peaking at E20, and concludes on the day of birth (Weisz and Ward, 1980). In mice the brain is still considered to be in a critical period, where microglia are sensitive to the programming effects of hormones (Lenz and Nelson, 2018). This window of a critical period could explain why we see no sex differences within our E19 study. Interestingly within all of our measurements we see overall that males are more effected by the COX-2 disruption based on the amount of deviations noted from the WT compared to the females. This may speak to large sex-biasing that is seen within ASD.

6.7 Developmental differences due to COX-2$^-$ disruption

We investigated the effect of the COX-2 disruption on microglia density and morphology within the E19 and P25 stage mouse brain and compiled the data into progression trajectories to better view the overall effect. Interestingly, it has been noted that microglia within ASD individuals are maturing faster than healthy age matched controls (Hanamsagar et al, 2017). Hanamsagar et al (2017) generated a microglial developmental index (MDI) in mice used to identify microglial developmental gene expression. They then applied this index to a human brain transcriptome dataset to examine for abnormalities the MDI within various neurological pathologies. They found that the MDI was altered in both Alzheimer's disease and ASD, suggesting the acceleration of microglial development within these pathologies. Interestingly Werling et al (2016), using the same data set, showed that a variety of autism-susceptibility gene (which were largely microglial specific) were upregulated significantly in adult males compared to females. This is of particular significance as males are more likely to be diagnosed with ASD compared to females, at a rate of 4:1. The concept of hyper ramification is shown in our work

through the increases in branch length as well as the total counts of primary and total branches, indicating a more complex branching network compared to the WT mouse.

Our progression trends show that within the developing brain that the COX-2 disruption resulted in altered projections in microglial density and morphology from the WT brain for both males and females. This was shown to occur in a region-specific manner. The differences that are determined region to region, as well within the different sexes, may speak to the fact that ASD is a spectrum disorder and that there are a wide variety of behavioral characteristics associated between males and females of those affected (Frazier et al, 2014; Lord et al, 1981). Recent behavioral studies completed in the COX-2^{-}KI mouse by Wong et al (2018) found that while both COX-2^{-}KI sexes showed greater hyperactivity compared to the sex-matched WT, COX-2^{-}KI males showed increased hyperactivity compared to their female counterparts. This is similar to traits we see of ADHD we see in males that have ASD compared to females (Willcutt, 2012). In marble burying tests, used to assess anxiety-linked and repetitive behaviors, it was seen that both the COX-2^{-}KI exhibited increases in the two behaviors, but males demonstrated increased anxiety-like and repetitive behaviors through increased marble burying. This increase in these behaviors in males has been noted within the ASD-affected human population as well (Evans et al, 2018; Supekar and Menon, 2015).

6.8 Future studies

In order to gain further knowledge about abnormalities in the COX-2/PGE2 signaling and its effect on microglial morphology in ASDs we propose that similar studies in our PGE2-injected ASD mouse model should also be conducted at E19 and P25 for comparisons. Our recent work already showed differences in microglial density, activity and branching patterns at P8 within the PGE2-injected mouse model (Wong et al, unpublished). As well we would suggest examining microglial changes at the P14 time point in mouse brain in both the PGE2-injected

and COX1-KI model. This time frame is suggested as this is the peak of microgliosis as well as where microglia are said to reach their mature form from pre-microglia (Matovich-Natan et al, 2016).

Using alternative models of PGE2 level alteration that can be applied to more real-life applications, such as acetaminophen administration and influenza A for decreased PGE2 levels, and LPS or misoprostol injections for increased PGE2 is of particular interest as well (Dudek et al, 2016; Oishi et al, 2014; Greco et al, 2003).

We were able to show that branching behaviors and activity levels were significantly different within our COX-2^{-}KI autism model, which may suggest accelerated microglial aging. Where this has been found to occur by Hanamsagar et al (2017) in ASD individuals, it would be of interest to complete gene and protein expression of microglial development markers to accompany our currently immunohistochemistry studies. In particular, Matovich-Natan et al (2016) determined that microglia follow a stepwise development, with three distinct temporal phases, known as early-, pre- and adult microglia each having their own particular markers establishing the phases. These markers could serve as candidates to see if the COX-2 disruption may be resulting in an accelerated aging process within microglia. As well the microglial developmental index created by Hanamsagar et al (2017) could serve as a data base for targets to examine the microglial aging process in our ASD model. Future expression studies for both protein and gene expression would be beneficial to complete within our specific regions of interest where the microenvironments of each region have such influence on the microglia.

6.9 Conclusion

The results of this novel study provide further support to the importance of the COX2/PGE2 pathway in regulation of microglial density and morphology in the developing brain. First, we show for the first time that in the healthy brain at E19 and P25 microglia density

and their morphological characteristics are sex-dependent and specific for different developmental stages in our regions of interest. Moreover, we also show that the disruption of the COX-2/PGE2 signaling resulted in increased microglial density, a decrease in the active morphology with a reciprocal increase in ramified morphologies, and an overall more complex branching network due to increased branch lengths, primary branch counts and total branch counts. Interestingly these microglial modifications regulated by PGE2 levels are region specific suggesting potentially diverse effects on brain function. Dysregulated microglial density has been linked to abnormalities with neural progenitors and neuronal differentiation; in human ASD studies it has been found that neuronal progenitor cells have abnormal growth rates as well as oddities with neurogenesis, which may suggest a tie between microglial density and ASD. As well the active morphology of microglia is known to assist in synaptic pruning, axonal guidance, and inflammation, three factors important for the development of a healthy brain. Additional human studies have shown ASD brains have abnormalities in all three mentioned roles of the active morphology. Ramified microglia have been recently implicated in learning and memory based on their ability to influence synaptic pruning, creating another potential link to ASD, as the dysregulation of ramified microglia could relate to the altered learning abilities within those with the disorder. Finally, hyper-ramification of microglia has been recently shown in mouse models of pathologies, such as Alzheimer's disease and chronic-stress, although the function is currently unknown. Further research is required to determine what the increased branching network we see within the ramified microglia could mean for the brain. The findings presented in this thesis complement previous post-mortem human studies, completed on the brains of those with ASDs, which found abnormal microglial activation and density and abnormalities in synaptic connections typically regulated by microglia during development. This study provides novel

molecular evidence for the role of the COX-2/PGE2 pathway in regulation of microglial morphology throughout development, and how its abnormal signaling may influence sex-dependent brain pathologies and result in ASD.

7.0 Bibliography

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