EXPLORING PERIPHERAL FACTORS IMPACTING SEXUAL DIMROPHISM OF THE BED NUCLEUS OF THE STRIA TERMINALIS

EXPLORING PERIPHERAL FACTORS IMPACTING SEXUAL DIMROPHISM OF THE BED NUCLEUS OF THE STRIA TERMINALIS

By:

Roksana Khalid, BAS (hon)

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Masters in Science

McMaster University

MASTER OF SCIENCE (2016) McMASTER UNIVERSITY
(Neuroscience Graduate Program) Hamilton, Ontario, Canada

TITLE: Exploring Peripheral Factors on Sexual Dimorphism of the Bed Nucleus of the Stria Terminalis

AUTHOR: Roksana Khalid, H.BAS (McMaster University)

SUPERVISOR: Dr. Jane A. Foster, PhD

NUMBER OF PAGES: Preliminaries: 9
Thesis: 52
Figures: 9

ACKNOWLEDGEMENTS

I would like to acknowledge Dr. Jane A. Foster for giving me the opportunity to grow. Her willingness to discuss ideas and inclusive attitude allowed me to surpass my expectations of learning and achievement in this program. I am also grateful to the graduate students who were in the lab before me, Dr. Jonathan Lai and Shawna Thompson, for taking the time to provide me with instruction and orientation when I started in the lab, and thank you to Sarra Bahna for helping near the end.

Thank you to the undergraduate members of the Foster lab as well, especially Douglas Chung and Owen Luo for contributing time towards troubleshooting procedures. I would like to thank Daiana Pogacean and Sureka Pavalagantharajah for being so organized and helpful around the lab, as well as for the pleasant chats. Also thank you to Rober Boshra and Aadil Bharwani for the intellectual discussions and coffee breaks.

I would also like to thank my family and friends in the program and outside for their support and caring.

Finally, thank you to my Alex, for everything.

Table of Contents

FIGURES:

STATEMENT OF CONTRIBUTIONS:

Dr. Jane A. Foster trained the author in tissue collection from postnatal mice. Marg Coote at the Brain Body Institute trained the author to run the AMH ELISA kits. Dr. Jonathan Lai trained the author to perform perfusions. Shawna Thompson demonstrated the immunohistochemistry procedure and collected tissue for adult microglia analysis.

All the work contributing to the collection of data, its analysis, and the collection of postnatal tissue was completed by the author.

ABSTRACT

Immune-brain-endocrine communication influences behaviour and contributes to the development of the central nervous system (CNS) in a sexually dimorphic manner. The bed nucleus of the stria terminalis (BST) is a highly sexually dimorphic brain region; in most mammalian species the male BST is larger than the female BST. Previously, our lab has shown that male and female mice lacking T cells due to knock out of the β and δ chains of the T cell receptor (*TCRβ-/-δ-/-)* have reduced anxiety-like behaviour. This was shown with increased time spent in the open arms of the elevated plus maze by *TCRβ-/-δ-/-* mice compared to wild type (WT) mice of both sexes. T cell deficient mice also show differences in brain volume compared to WT, including a lack of sexual dimorphism in volume of the BST. The present study explored the impact of T cell deficiency on immune and endocrine factors implicated in sex differences of the CNS. The first analysis was of serum Anti-Müllerian hormone (AMH). AMH is a key determinant of the male phenotype during fetal development. It has also been shown by others to contribute to sexual dimorphic development of the BST. Our postnatal analysis of serum AMH using ELISA demonstrated an age and genotype effect, where a peak in serum AMH levels in WT mice of both sexes was absent in both male and female *TCRβ-/-δ-/-* mice at postnatal day (P) 7. These results suggest that T cells have an impact on the endocrine system in early life but the process does not appear to be sexually dimorphic. The present study also explored the impact of TCR knockout on microglia, the resident

immune cells of the brain. Other have shown microglia contribute to sexual dimorphic brain development. This contribution occurs through interaction with endocrine factors, making them a key player in the immune-brain-endocrine crosstalk. Using immunohistochemistry and the microglial marker, anti-Iba1, microglia were examined in adult and P7 WT and *TCRβ-/-δ-/* mice. To quantify microglia, soma were traced using AxioVision microscope software, and microglia cell number, perimeter, radius, feret ratio, and area in dorsal and ventral BST were assessed. Our results show sex differences in microglia number in dorsal BST in adult WT mice, where female WT mice had a lower number of microglia compared to WT males, however this difference was absent in *TCRβ-/-δ-/-* adult mice. There were no effects on microglia number in the ventral BST and morphology analysis did not reveal any effects in the dorsal or ventral BST. Furthermore, the difference in microglia number was absent in all groups of P7 mice and analysis of soma morphology did not reveal any significant effects. This study explored the impact of TCR knockout on the BST by exploring the immune and endocrine factors shown to contribute to its sexual dimorphic development. The results suggest a non-dimorphic impact on the endocrine system in the postnatal period and a dimorphic impact on microglia that is age and region-specific. The findings reveal a complex network emphasizing the importance of a systems-wide approach to the study of sex differences in the CNS.

1. Introduction and Literature Review

1.1 Sexual Dimorphism

Sexual dimorphism is the difference in physical characteristics, such as size and morphology, between males and females. Sexual dimorphism in the central nervous system (CNS) is reflected in differences in brain structure between males and females and in differences in behaviour (Hedrick & Temeles, 1989). Evolutionary theories provide some explanations for dimorphic traits. Dimorphism provides advantages to males and females in carrying out their relative reproductive roles, and it could arise from competition for resources between the two sexes during non-breeding seasons (Hedrick and Temeles, 1989). Essentially, genes and environment are both at play in the establishment of sex differences.

Dimorphism in mammals is exemplified by the endocrine system. Sex differences in relation to hormones are well indoctrinated into our knowledge of sexual dimorphism, especially in humans, such that hormones are often referred to as being male- or female-typical even in colloquial conversation. In this regard, a stable testosterone level is predominantly referred to as influencing "masculinity" and cyclical releases of hormones such as estrogens are referred towards the "feminine". Alongside these dramatic differences, many disorders of the nervous system show a sex-bias in the afflicted population, e.g. the male bias in autism spectrum disorders (Werling & Geschwind, 2013), and the female bias

in multiple sclerosis (Khalid, 2014) and in anxiety disorders (Lewinsohn, Gotlib, Lewinsohn, Seeley, & Allen, 1998). This involvement of dimorphic traits in complex disease processes suggests that sexual dimorphism extends beyond one system alone and is much more complex.

Recent research from our lab and others has shown that the adaptive immune system is involved in sexual dimorphism in the CNS. The current work aims to expand our understanding of immune-CNS interaction. The following sections will first outline established knowledge regarding the development of sexual dimorphism during early developmental periods and the subsequent establishment of endocrine systems that support the expression of sex differences throughout life. In the context of this known literature, an overview of recent work demonstrating that peripheral immune signals are key players in the development and maintenance of sex differences will be provided, making the argument that they should be assessed alongside the endocrine system.

1.2 Mammalian Determination of Sex

Mammalian sex determination begins early in embryogenesis. In mammals, the female XX chromosome pair and the male XY chromosome pair determine sex. The sex-determining region Y, or *Sry,* is an intronless gene on the short arm of the human Y chromosome considered the male determining factor, that induces the development of the male gonadal and hormonal phenotype (Hacker, Capel, Goodfellow, & Lovell-Badge, 1995).

Prior to *Sry* expression, male and female embryos show gonadal ridges, composed of somatic cell precursors for gonads. Gonadal ridges are bipontential primordium, meaning they can develop into testes or ovaries (Tilmann and Capel, 1999). Both XX and XY embryos also contain Wolffian ducts that form from the intermediate mesoderm and Müllerian ducts that elongate from the mesonephros to the urogenital sinus. Downstream events lead to the persistence of Müllerian ducts in females that later differentiate into the uterus, cervix, uterine tubes, and upper vagina. The Müllerian ducts degenerate in XY males (Orvis and Behringer, 2007) but the Wolffian ducts persist and differentiate into the epididymis, vas deferens, and seminal vesicles (Brambell, 1927; Hannema & Hughes, 2007).

The differentiation process in the mouse embryo starts at 9 days post coitum (d.p.c.) and by 12 d.p.c. the gonads are differentiated into developing ovaries or testes (Brambell, 1927). Investigation of events in mouse gonadal ridges has shown that the high expression of *Sry* at 11.5 d.p.c. for 24 hours determines the development of testes by activating the expression of *Sox9* (Sekido, Bar, Narvàez, Penny, and Lovell-Badge, 2004). *Sox9* activity leads to the alignment of epithelialized Sertoli cells necessary for further gonadal development (Koopman, Münsterberg, Capel, Vivian, & Lovell-Badge, 1990). Increase in *Sry* activity is closely followed by the expression of *Amh*, the gene for anti-Müllerian hormone (AMH), also known as Müllerian inhibiting substance. AMH is secreted from Sertoli cells and it is upregulated 20 hours after the onset of *Sry* activity (Hacker et al., 1995). AMH activity further contributes to gonadal

differentiation by inducing regression of the Müllerian ducts, creating the male gonadal phenotype, and its expression persists into the early postnatal period in males (Al-Attar et al., 1997; Greco, 1993).

In XX females, gonadal differentiation follows a different trajectory, which is slower than in XY mice. There is an absence of Sertoli cells; instead the bipotential somatic precursor cells in XX females differentiate into granulosa cells (Krentz, Murphy, Sarver, Griswold, Bardwell, and Zarkower, 2011). Between embryonic day (E) 11.5 and E12.5 the Müllerian ducts develop in XX mice (Hannema and Hughs, 2006). At 12 d.p.c. the ovary is still undifferentiated and is composed of epithelial and germ cells. It matures until approximately 7 weeks post-partum, when puberty is attained (Brambell, 1927). AMH levels have not previously been reported as occurring in significant levels in embryonic females, however low levels expressed by granulosa cells have been found at the end of fetal life and in the neonatal period (Hazout et al., 2004).

Around the time when gonadal differentiation is occurring, various regions determined to be sensory, limbic, and motor structures of the brain are undergoing peak neurogenesis (Finlay & Darlington, 1995; Hannema & Hughes, 2007; Sekido, Ryohei; Bar, Isabelle; Narvaez, Veronica; Penny, 2004). The balance of neurogenesis and neuronal death during development establishes sexual dimorphism of the adult CNS and are tied to factors produced from the differentiated gonads (Tsukahara, 2009) as explained in the following sections.

1.3 Gonadal Hormones in Mammalian Sexual Differentiation

The above section briefly outlined the events in phenotypic differentiation between XY male and XX female mice, which are primarily driven by the expression of *Sry* by the Y chromosome at 11.5 d.p.c. in the male (Koopman et al., 1990; Hacker et al., 1995; Sekido et al., 2004). Androgen starts to be expressed around the time *Sry* induces male-type organization thus supporting the ongoing masculinization of the XY embryo.

The synthesis of endogenous androgens depends on several enzymes from the cytochrome P450 family of oxidases (Miller, 1988). The process starts from cholesterol and the activity of cholesterol side-chain cleavage enzyme, P450scc, converting cholesterol to pregnenolone. Downstream of this, androstenedione and testosterone are synthesized, and further activity by aromatase (P450arom) catalyzes the biosynthesis of estrogens from androstenedione and/or testosterone (Greco & Payne, 1994; Greco, 1993). Thus these hormones are interrelated and the effects of their activities are governed by the levels of each circulating in the relative sex.

1.3.1. Masculinizing Factors in Early Development

Masculinizing factors in early development are testosterone, the levels of which are regulated by Leydig cells, and AMH released by Sertoli cells. Leydig cells are the source of androgen in the masculinizing fetus and high levels of androgens persist until birth. This supports the perseverance of Wolffian ducts

that develop into organs of the male reproductive system. The androgen secreted by fetal Leydig cells is androstenedione, which is converted by 17*β*-hydroxysteroid dehydrogenase (17*β*HSD) to testosterone in the seminiferous tubules. Adult mouse Leydig cells secrete already converted testosterone, which suggests that there are two phases of Leydig cell development with fetal and adult forms (O'Shaughnessy et al., 2000).

As mentioned above Sertoli cells secrete AMH closely following upregulation of *Sry* activity, stimulating degeneration of Müllerian ducts and determining the male phenotype (Hacker et al., 1995). AMH is a dimeric 140 kDa glycoprotein and a member of the transforming growth factor (TGF)-β superfamily of signaling proteins involved in growth and differentiation. This superfamily is highly conserved across the animal kingdom (Al-Attar et al., 1997; Durlinger, Visser, & Themmen, 2002) and AMH occurs in all vertebrates (McLennan & Pankhurst, 2015). The product of *Amh* transcription is a theorized 560kDa preproprotein, which is cleaved into a proprotein, proAMH, which does not appear to activate its receptors but is further cleaved during protein synthesis into N- and C-terminus fragments. The noncovalent association of these two fragments produces bioactive AMH (McLennan & Pankhurst, 2015). Müllerian duct regression occurs during the critical window through AMH ligand receptor (AMHR) II activity. At this time the duct is not composed of a true epithelium, rather it is a mesoepithelium that has been suggested to be more susceptible to

regression and if the window of opportunity is missed the duct will not regress (Josso, Picard, & Tran, 1977; Orvis & Behringer, 2007).

1.3.2. Early Life Gonadal Events in Males versus Females

In males, AMH appears to be regulated by testosterone. Al-Attar *et al.* (1997) compared intratesticular and serum hormone levels through hydridization and enzyme-linked immuno assay (ELISA) in normal postnatal mice of the B6/CBA strain and showed that intratesticular, but not serum, testosterone repressed AMH. Interestingly, this repression did not occur before postnatal day (P) 4, due to a lack of androgen receptor expression on Sertoli cells. Since Sertoli cells do not usually develop in females AMH levels are not present in especially significant levels, but AMH is expressed in females and it does exert effects (Durlinger et al., 2002; Halpin, Jones, Fink, & Charlton, 1986; Hazout et al., 2004). In the ovary AMH and AMHRII signaling appears to be inhibitory on primordial follicle recruitment. These follicles are formed just after birth, and constitute the entire ovarian follicular pool during reproductive life (Durlinger et al., 2002).

Androgen expression also shows sex differences in the earliest stages of development. It is upregulated in the male fetus at 12 d.p.c., supporting and driving the masculinization process, but the female fetal gonad does not show a similar effect. The enzymes P450scc, 3β-hydroxysteroid dehydrogenase (3βHSD), 17α-hydroxylase/$C_{17\text{-}20}$ lyase (P450c17), and 17β-hydroxysteroid dehydrogenase (17βHSD), necessary for steroidogenesis, were investigated by

Greco and Payne (1994) in male and female fetal gonads at 13, 15, 17, and 20 d.p.c. using reverse transcriptase – polymerase chain reactions (RT-PCR). The levels of enzymes necessary for cholesterol to steroid hormone conversion were detected in male fetal testes relatively consistently. Meanwhile in female fetal ovaries, only the levels of 3βHSD were sometimes detectable, except at 20 d.p.c., when P450scc levels rose to a detectable range. Thus the steroidogenesis process that drives the expression of the male phenotype is relatively absent in the female fetus, marking the development of dimorphism early in fetal life.

1.4 Gonadal Hormones Effect Sex Differences in the Central Nervous System

The development of brain structures with sex-specific morphology involves mediators from different signaling systems. A well characterized impact on CNS dimorphism is that of gonadal hormones. Androgens and their metabolites lead to masculinization of the brain without which it develops in a "feminized" pattern (MacLusky & Naftolin, 1981). Although the contribution of gonadal hormones to the development of sex differences is appreciable, it is important to note that levels of expression of other genetic factors between males and females also show dimorphism and have the propensity to contribute to sex differences independently. This effect was shown in mice 10 d.p.c. using RT-PCR and microarray systems, revealing differential levels of expression in over 50 genes involved in cellular differentiation and proliferation, transcriptional regulation and signaling (Dewing, Shi, Horvath, & Vilain, 2003). Similarly, our work also shows

the contribution of the immune system on sexual dimorphism in the brain (Rilett et al., 2015) which will be discussed in later sections.

Classically masculinization of the CNS is considered to occur due to excessive androgens in the brain during the critical period of postnatal life. Androgens are aromatized to estrogens in the CNS, perhaps at a higher rate locally in sexually dimorphic regions (MacLusky & Naftolin, 1981; Toran-Allerand, 1976). Treatment of hypothamalic culture from newborn mouse pups with estradiol, testosterone, or BSA for controls has a potent effect with significant differences between treatment groups becoming evident after only 4 days, regardless of sex. Cultures receiving the steroid hormones show increased proliferation of neural processes with neurite outgrowth producing arborized structures, as well as differences in synaptic structures, visualized with silver nitrate (Toran-Allerand, 1976). The expression of higher rates of androgens in males in early life and the conversion of these androgens to estrogens (Greco & Payne, 1994) pushes the brain to develop in a male-typical manner.

1.5 Anti-Müllerian Hormone: The Overlooked Gonadal Hormone

Work in the 20th century with steroid hormone treatment revealed that a masculinized brain is one receiving higher doses of androgens during critical periods of development (Toran-Allerand, 1976). However AMH is also involved in masculinization in the periphery during the critical period of development (Al-Attar et al., 1997). Like the steroid hormones secreted from gonads in a continuous

time- and sex-dependent manner, the glycoprotein AMH is also continuously secreted throughout mammalian life and in much higher levels in developing males than females (Al-Attar et al., 1997; Wittmann & McLennan, 2013b). Although AMH production in the testis is stimulated by FSH in postnatal male mice, these high levels of AMH drop significantly with increased expression of its inhibitor testosterone as development progresses. This leads to the lower levels found in adult males (Al-Attar et al., 1997). Lower levels of AMH in adult males are similar to those in adult females after the peri-pubescent period when AMH expression is considered to begin in the ovaries (Wittmann & McLennan, 2013b). This pattern of postnatal vs. adult activity reveals that AMH is a critical marker of sexual differentiation. Historically, AMH has been overlooked in analysis of gonadectomized mice, with the targets being only testosterone and estrogen, therefore its impact on sexual dimorphism of the CNS remains to be investigated in detail.

1.5.1. Impact of Anti-Müllerian Hormone on the Central Nervous System

AMH is dimorphic during early periods of development, where higher levels induce the development of the male phenotype by triggering the degeneration of the Müllerian ducts in the bipotential fetal primordium (Hacker et al., 1995). In mice, its levels increase around 12 d.p.c. of fetal development and persist into the postnatal period, where they are inhibited by increasing levels of testosterone expression (Al-Attar et al., 1997). Although its impact on the CNS

has been explored to a much lesser degree, it was suspected to impact CNS development and/or function since AMH receptors are expressed in most developing neurons of 13, 14, 16, or 20 day old fetuses as demonstrated by Wang *et al.* (2009) using Cre-lacZ transgenic mice. Furthermore, AMH is expressed in postnatal male mice in variable levels dependent on upregulation of gonadal testosterone (Al-Attar et al., 1997). This work raises an interesting question: since gonadal differentiation is an embryonic event, and if the classically considered function of AMH as being solely a degenerating factor of the Müllerian ducts is true, then why does its expression persist into the postnatal period? Since AMH shows such a potent dimorphism, it can be predicted that it would be involved in determining sex-linked biases or even anatomical differences between males and females extending beyond gonadal differentiation. Investigations of AMH effects on the CNS have revealed such effects. Adult murine motor neurons have been shown to contain significant quantities of AMH mRNA, and AMH acts as a motor neuron growth factor *in vitro* (Wang et al., 2005). *Mis-/-* mice are AMH-deficient and the males have motor neuron numbers that are similar to females. Furthermore the heterozygote has an intermediate motor neuron number, suggesting a dose-dependent effect of AMH on phenotype (Wang et al., 2009). The effect of AMH knockout is also evident in behaviours. Normally, wild type (WT) male mice show greater tendencies towards exploratory behaviours compared to females (Rilett et al., 2015; Wang et al., 2009). This sex-biased behaviour can be demonstrated in the open-field

chamber and parameters that can show these differences include area explored and number of rearing. Male mice with the AMH knockout show a loss of sex differences in the open-field chamber, with rearings showing no differences from WT and knockout females, while WT males explore almost twice as much as females (Wang et al., 2009). Furthermore, behaviours that do not show a sex-linked bias do not appear to be influenced by AMH, such as in the rotarod test which measures the maximum length of time a mouse runs on an accelerating rotating rod. Male mice with the AMH knockout showed no changes in run-time compared to WT males, WT females, or females with the knockout (Wang et al., 2009).

Further exploration into the CNS reveals more impact of AMH. As mentioned, the bed nucleus of the stria terminalis (BST) shows significant sex differences; WT males have a BST volume that is almost twice the female volume (Rilett et al., 2015; Walter Wittmann & McLennan, 2013b). This brain region is implicated in anxiety-like behaviours in mammals (Dumont, 2009). The BST is most likely involved in persistent response rather than acute (Walker, Toufexis, & Davis, 2003), thus being implicated in trait anxiety rather than state. The BST has also been shown to be involved in sexual and reproductive behaviours in mice through the exploration of behaviours such as sniffing of soiled bedding.

Wittmann and McLennan (2013) implicated AMH as influencing the dimorphic form of the BST. Their exploration employed WT and AMH-deficient mice. Immunohistochemistry techniques were used with the antibody to calbindin-D28k, a calcium binding protein present in most neuronal types in the limbic system (Celio, 1990) and frequently used as a neuronal marker. They showed that at P20, WT, pre-pubescent, male mice have 25% more calbindin-positive neurons in the principal nucleus of the BST (pBST). Furthermore, the neurons of the pBST were also 4% larger in WTmales compared to females, but both the differences in number and size were absent in the *Amh-/-* mice. With further development, the pBST of the knockout males increased in size to be larger than the pBST of knockout females, but it never reached the WTmale size. This lead to a neuronal phenotype that was somewhere in the middle of the range in pBST sizes across the groups. Finally, the impact on CNS also had a functional component with *Amh-/-* males showing the least interest in soiled bedding, compared to all other groups (Wittmann & McLennan, 2013a).

Additionally to the BST, AMH activity has also been shown to influence other brain regions in a sexually dimorphic manner, such as the cerebellum, through size and the number of Purkinje cells. The cerebellum of *Mis-/-* mice, deficient in AMH, showed a loss in number of Purkinje cells in adult male cerebelli and a reduction in Purkinje cell size as well (Wittmann & Mclennan, 2011). A similar pattern of AMH knockout emerged with an investigation of the sexually dimorphic nucleus of the preoptic area (SDN-POA). The SDN-POA in

WTmale mice contains almost twice the calbindin-positive neurons compared to WTfemales, however the number of neurons as well as their size was reduced with AMH knockout (Wittmann & McLennan, 2013b).

The studies by Wittmann and McLennan also discuss the processes occurring in female conspecifics. After 20 days, female animals of both WTand AMH deficient groups showed an atrophy of the sexually dimorphic brain regions BST and SDN-POA that occurred independent of AMH (Wittmann & McLennan, 2013a, 2013b). In summation this data supports the hypothesis that AMH, a gonadal hormone secreted during fetal brain development and extending into the neonatal period, supports the development of sexually dimorphic nuclei in a sex-biased manner. The pattern of expression suggests that higher levels of AMH may predispose the development of a male CNS phenotype that higher levels of testosterone then continue to support.

1.5.2. Anti-Müllerian Hormone: Mechanism of Action

The mechanism of AMH action has proven elusive due to the redundancy in the TGFβ superfamily and so far only AMHRII has been demonstrated as a relatively distinct receptor type of AMH. AMHRI has been hypothesized to exist, however compelling evidence has yet to be presented to support this idea. The ligands in the TGFβ superfamily signal through binding to type 2 and type 1 serine/threonine kinase receptors on the cell membrane, associated with Smad proteins, and they share the type 1 and type 2 receptors across the various

ligands (Shi & Massagué, 2003). AMHRII signaling occurs through association with the type 2 receptor bone morphogenic protein receptor (BMPR) 2, and two type 1 receptors, Alk2 and Alk3, which form a kinase complex that induces Smad1/5/8 signaling through phosphorylation. The downstream effect is nuclear translocation of the phosphorylated Smad proteins and regulation of transcription of target genes (McLennan & Pankhurst, 2015; Shi & Massagué, 2003). Although this pathway is relatively well established in terms of AMH effects, due to the sharing of ligands and receptors in the TGFβ superfamily, there are novel mechanisms of AMH activity still to be revealed.

One study demonstrating the effect of AMH activity on the CNS showed the impact of this hormone on neuroserpin, a serine-protease inhibitor endogenous to the CNS that plays a protective role during excitotoxic injury situations. Lebeurrier *et al.* (2008) explored signaling of Alk proteins and found Alk6 to be a relatively strong stimulator of neuroserpin expression *in vitro.* Furthermore, only Smad5 significantly increased transcriptional activity of a neuroserpin promoter construct, suggesting that BMPs and AMH could regulate neuroserpin expression. Administration of BMPs to neuron and astrocyte cultures produced some effects, but the most robust promotion of neuroserpin expression was found with recombinant AMH exposure. Furthermore, analysis of the cortex, striatum, and hippocampus from AMHRII knockout and WTmice using RT-PCR revealed AMH deficiency to be correlated with reduced neuroserpin mRNA levels. Sex-differences in AMHRII and AMH were also revealed in nervous tissue

from WTP7 mice. Postnatal female mice showed lower mRNA levels of both AMHRII and AMH in cortical and spinal tissue compared to males. The lower levels of AMH corresponded with lower levels of neuroserpin (Lebeurrier et al., 2008).

Although the study by Lebeurrier and colleagues holds most implications for excitotoxic situations, the novel mechanism of AMH effects presented in their study - alongside the evidence for AMH impact on CNS dimorphism from McLennan and colleagues – reveals novel venues for investigation of sex differences. Furthermore AMH implication in both pathological and basal conditions of the CNS suggests that it may interact with immune factors that impact the outcome of both those conditions although these have yet to be found. The following sections will explore how the immune system is implicated in sex differences of the CNS and draw parallels and intersections with the outcomes of endocrine activity.

1.6 Quantifying Immune Cells Using Immune Cell Markers

The purpose of this section is to give a brief overview of immune cell population measurement. The particular markers expressed on immune cells used to quantify them in research reflect their biological functions.

The immune system can be organized into the adaptive and the innate systems. Hematopoietic stem cells in the bone marrow of adult mammals differentiate into leukocytes (white blood cells) with myeloid and lymphoid

lineages. Myeloid cells comprise the innate immune system and lymphoid cells comprise the adaptive immune system. One of the downstream differentiated products of myeloid cells are monocytes, which can differentiate into macrophages and are capable of phagocytosis.

In the brain, the resident immune cells called microglia also have phagocytic capability and derive from myeloid precursors (Murphy, 2011). Myeloid precursors destined to be microglia differentiate in the embryonic yolk sac and populate the developing CNS around E9.5 in mice, developing a pattern of cell surface markers unique from peripheral macrophages (Ginhoux et al., 2010). Due to the shared hematopoietic ancestry, macrophages in the periphery and microglia in the CNS can be identified with antibodies to the membrane protein F4/80, a member of the adhesion G-protein coupled receptor (GPCR) family (Lin et al., 2005), but microglia can also be quantified using antibodies to the ionized calcium-binding adapter molecule 1 (Iba1) as well as the fractalkine receptor, CX3CR1. In the CNS, both Iba-1 and CX3CR1 are solely expressed on microglia. Iba1 has been shown to be involved in membrane ruffling, cell migration and phagocytosis of microglia (Ohsawa, Imai, Sasaki, & Kohsaka, 2004). CX3CR1 binds the chemokine CX3CL1 or fractalkine, which is produced by neurons, and is a modulator of neuron-microglia crosstalk (Wolf, Yona, Kim, & Jung, 2013).

Antibodies can be produced or are commercially available for several other microglial markers and depending on the marker chosen, can reveal on the

activational state of the cell. Microglia exist on a spectrum of activation demonstrating morphologies that range from ramified, with thin, long processes and relatively small cell bodies, to amoeboid, with large cell bodies and smaller processes. These morphological differences are associated with functional changes as well as differences in quantity of expressed cell markers (Glenn, Ward, Stone, Booth, & Thomas, 1992; Karperien, Ahammer, & Jelinek, 2013; Ohsawa et al., 2004).

Although there are several other cell surface proteins that can be used to identify a particular cell type, all immune cells show specificity and distinction in the expression of cell surface molecules called clusters of differentiation (CD) molecules. CD are groups of molecules responsive to specific antibodies, therefore making them specific markers of cells. The cell surface molecule that expresses the target for a particular set of antibodies is designated CD, followed by a specific number (Bernard & Boumsell, 1984). For example, all T lymphocytes express CD3, while T-helper 1 (Th_1) and Th_2 cells also express CD4. Therefore in literature they are designated $CD3^+CD4^+$. Cytotoxic T lymphocytes express CD3, being from the same lymphoid lineage as T-helper cells, and they also express CD8. Thus they are designated as $CD3^+CD8^+$. Like monocytes, lymphocytes also show variability in expression of cell surface molecules as they range in activational state.

1.6.1. Activation of Adaptive Immune Cells

The activation of the adaptive immune response is highly regulated, especially by the innate immune system. Under pathological conditions macrophages and neutrophils are often the first-responders to an infection, initiating an inflammatory response that triggers the members of the innate and adaptive immune system into action. This effect is mediated by signaling molecules called cytokines and chemokines. Cytokines bind to receptors expressed on cell surfaces and chemokines attract other immune cells, such as more macrophages, from circulation (Murphy, 2011). The response of the mammalian innate immune system to infection is initiated by pathogen recognition receptors (PRRs) that respond to structurally conserved regions of microbes called pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) are transmembrane members of the PRR family (Iwasaki & Medzhitov, 2010). The innate immune system is also activated by cytokine and chemokine receptors.

The bridge between innate immune cell activation and an adaptive immune response is best represented by the presentation of antigens – substances that induce an immune response – by phagocytic cells to lymphocytes. An example is the dendritic cell of the innate immune system in which uptake of a microbial factor induces the production of inflammatory cytokines, as well as upregulation of TLRs and costimulatory molecules. The dendritic cell then migrates into lymph nodes and presents the antigens to naïve

T lymphocytes. This process is the initial activation of the adaptive immune response and essentially it involves the innate immune cell presenting the antigen via major histocompatibility complex (MHC) molecules to the T cell receptor (TCR) complex (Mogensen, 2009; Takeda, Kaisho, & Akira, 2003). The TCR of T helper cells is designated $CD4^+$ and the TCR of cytotoxic T lymphocytes is designated $CD8^+$. Furthermore, $CD4^+$ and costimulatory molecules bind to MHCII molecules, which are expressed on innate immune cells. $CD8^+$ with its costimulatory molecules binds to MHCI molecules, which are expressed on virtually all nucleated cells (Murphy, 2011).

Pathogenic conditions are not necessary for interaction between the adaptive and immune systems, e.g. MHCI consistently interacts with CD8+ T lymphocytes in the process of immune surveillance. It is in the specific scenario of the presentation of an aberrant antigen that the T cell becomes activated to perform cytotoxic functions. The interaction of immune cells with parenchymal cells is omnipresent; therefore it is surprising that they are neglected in consideration of basal processes such as the development of sexual dimorphism in the CNS. Furthermore the immune system also shows sex differences, providing evidence for its responsiveness to sex and its effects.

1.7 Sexual Dimorphism in the Immune System

Cells of the immune system exist in circulation but also reside in various areas of the body, for example distinct leukocyte populations can be found in the

pleural and peritoneal cavities (Scotland, Stables, Madalli, Watson, & Gilroy, 2011). Microglia residing in the CNS play various classic immune roles, such as regulating inflammatory responses, but recent research has also shown that they are involved in processes regulating neuronal structure and function (Kipnis, Gadani, & Derecki, 2012; Lenz, Nugent, Haliyur, & McCarthy, 2013; Morris, Clark, Zinn, & Vissel, 2013; Sidor, Halgren, & Foster, 2014), thus challenging the historical misconception of the CNS being an immune privileged cite.

In the periphery, sex differences in the immune system have been shown with investigation of the peritoneal and pleural cavities of adult mice using flow cytometry. Females were shown to contain more immune cells compared to males. Specifically, females had more $F4/80^+$ macrophages, $CD3^+$ T lymphocytes, and $CD19^+$ B lymphocytes. Further investigation into the percentage of resident cells per cavity revealed a bias towards more T lymphocytes in females compared to males. Females had higher numbers of both $CD8^+$ and $CD4^+$ T lymphocytes and they also mounted a higher immune response to immune stimulating factors. Finally, sham operated females had a higher number of total immune cells compared to ovariectomized females, revealing the immunomodulatory impact of female gonadal hormones (Scotland et al., 2011).

1.8 Impact of the Immune System on Stress-Reactivity and Anxiety-like Behaviours

The prevalence of anxiety disorders shows a sex bias with girls more likely to develop an anxiety disorder compared to boys (Lewinsohn et al., 1998; Remes, Brayne, Linde, & Lafortune, 2014). Stress and anxiety responses are an excellent example of bidirectional endocrine-CNS communication. They are most commonly attributed to the hypothalamic-pituitary-adrenal (HPA) axis where a perceived stressor elicits the hypothalamic corticotrophin-releasing factor (CRF) response inducing the pituitary release of adrenocorticotrophic hormone (ACTH). ACTH enters circulation and induces the adrenal corticosterone (CORT) – or cortisol in humans – response. The effects of CORT are widespread – CORT is a glucocorticoid having effects on the metabolism of proteins, fats, carbohydrates and inflammatory activity through the relative cytoplasmic glucocorticoid receptors (Ballard, Baxter, Higgins, Rousseau, & Tomkins, 1974; Barnes, 1998).

Although the HPA axis is heavily investigated in stress research, a contributor to its function is a member of the immune system, the MHCI molecule. Fully formed and functional MHCI molecules expressed on cell surfaces depend on endoplasmic reticulum (ER) processing prior to membrane insertion. Peptides involved in MHC formation are transferred into the ER via transporter associated with antigen processing (TAP) protein. Another molecule involved in the complete MHCI complex is β2-microglobulin (β2M) that forms the peptide-binding cleft with MHCI for a fully functioning complex. *β2M-/-TAP-/-* mice lacking both these

factors involved in MHCI production lack a functional MHCI, and do not associate with cytotoxic T lymphocytes that they would normally respond to via cell to cell communication (Sankar, MacKenzie, & Foster, 2012).

As mentioned, MHCI molecules are expressed by virtually all nucleated cells (Murphy, 2011), and in the CNS they have been found to not only be expressed but also respond to systemic stressors. Following acute and chronic systemic infections, MHCI mRNA was shown to be expressed in the PVN of rats (Foster, Quan, Stern, Kristensson, & Herkenham, 2002). Furthermore using *β2M-/-TAP-/-* mice it was shown that MHCI is not only responsive to stressors; it is also involved in basal behaviours. Different levels of CORT were found in both male and female knockout groups compared to WTin response to a lipopolysaccharide (LPS) injection 2h and 6h post-injection. Furthermore, *β2M-/-TAP-/-* mice showed significant HPA activation in response to just a saline injection compared to WTmice, demonstrated by higher levels of plasma CORT in *β2M-/-TAP-/-* females 2 hours (h) after injection. The response to a saline injection is indicative of the involvement of the MHC complex in basal activity (Sankar et al., 2012).

The above findings reveal that the immune system is involved in shaping the underlying circuitry of limbic systems. More evidence to demonstrate that immunity plays a role in setting the limbic tone comes from germ free (GF) mice. These mice are never exposed to non-sterile conditions and come from mothers

under similar conditions. Due to the lack of exposure to microbes, their gut is never populated by the normal gut flora that forms the microbiota in normal mice that are bred in the usual specific-pathogen free (SPF) conditions. GF mice essentially have an "uneducated" immune system, meaning the factors that the immune system responds to postnatally are absent, and any circulating immune system factors from the pregnant dams that may interact with the fetus are absent as well. GF mice show reduced anxiety-like behaviours in the elevated plus maze (EPM) compared to conventionally reared SPF (Neufeld, Kang, Bienenstock, & Foster, 2011a) mice that persists even after their guts are colonized with microbiota from SPF mice (Neufeld, Kang, Bienenstock, & Foster, 2011b). These results show that there is a critical period during which immune activity sets the tone for development of anxiety-related circuitry.

The impact of the immune system on limbic circuitry becomes clearer with further investigation of the adaptive immune system. T lymphocytes in particular have been implicated in influencing the tone for development of anxiety-related circuitry. *TCRβ-/-δ-/-* mice have nonfunctional T cells due to mutations of the *β* and *δ* chains of the TCR. Normally WTmice show sex differences in tests of stress and anxiety, represented by parameters such as open and closed arm entries and activity in the EPM, transitions between light and dark chambers of the light-dark tests, open field time and activity in the open field test, among others. *TCRβ-/-δ-/-* mice tested in the EPM, open field, and light-dark test showed reduced anxiety-like behaviours, compared to WTconspecifics, and with sex

differences. Female *TCRβ-/-δ-/-* mice spent more time in the light chamber compared to the WTfemale mice, and male *TCRβ-/-δ-/-* mice spent more time in the center of the open field compared to WTmales. The light chamber and center of the open field are normally aversive situations since mice inherently prefer to be in covered, darker spaces. Thus the increased entrance and exploration of open and lit areas in *TCRβ-/-δ-/-* mice is a marker of reduced anxiety-like behaviours. Additionally, *ex vivo* brain imaging with 7 Tesla MRI comparing brain volume differences in *TCRβ-/-δ-/-* male and female mice against WTrevealed volume changes in limbic regions. This analysis included the hypothalamus, amygdala, hippocampus, and the BST among others. The changes in the BST in *TCRβ-/-δ-/-* mice were particularly striking since the normal sex difference was lost (Rilett et al., 2015). This data revealed that T lymphocytes, cells of the adaptive immune system, play a role in development of sex differences in the brain. Furthermore, immunomodulation of the CNS has functional consequences as evidenced by behavioural changes in *TCRβ-/-δ-/-* mice compared to WTconspecifics.

1.8.1. The Sexual Dimorphic Development of the Bed Nucleus of the Stria Terminalis is Responsive to both Endocrine and Immune Signals

The current work aims to explore the mechanism by which the adaptive immune system, specifically T lymphocytes, may be exerting their effects on brain anatomy. The brain region targeted in the analysis is the BST due to the particularly strong response in its development in *TCRβ-/-δ-/-* females. In

TCRβ-/-δ-/- female mice, the BST volume increased to match that of *TCRβ-/-δ-/-* and WTmales (Rilett et al., 2015). This suggests that the early life effect of T lymphocytes on this brain region in WTfemales is likely involved in the apoptotic processes that leads to a smaller BST in WTfemales compared to WTmales, although the rate of neurogenesis has yet to be assessed in *TCRβ-/-δ-/-* mice.

The known mechanism for the development of sex differences in the brain involves the actions of the B-cell leukemia/lymphoma -2 (bcl-2) proto-oncogene that blocks apoptosis in B cells (Lu, Poulsom, Wong, & Hanby, 1993). *Bax* is a proapoptotic gene in the Bcl-2 family of apoptotic genes and it is known to be regulated by gonadal hormones, especially by estrogen converted from gonadal androgens acting in the brain (Garcia-Segura, Cardona-Gomez, Naftolin, & Chowen, 1998; Zhao, Wu, & Brinton, 2004). Levels of bcl-2 have been shown to increase in cultured rat hippocampal neurons that received ER agonists and in vivo this supports neuronal survival (Zhao et al., 2004). In contrast, *Bax-/-* mice show a loss of sex differences in the number of apoptotic cells in the BNSTp, demonstrated with TUNEL staining (Gotsiridze, Kang, Jacob, & Forger, 2007).

Mitra *et al.* (2003) used immunolocalization techniques to determine the presence of the best characterized estrogen receptors (ER), ERα and ERβ, in the mouse brain. Young adult female mice were ovariectomized, and the ERs localized two weeks later. Their findings show both ERα and ERβ expression across major regions of the brain, albeit with varying degree in specific nuclei

within those regions. Particularly strong expression of both receptors was seen in sexually dimorphic regions of the brain including the BST and medial nucleus of the amygdala in the forebrain. In addition to steroids, the work presented in above sections outlining the impact of AMH is evidence of the BST's responsiveness to yet another endocrine factor.

Essentially the work with ER, AMH and the impact of T lymphocyte knockout reveals that there is a synergistic effect of neurogenesis and apoptosis that drives the development of sexual dimorphism in the brain. This effect involves gonadal hormones and immune signals, but the point of convergence of these two factors on BST dimorphism is not yet clear. Since AMH is one of the earliest factors expressed in embryonic development of dimorphic traits and it impacts the dimorphic development of the BST, the current work aimed to quantify AMH in *TCRβ-/-δ-/-* mice in order to provide novel insight into the complex neurogenesis-apoptosis synergy in early life that leads to the development of sex differences in the BST.

1.8.2. Microglial Modulation of Sex Differences in the Brain

The interaction of proapoptotic and antiapoptotic factors in the development of sex differences, especially in the BST, involves the endocrine and immune systems (Gotsiridze et al., 2007; Rilett et al., 2015). Interestingly it is an immune system factor that shows a synergistic effect involving both these systems. Microglia have been shown to be essential to the prostaglandin E2 (PGE2) mediated masculinizing process that contributes to male-typical neuronal

features, such as more dendritic spines and thicker branching, through the conversion of PGE2 from estradiol in the brain. Microglial involvement in this process was demonstrated by several lines of evidence: (1) estradiol added to neuronal culture increased PGE2 and also induced male-typical neuronal differentiation; (2) microglia inhibition alongside estradiol administration did not lead to masculinization as it normally would with administration of this hormone alone; (3) PGE2 upregulation was inhibited with microglia inhibition alongside estradiol administration, suggesting that microglia are involved in the feed-forward process which increases PGE2 in the brain (Lenz et al., 2013).

The implication of this evidence for the current work is that microglia cells are involved in the development of sex differences, integrating the endocrine and immune systems, so it is possible they are also affected in the loss of sex differences seen in the BST of *TCRβ-/-δ-/-* mice. Under nonpathological conditions, T lymphocytes do not appear to populate the brain parenchyma. Instead they populate the meningeal layers surrounding the CNS (Derecki et al., 2010). Microglia are dynamic immune cells that are able to respond to T lymphocytes and cytokines produced by them (Ebner et al., 2013; Hanisch & Kettenmann, 2007; Wlodarczyk, Løbner, Cédile, & Owens, 2014) although the majority of literature has explored their response under pathological conditions. Therefore it seems likely that instead of just being affected by the T cell knockout, they might be the mediators of T cell communication with the CNS. Based on this evidence, the current work investigated the changes in microglia cells of *TCRβ-/-*

δ-/- mice in order to gauge the direction of effects and shed some light on the implication of the immune system in the development of a sexually dimorphic BST.

2. Hypotheses and Aims

I. Aim: to assess and quantify changes in levels of AMH in *TCRβ-/-δ-/-* and WT mice.

HYPOTHESIS: there will be higher levels of AMH in WT males and *TCRβ-/-δ-/-* mice that show larger BST volumes in adulthood.

II. Aim: to assess and quantify changes in microglia number in *TCRβ-/-δ-/-* and WT mice.

HYPOTHESIS: there will be more microglia in WT males and *TCRβ-/-δ-/-* mice that show larger BST volumes in adulthood.

3. Methods

3.1 Tissue Collection

Eleven week old male and female mice (n=6/sex/genotype) and postnatal day 7 male and female mice (n=6/sex/genotype) were intracardially perfused with heparin dissolved in phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. Brains were collected and post-fixed and then transferred to 1XPBS at 4°C until further processing. Brains were sent to

Neuroscience Associates (Knoxville, TN) for sectioning and immunohistochemical processing.

For P7 tissue, each brain was cryoprotected in 10% sucrose, followed with emersion in 30% sucrose. The brains were frozen in cold isopentane and stored at -70^{o}C until cryostat sectioning. A series of 20 µm coronal sections were collected through the BST (Bregma 3.27-4.11mm) according to the stereotaxic atlas of Paxinos *et al.* (Paxinos *et al.,* 2007). All sections in the BST were collected, 2 sections per slide. Sections were stained with toluidine blue as reference and compared to the mouse stereotaxic atlas for identification. Sections were water-mounted onto gelatin-coated glass slides, dried at room temperature for 10 min, and stored at -35 ^{o}C until processing.

3.2 Tissue Collection for Hormone Analysis

At P0, P2, P7, P14, and P21, cord blood was collected following decapitation. The number of mice used per group are listed in Table 1. The blood was transferred to 4 ^{o}C to clot for 1 hour. Samples were centrifuged for 15 min at 3000 rpm. The serum was collected and stored at -70 ^{o}C. Serum was analyzed with ELISA kits for AMH (Elabscience, Beijing, China).

	P0	P2	P7	P14	P21
WT F	8	10	7	6	5
WT M	7	9	7	5	5
TCR F	10	8	5	5	5
TCR M	11	9	8	5	5

Table 1 Number of postnatal mice used per age, genotype, and sex in AMH analysis.

3.3 Immunohistochemistry

For adult tissue, immunohistochemical processing of 35 µm sections with anti-Iba1 antibody (Wako Ltd., Osako, Japan) was conducted. Every 8th section was processed and the sections with the BST (Bregma 1.32 to -1.28mm) were used in this study. The mouse brain atlas by Paxinos and Franklin (Paxinos and Franklin, 2001) was used for reference. As mentioned, adult tissue was processed by Neuroscience Associates.

P7 tissue was analyzed using immunohistochemistry and anti-iba-1 (Wako Ltd., Osako, Japan). Every 2nd pair of sections was stained with anti-Iba1 and the 1st pair was used for reference with toluidine blue staining. Tissue sections were thawed at room temperature for 1 hour and fixed in 4% formaldehyde in 1xPBS for 5 min. Next the sections were rinsed 3 times in 1XPBS for 5 min, incubated in 0.3% hydrogen peroxide in 100% methanol for 30 min to quench endogenous peroxidase activity, rehydrated in 1xPBS with 3 washes of 5 min each, followed by 1 hour incubation in blocking solution consisting of 10% Casein (Vector, Burlington, ON, Canada) solution in 1XPBS 0.1% Triton-X (TX). Following the

blocking step, the tissue was incubated for 24h at 4 ^{o}C in primary antibody, rabbit anti-mouse Iba1, at a 1:500 dilution. Next, the sections were rinsed for 5 min in 1XPBS, followed by two 5 min rinses in PBS-TX. The sections were then incubated at 4 ^{o}C for 18h in biotinylated anti-rabbit secondary antibody (Vector, Burlington, ON, Canada) at a 1:200 dilution. Following two 5 min 1XPBS washes, the sections were incubated for 30 min with avidin-biotin peroxidase complex (Vectastain ABC kit; Vector, Burlington, ON, Canada) prepared according to manufacturer instructions. Next sections were washed 2 times for 5 min in 1XPBS and then the tissue was incubated in diaminobenzidine contained in the DAB peroxidase Substrate Kit (Vector, Burlington, ON, Canada) for 4 min to develop the peroxidase colour. Following a 5 min rinse in tap water, tissue was dehydrated using increasing concentrations of ethanol. Slides were dipped in 20%, 50%, and 70% ethanol for 20 sec each, and held in 90% and 100% for 1 min each. The ethanol was cleared using xylene, and the slides were coverslipped with permount.

Reference slides were thawed at room temperature for 1 hour and fixed in 4% formaldehyde in 1xPBS for 5 min. Next the sections were rinsed 3 times in 1XPBS for 5 min, followed by a 5 min rinse in sterile water. Tissue was stained for 17 sec in 0.05% toluidine blue. Toluidine blue was cleared and tissue was dehydrated using increasing concentrations of ethanol with quick dips (2 sec each) of the slide in each concentration. The slides were rinsed in distilled water for 5 sec, dried at room temperature and coverslipped with permount.

3.5 Visualization and Image Analysis

Stained tissue sections were visualized using a Zeiss Axioskop 2 Plus microscope and photographed using the AxioCam MRc microscope camera (Zeiss, Toronto, ON, Canada). The BST was identified at 2.5X magnification using the mouse brain atlas by Paxinos and Franklin (Paxinos and Franklin, 2001) for reference. The target regions were analyzed at 40X magnification with the images taken in black and white. AxioVision microscope software (Zeiss, Toronto, ON, Canada) was used for the analysis of the stained sections. Cell counts and parameters were obtained for each tissue section and the parameters analyzed were area, feret ratio (a measure of the length and width creating the oblong shape of a soma), perimeter and radius. Cell soma were manually outlined and criteria for inclusion included circularity, connection to processes and pixilation. Fig. 1 and Fig. 2 are representative images of the analysis procedure.

3.6 Statistical and Data Analysis

AMH levels were assessed using 2-way ANOVA with genotype and sex as factors for each time point and interactions were followed up with *post-hoc* analyses with $\alpha = 0.05$. Microglia cell counts were analyzed using 2-way ANOVA with sex and genotype as factors and significant interactions were followed up with *post-hoc* analyses.

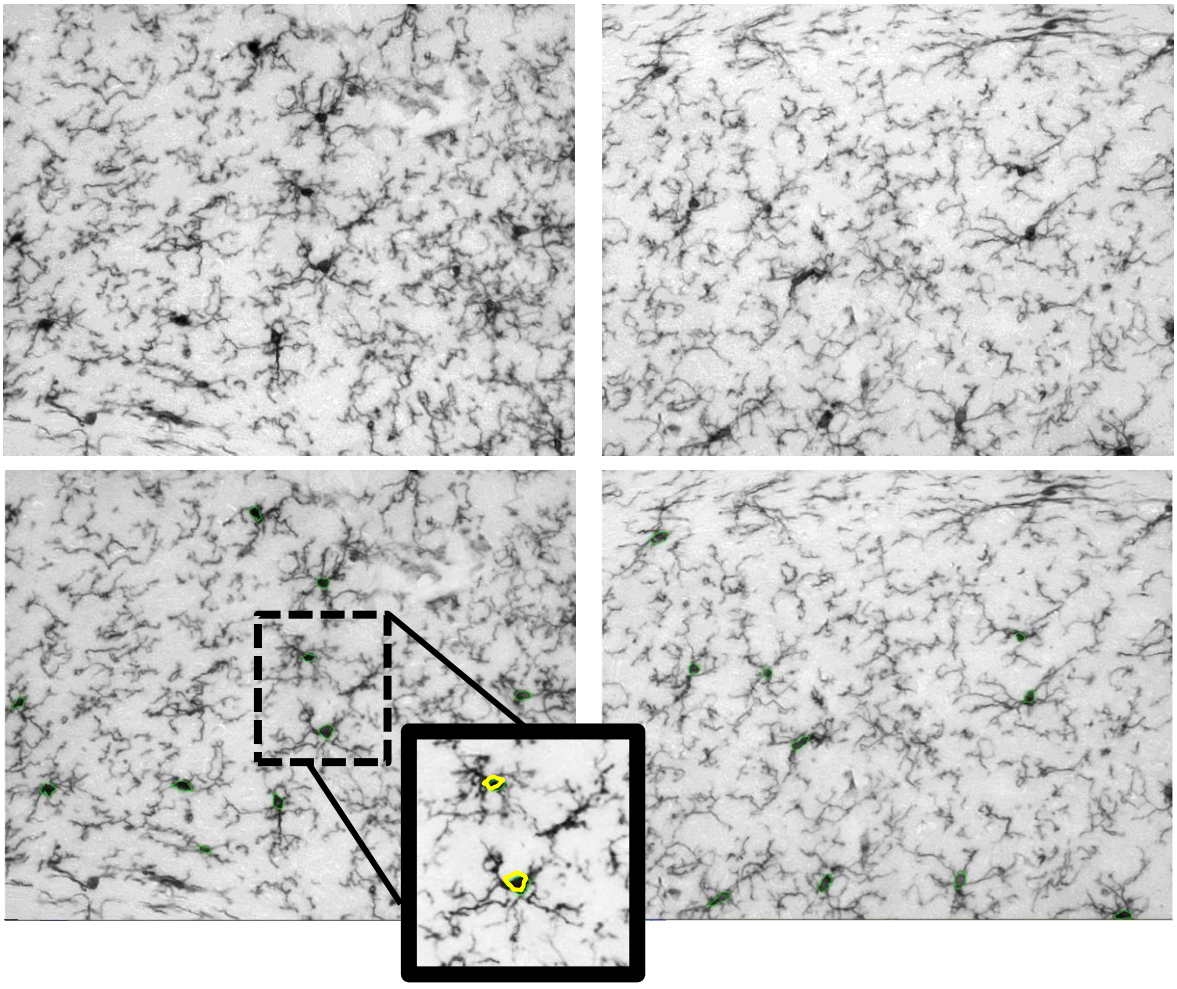

Fig. 1 *Representative images for analysis of adult microglia*. The images on the left are from the dorsal BST and the images on the right are from the ventral BST. The green outlines on the bottom images are marking the cell soma for analysis. The anterior commissure (AC) was used as a marker region and can be distinguished from the BST. Microglia in the AC have long projections extending horizontally on the plane of the tracts.

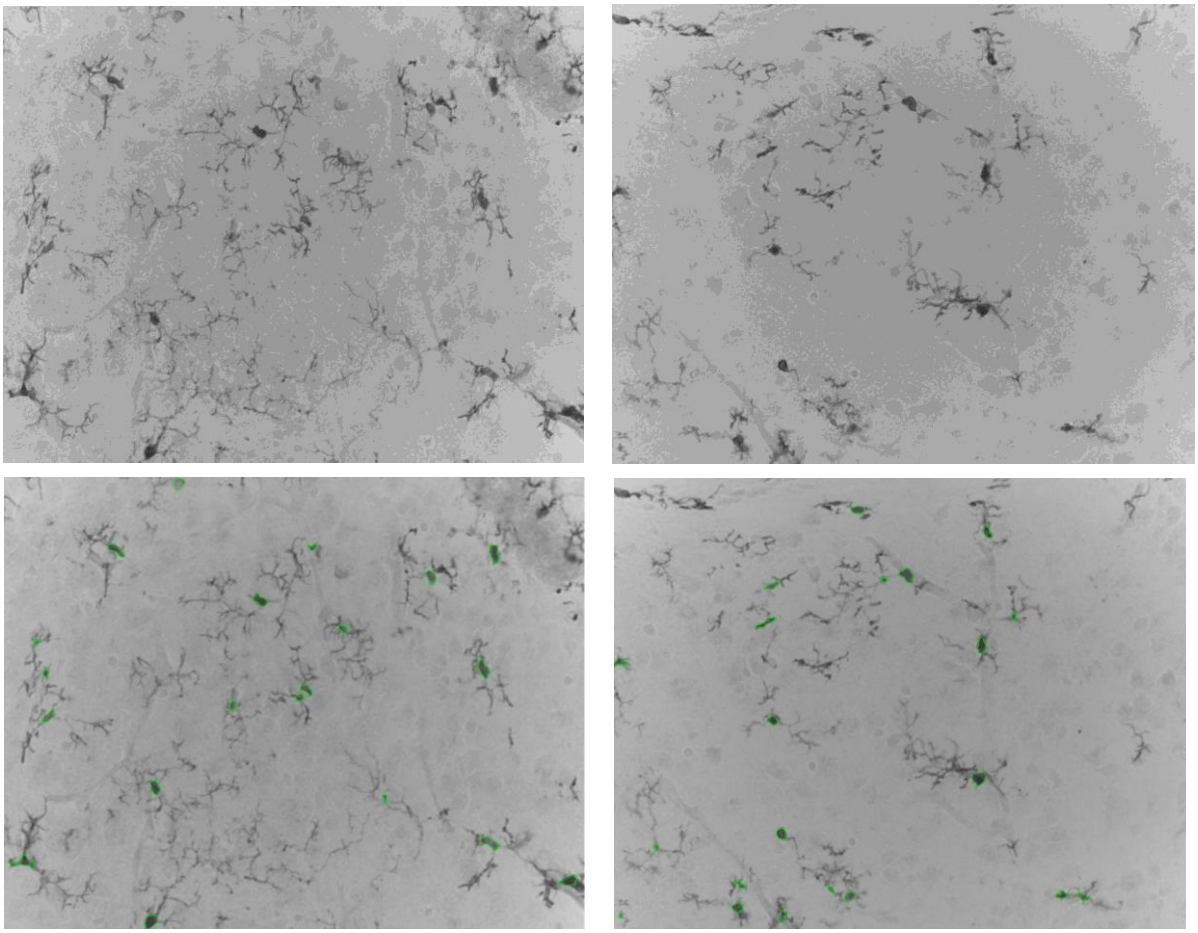

Fig. 2 *Representative images for analysis of P7 microglia*. The images on the left are from the dorsal BST and the images on the right are from the ventral BST. The outlines on the bottom images are marking the cell soma for analysis. The anterior commissure (AC) was used as a marker of the relative region and can be distinguished from the BST. Microglia in the AC have projections extending horizontally on the plane of the tracts. Additionally the lateral ventricles were used at markers of the dorsal BST (top right corners of image in the left panel).

4. Results

4.1 Trajectory of Anti-Müllerian Hormone Levels in Postnatal Mice

AMH levels were measured across the first 3 weeks of postnatal life in WT and *TCRβ-/-δ-/-* mice. There was a significant effect of age ($F_{(4, 120)} = 3.78$; $p = 0.0064$) and an age X genotype interaction ($F_{(12, 120)} = 2.80$; $p = 0.0021$). An age effect was present in WT females with a peak in AMH levels at P7 compared to P0 ($p = 0.0005$) and a similar effect was present in WT males with a peak in AMH levels at P7 compared to P0 ($p = <0.0001$). *TCRβ-/-δ-/-* male mice showed higher levels of AMH at P2 compared to P0 ($p = 0.0227$). A genotype effect was present in the *TCRβ-/-δ-/-* female mice compared to WT at P7 ($p = 0.0014$) as well as in *TCRβ-/-δ-/-* male mice compared to WT at P7 ($p = 0.0014$). This genotype effect was the absence of the P7 peak in serum AMH levels in both female and male *TCRβ-/-δ-/-* mice.

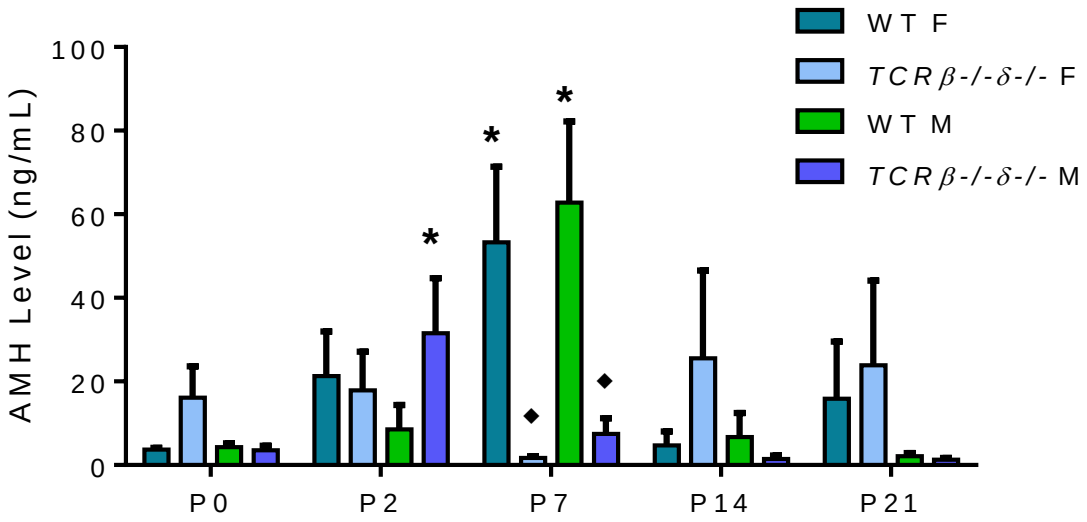

Fig. 3 *Serum AMH levels at P0, P2, P7, P14, and P21 in TCRβ-/-δ-/- and WT mice*. An age effect (asterisks) was present in WT females (p = 0.0005) and males (p = <0.0001) at P7 demonstrated by a peak in AMH levels at P7 compared to P0. An age effect was also present in *TCRβ-/-δ-/-* male mice at P2 (p = 0.0227). A genotype effect (diamonds) was present in the *TCRβ-/-δ-/-* female mice compared to WT at P7 (p = 0.0014) as well as in *TCRβ-/-δ-/-* male mice compared to WT at P7 (p = 0.0001).

4.2 Microglia Analysis in Adult Mice

This study aimed to assess and quantify changes in microglia number in *TCRβ-/-δ-/-* and WT mice. Adult mice showed an effect in the dorsal BST of WT mice only. This was a main effect of sex ($F_{(1, 20)} = 5.40$, p = 0.031) with female WT mice presenting significantly fewer microglia cells in the dorsal BST

compared to WT males ($p < 0.05$; Fig. 4A), while the *TCRβ-/-δ-/-* mice did not show a sex difference in microglia cell number in the dorsal or the ventral BST (Fig. 4B; all $p > 0.05$).

Analysis of the morphology of microglia cells did not reveal any sex or genotype differences in cell soma area, feret ratio, perimeter or radius in WT or *TCRβ-/-δ-/-* mice in the dorsal BST (Fig. 5 A-D; $p > 0.05$) or ventral BST (Fig. 6 A-D; $p > 0.05$). These results suggest an effect of T lymphocytes on microglia that corresponds to a difference in BST volume. This is based on the finding that the lowest number of microglia cells were in WT females that have previously been reported to have a smaller BST compared to WT males and *TCRβ-/-δ-/-* mice of both sexes (Rilett et al., 2015).

4.3 Microglia Analysis in Postnatal mice

At P7, no difference in microglial number between male and female WT mice was observed in the dorsal BST (Fig. 7A). Analysis of the morphology of microglia cells did not reveal any sex or genotype differences in cell soma area, feret ratio, perimeter or radius in WT or *TCRβ-/-δ-/-* mice in the dorsal BST (Fig. 8 A-D; $p > 0.05$) or ventral BST (Fig. 9 A-D; $p > 0.05$).

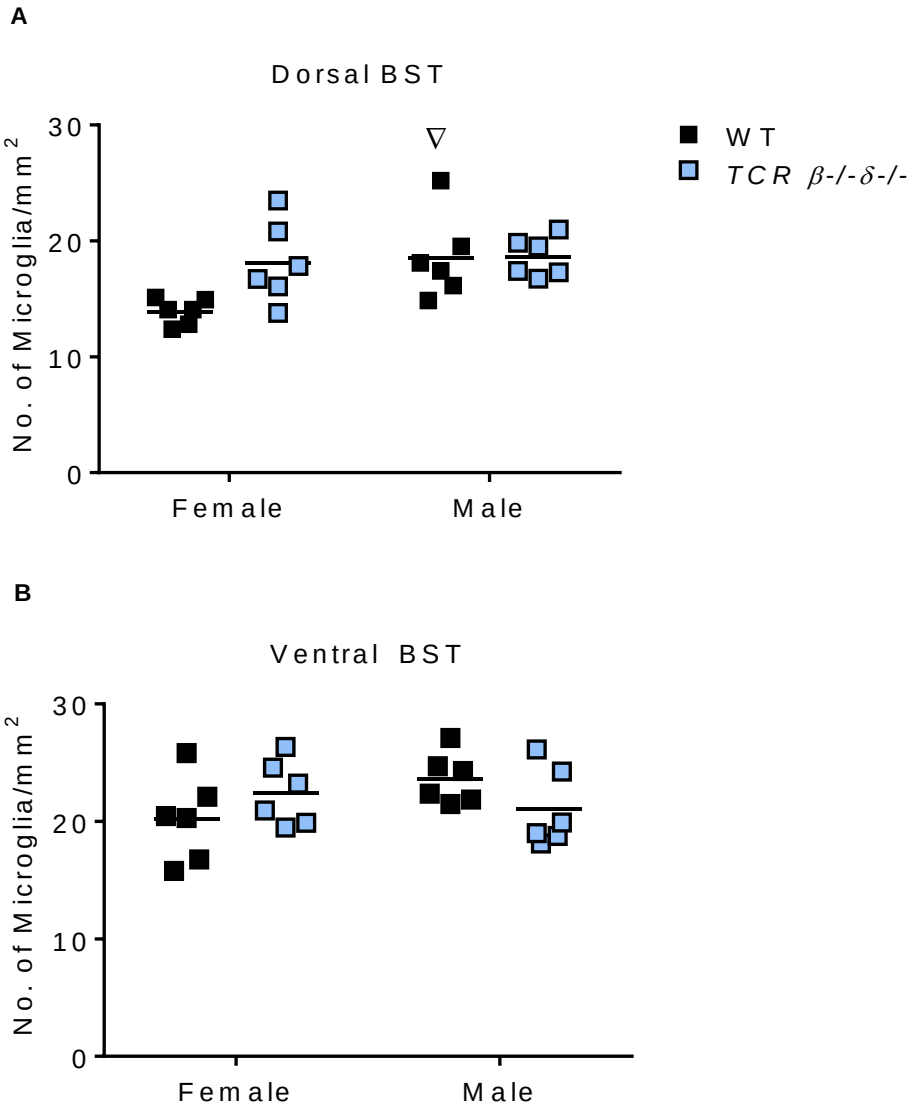

Fig. 4 *Microglia cell counts in dorsal and ventral BST in adult WT and TCRβ-/-δ-/- mice*. Adult mice showed a sex effect in the dorsal BST of WT mice only. Female WT mice had significantly less microglia in the dorsal BST ($F_{(1, 20)} = 5.399$, $p = 0.0308$) compared to WT males (A; inverted triangle). *TCRβ-/-δ-/-* mice did not show any sex differences (all $p > 0.05$).

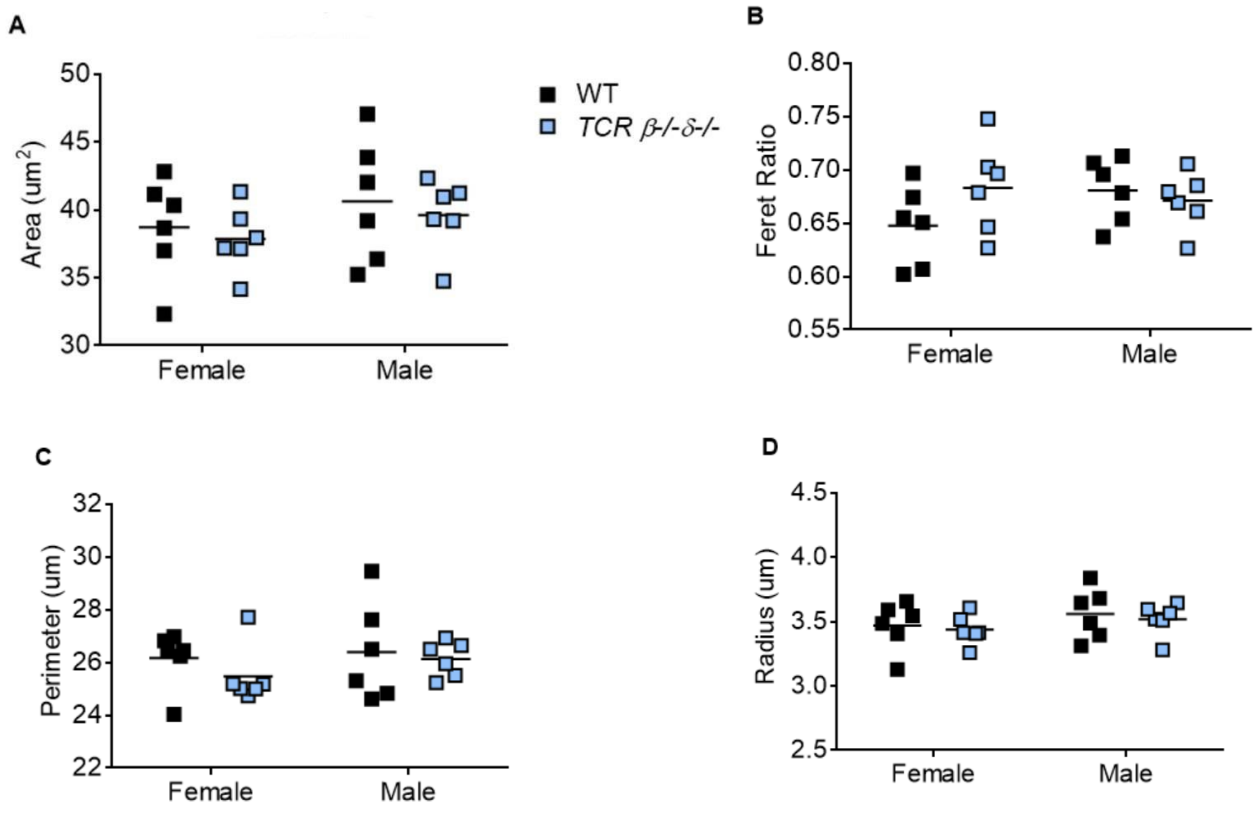

Fig. 5 *Microglia morphology in dorsal BST of adult WT and TCRβ-/-δ-/- mice*. Analysis of microglia morphology did not show any sex or genotype differences in cell soma area (A), feret ratio (B), perimeter (C), or radius (D) in the dorsal BST of any group in adult mice (all $p > 0.05$).

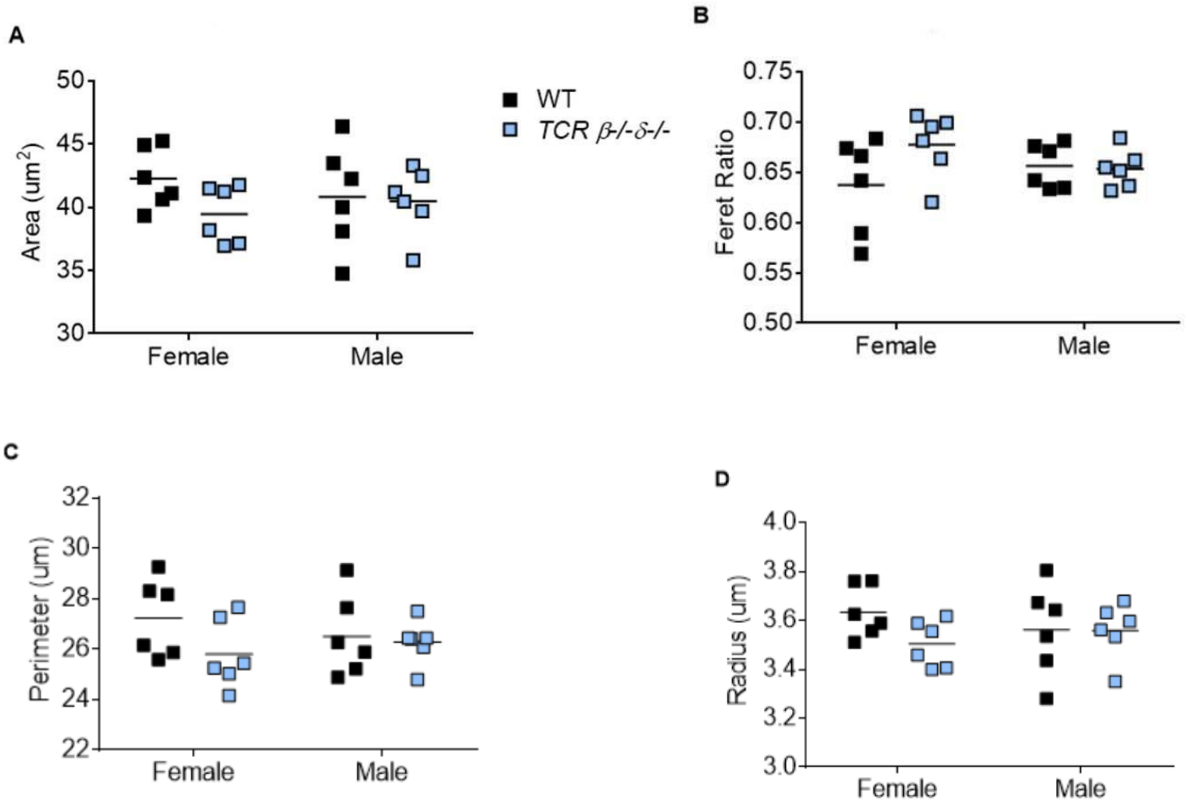

Fig. 6 *Microglia morphology in ventral BST of adult WT and TCRβ-/-δ-/- mice*. Analysis of the morphology of microglia cells did not reveal any sex or genotype differences in cell soma area, feret ratio, perimeter or radius in WT or *TCRβ-/-δ-/-* mice in the ventral BST (Fig. 4 A-D; $p > 0.05$) or ventral BST (Fig. 5 A-D; $p > 0.05$).

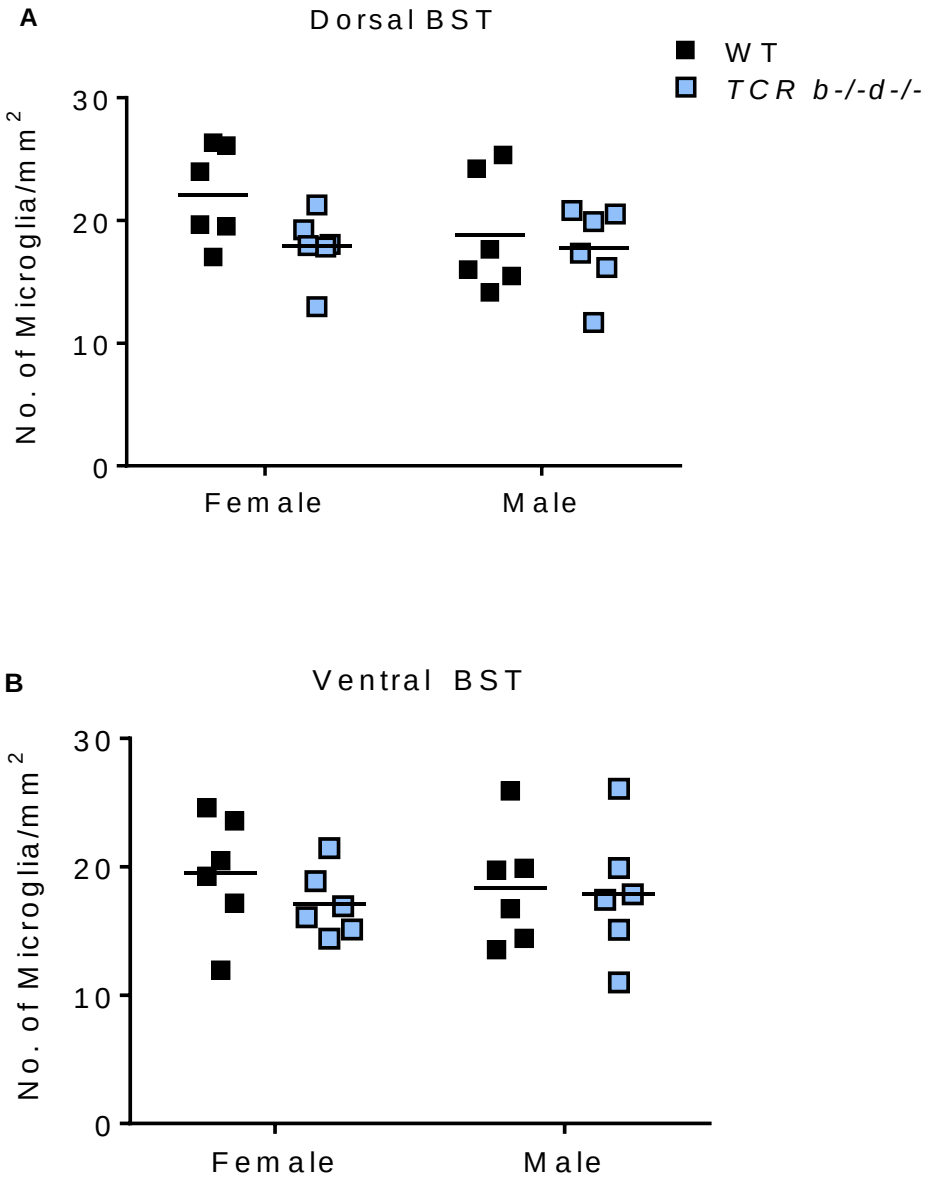

Fig. 7 *Microglia cell counts in dorsal and ventral BST in WT and TCRβ-/-δ-/- mice at P7*. P7 mice did not show a significant sex or genotype effect in number of microglia cells in dorsal or ventral BST (all $p > 0.05$).

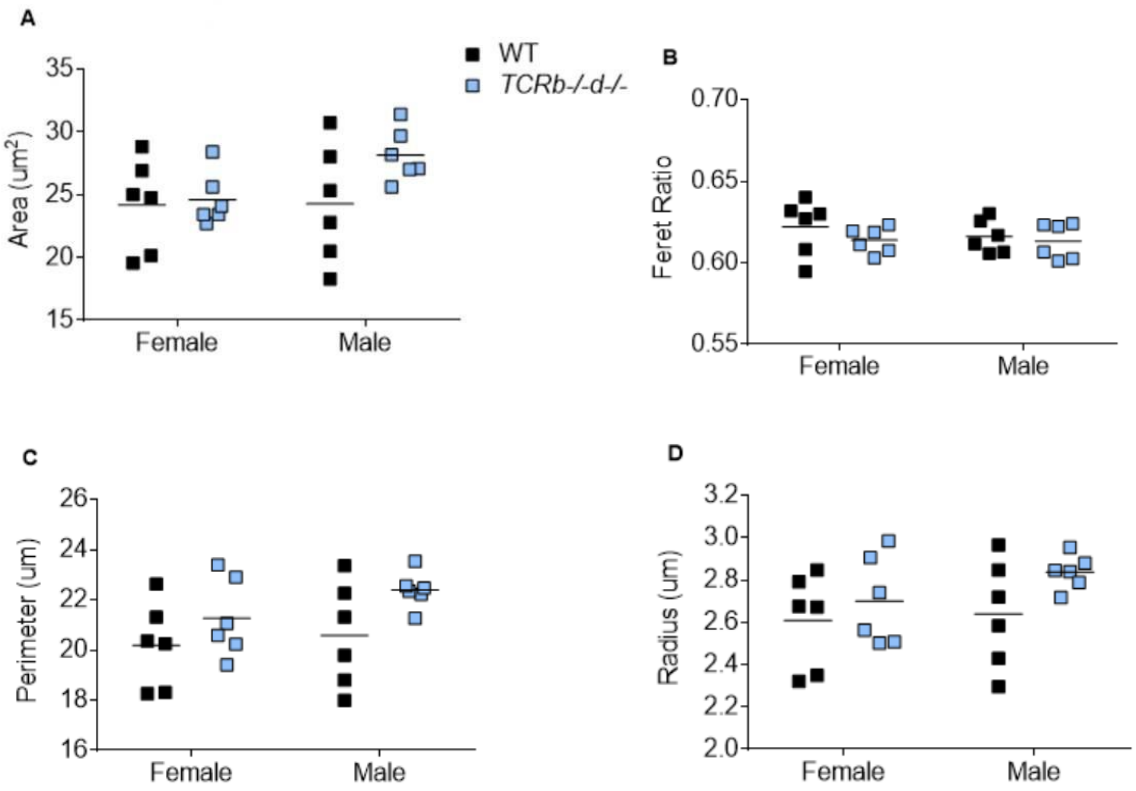

Fig. 8 *Microglia morphology in dorsal BST of WT and TCRβ-/-δ-/- mice at P7.* Microglia cell body was analyzed for area (A), feret ratio (B), perimeter (C), and radius (D) in the dorsal BST of P7 mice. Microglia cell bodies did not show any sex or genotype effects in any of the parameters in the dorsal BST of any group in P7 mice (all $p > 0.05$).

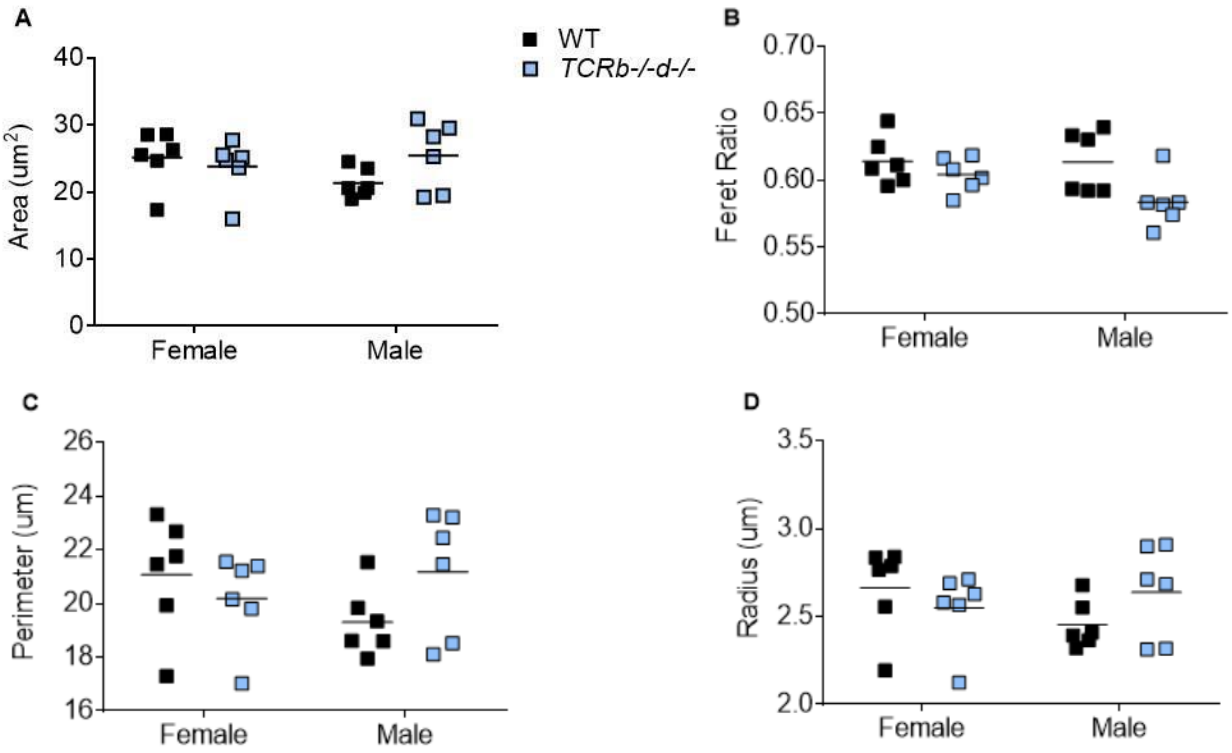

Fig. 9 *Microglia morphology in ventral BST of WT and TCRβ-/-δ-/- mice at P7.* Microglia cell body was analyzed for area (A), feret ratio (B), perimeter (C), and radius (D) in the ventral BST of P7 mice. Microglia cell bodies did not show any sex or genotype effects in any of the parameters in the ventral BST of any group in P7 mice (all $p > 0.05$).

5. Discussion

This study aimed to assess and quantify changes in levels of AMH in *TCRβ-/-δ-/-* and WT mice. It was hypothesized that there will be higher levels of AMH in groups that show larger BST volumes in adulthood. This is based on literature demonstrating an effect of AMH that predisposes a larger BST volume (Walter Wittmann & McLennan, 2013b). Significant effects were found with analysis of both age and genotype, however they were unexpected. Previous research has reported a peak in AMH levels in male mice at P0 with a decline in levels corresponding to increasing postnatal age (Al-Attar et al., 1997). Our analysis did not reveal high levels of AMH in the WT or *TCRβ-/-δ-/-* male groups at P0, but a peak was present in WT mice at P7. This peak in serum AMH levels was absent in the *TCRβ-/-δ-/-* mice. The absence of a peak at P0 in WT male mice may be representative of strain differences as the previous report of postnatal AMH levels was not in WT C57Bl/6 mice. Furthermore, AMH has been reported in literature to contribute more towards male-typical rather than female-typical brain development, therefore the peak at P7 in WT mice of both sexes is a novel finding. It is necessary to conduct further investigation of the non-dimorphic genotype impact on the endocrine system at P7 in order to draw a conclusion about its effect.

The mechanism by which the *TCRβ-/-δ-/-* genotype is contributing to differences in AMH may also be investigated in future work through analysis of additional endocrine factors. There are several reasons for suggesting this line of

investigation. The central regulatory regions contributing to a male- or female-typical endocrine profile show sex-dependent genotype effects in *TCRβ-/-δ-/-* mice. First, the hypothalamus shows a reduced volume in *TCRβ-/-δ-/-* female mice compared to WT females and an increase in volume in *TCRβ-/-δ-/-* male mice compared to WT male mice (Rilett et al., 2015). Second, FSH has been shown to stimulate AMH in gonads (Al-Attar et al., 1997) and FSH levels in the pituitary of WT postnatal mice have been shown to be high until P10 (Halpin et al., 1986). The contribution of pituitary factors versus hypothalamic factors, to sex differences in endocrine factors in *TCRβ-/-δ-/-* mice, remains to be assessed. Next, there are inhibitory/stimulatory processes also occurring at the level of the gonad that contribute to neuroanatomical sex differences and interact with AMH. Testicular testosterone has been shown to inhibit gonadal AMH (Al-Attar et al., 1997). A previous study reported first detecting AMH in mouse ovaries at P6, and AMH expression in granulosa cells was detectable after birth (Munsterberg & Lovell-Badge, 1991). Our analysis revealed AMH in serum as early as P0, albeit at low levels. Furthermore, activity of specific ER subtypes in human granulosa cell preparations has also been shown to impact AMH levels (Grynberg et al., 2012). The effects seen in *TCRβ-/-δ-/-* mice may involve one or more of these different endocrine factors and future work examining them individually or in combination will contribute to our understanding of the immune role in the development of sex differences in the brain.

This study also aimed to assess and quantify changes in microglia numbers in *TCRβ-/-δ-/-* and WT mice. It hypothesized that there will be more microglia in WT males and *TCRβ-/-δ-/-* mice that show larger BST volumes in adulthood. In male mammals, the BST is larger compared to female conspecifics (Rilett et al., 2015; Walter Wittmann & McLennan, 2013b). Microglia have been shown to effect male-typical brain development through a synergistic relationship with endocrine signals (Lenz et al., 2013) suggesting microglia may be mediators of a larger brain volume. Therefore this study hypothesized that a larger brain volume would require more microglia cells, thus there would be more microglia cells in animals that have previously been shown to have a larger BST (Rilett et al., 2015). This hypothesis was validated in the dorsal BST of adult mice where WT females had the smallest number of microglia cells while WT males, and *TCRβ-/-δ-/-* females and males, showed similarly higher numbers of microglia. Previously our lab has shown that *TCRβ-/-δ-/-* mice do not show sex differences in volume of the BST, while in WT mice the females have a BST that is smaller in volume compared to WT males (Rilett et al., 2015). The number of microglia in the dorsal BST demonstrated a similar trend where WT females had fewer microglia cells compared to WT males but the *TCRβ-/-δ-/-* mice showed no sex differences in microglia number (Fig. 4A). This data suggests that microglia cells may be involved in sexual dimorphism of the BST but future work assessing microglia depletion or inhibition alongside changes in brain volume is necessary to draw causal conclusions. The ventral BST did not show any significant sex or genotype

effects which may be representative of differences in cell populations in the dorsal and ventral BST. Indeed, a dorsal-ventral gradient of neuronal excitability has been reported in the BST using a slice preparation from adult mice (Egli & Winder, 2003). Future work examining the BST should continue to delineate the dorsal and ventral BST for regional accuracy when drawing conclusions about this region's contribution to circuitry.

The finding that members of the innate immune system – microglia – were impacted by alteration to the adaptive immune system factors, T lymphocytes, contributes to the proposition that the adaptive-innate communication within the CNS impacts brain parenchyma. Derecki et al. (2010) suggested that impact of T cells on brain function under physiological conditions is likely through meningeal myeloid cells rather than direct T cell influence. This conclusion followed several lines of reasoning: (1) T cells have not been shown to populate the brain parenchyma under physiological conditions; (2) T cells have been shown to populate the meningeal layers surrounding the CNS under physiological conditions; (3) depletion of T cells in the meningeal layer leads to a shift in inflammatory profile of the meningeal myeloid cells which also correlated with behavioural consequences such as impairment in cognitive function (Derecki et al., 2010). Our study followed a similar line of reasoning when assessing changes in microglia in the BST by manipulating the adaptive arm of the immune system to assess effects in the innate immune system however the analysis of microglia did not include assessment of their inflammatory profile. Therefore it is too early

to link innate-adaptive immune communication to the changes evident in brain volume of *TCRβ-/-δ-/-* mice.

At P7, mice did not show a sex or genotype effect in the number of microglia in the dorsal or the ventral BST. It was expected that a similar effect to adult mice would be found at P7 since this time point is past the peak age at which sex determining processes in the BST have previously been reported (Ahern et al., 2013). The lack in effects may be due to the small sample size and future work could re-asses the number of microglia cells in the BST at P7 with a larger number of mice. It would also be interesting to examine microglia in the BST of P5 mice, when apoptotic processes in the BST are at their peak in the postnatal period (Ahern et al., 2013). Correlations in microglia number or morphology with these signals would provide a more whole-some picture of immune cell involvement in brain development. There is also the possibility that there is lag in apoptotic signals and the actual formation of the dimorphic BST, which could extend into the second week of postnatal life. Previous work examining the development of sex differences in BST volume assessed the principle nucleus (BSTp) and it was found that sex differences in the BSTp emerged at P9 (Gotsiridze et al., 2007). Temporal accuracy can be achieved in future analysis of postnatal mice by assessing regional changes along a trajectory.

Although there is still research needed to comprehensively understand how T cells communicate with the CNS, the study of peripheral immune system impact

on central structure and function recently experienced a breakthrough with the finding that functional lymphatic vessels exist in the CNS. These vessels have the capacity to carry fluid and immune cells. These vessels were also found to line the dural sinuses, move cerebrospinal fluid (CSF), and connect to deep cervical lymph nodes (Louveau et al., 2015). Essentially, this finding revealed a "highway" for CNS-immune communication, illustrating that the previously held belief of immune responses being restricted to the periphery and to pathological conditions due to a lack of communication channels is exclusionist of the evidence demonstrating the contrary.

6. Summary and Conclusions

The work reported here demonstrated effects of an immune system factor, the T lymphocyte, on diverse systems in mice. The connections made between these peripheral cells and the CNS in this and previous studies from our lab have shown that the adoption of systems-wide approaches beyond the endocrine system alone are necessary in order to gain a deeper understanding of sexual dimorphism. This line of research holds implications for anxiety-related circuitry in the brain, and subsequently anxiety-related behaviours, which affect the human population in a sex-biased manner. This study also illuminated the importance of looking at circulating factors as a trajectory across different age points, as well as different mouse strains, since results from one study may not replicate across all others. This holds important consequences for interpretation of scientific findings.

7. References

Ahern, T. H., Krug, S., Carr, A. V., Murray, E. K., Fitzpatrick, E., Bengston, L., … Forger, N. G. (2013). Cell death atlas of the postnatal mouse ventral forebrain and hypothalamus: Effects of age and sex. *Journal of Comparative Neurology*, *521*(11), 2551–2569. http://doi.org/10.1002/cne.23298

Al-Attar, L., Noël, K., Dutertre, M., Belville, C., Forest, M. G., Burgoyne, P. S., … Rey, R. (1997). Hormonal and cellular regulation of Sertoli cell anti-Müllerian hormone production in the postnatal mouse. *The Journal of Clinical Investigation*, *100*(6), 1335–43. http://doi.org/10.1172/JCI119653

Ballard, P. L., Baxter, J. D., Higgins, S. J., Rousseau, G. G., & Tomkins, G. M. (1974). General presence of glucocorticoid receptors in mammalian tissues. *Endocrinology*, *94*(4), 998–1002. http://doi.org/10.1210/endo-94-4-998

Barnes, P. J. (1998). Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clinical Science (London, England : 1979)*, *94*(6), 557–72. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9854452

Bernard, A., & Boumsell, L. (1984). [Human leukocyte differentiation antigens]. *Presse médicale (Paris, France : 1983)*, *13*(38), 2311–6. Retrieved from http://europepmc.org/abstract/med/6239187

Brambell, F. W. R. (1927). The Development and Morphology of the Gondas of the Mouse. - Part I. The Morphogenesis of the Indifferent Gonad and of the Ovary. *Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character*, *101*(711), 391–409. Retrieved from http://www.jstor.org/stable/81227

Celio, M. R. (1990). Calbindin D-28k and parvalbumin in the rat nervous system. *Neuroscience*, *35*(2), 375–475. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2199841

Derecki, N. C., Cardani, A. N., Yang, C. H., Quinnies, K. M., Crihfield, A., Lynch, K. R., & Kipnis, J. (2010). Regulation of learning and memory by meningeal immunity: a key role for IL-4. *J Exp Med*, *207*(5), 1067–1080. http://doi.org/10.1084/jem.20091419

Dewing, P., Shi, T., Horvath, S., & Vilain, E. (2003). Sexually dimorphic gene expression in mouse brain precedes gonadal differentiation. *Molecular Brain Research*, *118*(1-2), 82–90. http://doi.org/10.1016/S0169-328X(03)00339-5

Dumont, E. C. (2009). What is the bed nucleus of the stria terminalis? *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, *33*(8), 1289–90.

http://doi.org/10.1016/j.pnpbp.2009.07.006

Durlinger, A., Visser, J., & Themmen, A. (2002). Regulation of ovarian function: the role of anti-Mullerian hormone. *Reproduction*, *124*(5), 601–609. http://doi.org/10.1530/rep.0.1240601

Ebner, F., Brandt, C., Thiele, P., Richter, D., Schliesser, U., Siffrin, V., … Nitsch, R. (2013). Microglial Activation Milieu Controls Regulatory T Cell Responses. *The Journal of Immunology* , *191* , 5594–5602. http://doi.org/10.4049/jimmunol.1203331

Egli, R. E., & Winder, D. G. (2003). Dorsal and ventral distribution of excitable and synaptic properties of neurons of the bed nucleus of the stria terminalis. *Journal of Neurophysiology*, *90*(March 2003), 405–414. http://doi.org/10.1152/jn.00228.2003

Finlay, B. L., & Darlington, R. B. (1995). Linked regularities in the development and evolution of mammalian brains. *Science (New York, N.Y.)*, *268*(5217), 1578–1584. http://doi.org/10.1126/science.7777856

Foster, J. A., Quan, N., Stern, E. L., Kristensson, K., & Herkenham, M. (2002). Induced neuronal expression of class I major histocompatibility complex mRNA in acute and chronic inflammation models. *Journal of Neuroimmunology*, *131*(1-2), 83 – 91. Retrieved from http://resolver.scholarsportal.info/resolve/01655728/v131i1-2/83_ineociiaacim.xml

Garcia-Segura, L. M., Cardona-Gomez, P., Naftolin, F., & Chowen, J. A. (1998). Estradiol upregulates Bcl-2 expression in adult brain neurons. *Neuroreport*, *9*(4), 593–597. Retrieved from http://ovidsp.tx.ovid.com.libaccess.lib.mcmaster.ca/sp-3.19.0a/ovidweb.cgi?QS2=434f4e1a73d37e8cc073476c0173649e725ac3b7ed089f52e243bda157bae60dd8a5becd70ff60e52c313cba7991e0763748e96db6f6a0cb8f7c1c4dbadb08832934a83b8d67f03bd86ee89b4178b7d4397c2f6766d9029c

Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., … Merad, M. (2010). Hematopoietic Waves of Microglial Recruitment and Differentiation Occur in the Central Nervous, *701*(2009).

Glenn, J. A., Ward, S. A., Stone, C. R., Booth, P. L., & Thomas, W. E. (1992). Characterisation of ramified microglial cells: detailed morphology, morphological plasticity and proliferative capability. *Journal of Anatomy*, *180 (Pt 1*, 109–18. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1259614&tool=pm

centrez&rendertype=abstract

Gotsiridze, T., Kang, N., Jacob, D., & Forger, N. G. (2007). Development of sex differences in the principal nucleus of the bed nucleus of the stria terminalis of mice: role of Bax-dependent cell death. *Developmental Neurobiology*, *67*(3), 355–62. http://doi.org/10.1002/dneu.20353

Greco, T. L. (1993). Estrogen receptors, estradiol, and diethylstilbestrol in early development: the mouse as a model for the study of estrogen receptors and estrogen sensitivity in embryonic development of male and female reproductive tracts. *Endocrine Reviews*, *14*(1), 59–71. http://doi.org/10.1210/er.14.1.59

Greco, T. L., & Payne, A. H. (1994). Ontogeny of expression of the genes for steroidogenic enzymes P450 side-chain cleavage, 3 beta-hydroxysteroid dehydrogenase, P450 17 alpha-hydroxylase/C17-20 lyase, and P450 aromatase in fetal mouse gonads. *Endocrinology*, *135*(1), 262–8. http://doi.org/10.1210/endo.135.1.8013361

Grynberg, M., Pierre, A., Rey, R., Leclerc, A., Arouche, N., Hesters, L., … Taieb, J. (2012). Differential regulation of ovarian anti-müllerian hormone (AMH) by estradiol through α- and β-estrogen receptors. *The Journal of Clinical Endocrinology and Metabolism*, *97*(9), E1649–57. http://doi.org/10.1210/jc.2011-3133

Hacker, a, Capel, B., Goodfellow, P., & Lovell-Badge, R. (1995). Expression of Sry, the mouse sex determining gene. *Development (Cambridge, England)*, *121*, 1603–1614.

Halpin, D. M. G., Jones, A., Fink, G., & Charlton, H. M. (1986). Postnatal ovarian follicle development in hypogonadal (hpg) and normal mice and associated changes in the hypothalamic--pituitary ovarian axis. *Reproduction*, *77*(1), 287–296. http://doi.org/10.1530/jrf.0.0770287

Hanisch, U.-K. K., & Kettenmann, H. (2007). Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nature Neuroscience*, *10*(11), 1387–1394. http://doi.org/10.1038/nn1997

Hannema, S. E., & Hughes, I. a. (2007). Regulation of Wolffian duct development. *Hormone Research*, *67*(3), 142–51. http://doi.org/10.1159/000096644

Hazout, A., Bouchard, P., Seifer, D. B., Aussage, P., Junca, A. M., & Cohen-Bacrie, P. (2004). Serum antimüllerian hormone/müllerian-inhibiting substance appears to be a more discriminatory marker of assisted

reproductive technology outcome than follicle-stimulating hormone, inhibin B, or estradiol. *Fertility and Sterility*, *82*(5), 1323–1329. http://doi.org/10.1016/j.fertnstert.2004.03.061

Hedrick, A. V., & Temeles, E. J. (1989). The evolution of sexual dimorphism in animals: Hypothesis and tests. *Trends in Ecology & Evolution*.

Iwasaki, A., & Medzhitov, R. (2010). Regulation of adaptive immunity by the innate immune system. *Science (New York, N.Y.)*, *327*(5963), 291–5. http://doi.org/10.1126/science.1183021

Josso, N., Picard, J. Y., & Tran, D. (1977). The anti-Müllerian hormone. *Birth Defects Original Article Series*, *13*(2), 59–84. Retrieved from http://europepmc.org/abstract/med/617047

Karperien, A., Ahammer, H., & Jelinek, H. F. (2013). Quantitating the subtleties of microglial morphology with fractal analysis. *Frontiers in Cellular Neuroscience*, *7*, 3. http://doi.org/10.3389/fncel.2013.00003

Khalid, R. (2014). Contributing factors in multiple sclerosis and the female sex bias. *Immunology Letters*, *162*(1), 223–232. http://doi.org/10.1016/j.imlet.2014.09.004

Kipnis, J., Gadani, S., & Derecki, N. C. (2012). Pro-cognitive properties of T cells. *Nature Reviews. Immunology*, *12*(9), 663–9. http://doi.org/10.1038/nri3280

Koopman, P., Münsterberg, a, Capel, B., Vivian, N., & Lovell-Badge, R. (1990). Expression of a candidate sex-determining gene during mouse testis differentiation. *Nature*. http://doi.org/10.1038/348450a0

Lebeurrier, N., Launay, S., Macrez, R., Maubert, E., Legros, H., Leclerc, A., … Vivien, D. (2008). Anti-Mullerian-hormone-dependent regulation of the brain serine-protease inhibitor neuroserpin. *Journal of Cell Science*, *121*(Pt 20), 3357–3365. http://doi.org/10.1242/jcs.031872

Lenz, K. M., Nugent, B. M., Haliyur, R., & McCarthy, M. M. (2013). Microglia are essential to masculinization of brain and behavior. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *33*(7), 2761–72. http://doi.org/10.1523/JNEUROSCI.1268-12.2013

Lewinsohn, P. M., Gotlib, I. H., Lewinsohn, M., Seeley, J. R., & Allen, N. B. (1998). Gender differences in anxiety disorders and anxiety symptoms in adolescents. *Journal of Abnormal Psychology*, *107*(November 2015), 109–117. http://doi.org/10.1037//0021-843X.107.1.109

Lin, H.-H., Faunce, D. E., Stacey, M., Terajewicz, A., Nakamura, T., Zhang-Hoover, J., … Stein-Streilein, J. (2005). The macrophage F4/80 receptor is required for the induction of antigen-specific efferent regulatory T cells in peripheral tolerance. *The Journal of Experimental Medicine*, *201*(10), 1615–25. http://doi.org/10.1084/jem.20042307

Louveau, A., Smirnov, I., Keyes, T. J., Eccles, J. D., Rouhani, S. J., Peske, J. D., … Kipnis, J. (2015). Structural and functional features of central nervous system lymphatic vessels. *Nature*, *523*(7560), 337–337–341. http://doi.org/10.1038/nature14432

Lu, Q. L., Poulsom, R., Wong, L., & Hanby, a M. (1993). Bcl-2 expression in adult and embryonic non-haematopoietic tissues. *The Journal of Pathology*, *169*(4), 431–437.

MacLusky, N. J., & Naftolin, F. (1981). Sexual differentiation of the central nervous system. *Science*, *211*(4488), 1294–1303.

McLennan, I. S., & Pankhurst, M. W. (2015). Anti-müllerian hormone is a gonadal cytokine with two circulating forms and cryptic actions. *Journal of Endocrinology*, *226*(3), R45–R57. http://doi.org/10.1530/JOE-15-0206

Miller, W. L. (1988). Molecular biology of steroid hormone synthesis. *Endocrine Reviews*, *9*(3), 295–318. http://doi.org/10.1210/edrv-9-3-295

Mogensen, T. H. (2009). Pathogen recognition and inflammatory signaling in innate immune defenses. *Clinical Microbiology Reviews*, *22*(2), 240–273. http://doi.org/10.1128/CMR.00046-08

Morris, G. P., Clark, I. A., Zinn, R., & Vissel, B. (2013). Microglia: A new frontier for synaptic plasticity, learning and memory, and neurodegenerative disease research. *Neurobiology of Learning and Memory*, *105*, 40–53. http://doi.org/10.1016/j.nlm.2013.07.002

Munsterberg, A., & Lovell-Badge, R. (1991). Expression of the mouse anti-mullerian hormone gene suggests a role in both male and female sexual differentiation. *Development*, *113*(2), 613–624. Retrieved from http://dev.biologists.org.libaccess.lib.mcmaster.ca/content/113/2/613.long

Murphy, K. M. (2011). *Janeway's immunobiology*. Garland Science.

Neufeld, K. M., Kang, N., Bienenstock, J., & Foster, J. A. (2011). Reduced anxiety-like behavior and central neurochemical change in germ-free mice. *Neurogastroenterology and Motility*, *23*(3), 255–265. http://doi.org/10.1111/j.1365-2982.2010.01620.x

Neufeld, K.-A. M., Kang, N., Bienenstock, J., & Foster, J. A. (2011). Effects of intestinal microbiota on anxiety-like behavior. *Communicative & Integrative Biology*, *4*(4), 492–4. http://doi.org/10.4161/cib.4.4.15702

Ohsawa, K., Imai, Y., Sasaki, Y., & Kohsaka, S. (2004). Microglia/macrophage-specific protein Iba1 binds to fimbrin and enhances its actin-bundling activity. *Journal of Neurochemistry*, *88*(4), 844–56. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14756805

Orvis, G. D., & Behringer, R. R. (2007). Cellular mechanisms of Mullerian duct formation in the mouse. *Dev Biol*, *306*(2), 493–504. http://doi.org/S0012-1606(07)00228-X [pii]\r10.1016/j.ydbio.2007.03.027

Remes, O., Brayne, C., Linde, R. Van Der, & Lafortune, L. (2014). A systematic review of reviews on the prevalence of anxiety disorders in adult populations. *Prospero*, *2*. http://doi.org/10.1002/brb3.497

Rilett, K. C., Friedel, M., Ellegood, J., MacKenzie, R. N., Lerch, J. P., & Foster, J. A. (2015). Loss of T cells influences sex differences in behavior and brain structure. *Brain, Behavior, and Immunity*, *46*, 249–260. http://doi.org/10.1016/j.bbi.2015.02.016

Sankar, A., MacKenzie, R. N., & Foster, J. A. (2012). Loss of class I MHC function alters behavior and stress reactivity. *Journal of Neuroimmunology*, *244*(1-2), 8–15. http://doi.org/10.1016/j.jneuroim.2011.12.025

Scotland, R. S., Stables, M. J., Madalli, S., Watson, P., & Gilroy, D. W. (2011). Sex differences in resident immune cell phenotype underlie more efficient acute inflammatory responses in female mice. *Blood*, *118*(22), 5918–27. http://doi.org/10.1182/blood-2011-03-340281

Sekido, Ryohei; Bar, Isabelle; Narvaez, Veronica; Penny, G. L.-B. R. (2004). SOX9 is up-regulated by the transient expression of SRY specifically in Sertoli cell precursors. *Developmental Biology*, *274*(2), 271 – 279. Retrieved from http://resolver.scholarsportal.info/resolve/00121606/v274i0002/271_siubttssiscp.xml

Shi, Y., & Massagué, J. (2003). Mechanisms of TGF-β Signaling from Cell Membrane to the Nucleus. *Cell*, *113*(6), 685–700. http://doi.org/10.1016/S0092-8674(03)00432-X

Sidor, M. M., Halgren, C. R., & Foster, J. A. (2014, February 15). The impact of early life immune challenge on behavior and microglia during postnatal development. *Inflammation and Cell Signaling*. Retrieved from

http://www.smartscitech.com/index.php/ICS/article/view/83

Takeda, K., Kaisho, T., & Akira, S. (2003). Toll-Like Receptors. *Annual Review of Immunology*, *21*(1), 335–376. http://doi.org/10.1146/annurev.immunol.21.120601.141126

Toran-Allerand, C. D. (1976). © Elsevier Scientific Publishing Company , Amsterdam - Printed in The Netherlands Sex steroids and the development of the newborn mouse hypothalamus and preoptic area in vitro : implications for sexual differentiation (Accepted January 12th , 1976) Sexu, *106*, 407–412.

Tsukahara, S. (2009). Sex differences and the roles of sex steroids in apoptosis of sexually dimorphic nuclei of the preoptic area in postnatal rats. *Journal of Neuroendocrinology*, *21*(4), 370–376. http://doi.org/10.1111/j.1365-2826.2009.01855.x

Walker, D. L., Toufexis, D. J., & Davis, M. (2003). Role of the bed nucleus of the stria terminalis versus the amygdala in fear, stress, and anxiety. *European Journal of Pharmacology*, *463*(1-3), 199–216. http://doi.org/10.1016/S0014-2999(03)01282-2

Wang, P.-Y., Koishi, K., McGeachie, A. B., Kimber, M., Maclaughlin, D. T., Donahoe, P. K., & McLennan, I. S. (2005). Mullerian inhibiting substance acts as a motor neuron survival factor in vitro. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(45), 16421–5. http://doi.org/10.1073/pnas.0508304102

Wang, P.-Y., Protheroe, A., Clarkson, A. N., Imhoff, F., Koishi, K., & McLennan, I. S. (2009). Müllerian inhibiting substance contributes to sex-linked biases in the brain and behavior. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(17), 7203–8. http://doi.org/10.1073/pnas.0902253106

Werling, D. M., & Geschwind, D. H. (2013). Sex differences in autism spectrum disorders. *Current Opinion in Neurology*, *26*(2), 146–53. http://doi.org/10.1097/WCO.0b013e32835ee548

Wittmann, W., & Mclennan, I. S. (2011). The Male Bias in the Number of Purkinje Cells and the Size of the Murine Cerebellum may Require M??llerian Inhibiting Substance/Anti-M??llerian Hormone. *Journal of Neuroendocrinology*, *23*(9), 831–838. http://doi.org/10.1111/j.1365-2826.2011.02187.x

Wittmann, W., & McLennan, I. S. (2013a). Anti-Mullerian hormone may regulate

the number of calbindin-positive neurons in the sexually dimorphic nucleus of the preoptic area of male mice. *Biology of Sex Differences*, *4*(1), 18. http://doi.org/10.1186/2042-6410-4-18

Wittmann, W., & McLennan, I. S. (2013b). The bed nucleus of the stria terminalis has developmental and adult forms in mice, with the male bias in the developmental form being dependent on testicular AMH. *Hormones and Behavior*, *64*(4), 605–610. http://doi.org/10.1016/j.yhbeh.2013.08.017

Wlodarczyk, A., Løbner, M., Cédile, O., & Owens, T. (2014). Comparison of microglia and infiltrating $CD11c^+$ cells as antigen presenting cells for T cell proliferation and cytokine response. *Journal of Neuroinflammation*, *11*(1), 57. http://doi.org/10.1186/1742-2094-11-57

Wolf, Y., Yona, S., Kim, K.-W., & Jung, S. (2013). Microglia, seen from the CX3CR1 angle. *Frontiers in Cellular Neuroscience*, *7*, 26. http://doi.org/10.3389/fncel.2013.00026

Zhao, L., Wu, T. W., & Brinton, R. D. (2004). Estrogen receptor subtypes alpha and beta contribute to neuroprotection and increased Bcl-2 expression in primary hippocampal neurons. *Brain Research*, *1010*(1-2), 22–34. http://doi.org/10.1016/j.brainres.2004.02.066