Curriculum Vitae

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Education

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PUBLICATIONS

VanRyzin, J. W., Arambula, S. E., Ashton, S. E., Blanchard, A. C., Burzinski, M. D., Davis, K. T., Edwards, S., Graham, E. L., Holley, A., Kight, K. E., Marquardt, A. E., Perez-Pouchoulen, M., **Pickett, L. A.,** Reinl, E. L., & McCarthy, M. M. (2021). Generation of an Iba1-EGFP Transgenic Rat for the Study of Microglia in an Outbred Rodent Strain. *eNeuro*, *8*(5), ENEURO.0026-21.2021.

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Pickett, L. A., Yourshaw, M., Albornoz, V., Chen, Z., Solorzano-Vargas, R. S., Nelson, S. F., Martín, M. G., & Lindberg, I. (2013). Functional consequences of a novel variant of PCSK1. *PloS one*, *8*(1), e55065.

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ABSTRACTS and PRESENTATIONS

Pickett, L.A., Lenz, K.M., Galan, A., & McCarthy, M.M. (2015). Mast cells and microglia mediate sexual differentiation of the preoptic area. Ninth Annual Meeting of the Organization for the Study of Sex Differences.

Perez-Pouchoulen M., VanRyzin J. W., **Pickett L. A**, & McCarthy M. M. (2014). Developmental profile of microglia and evidence of phagocytic activity in the postnatal developing rat cerebellum. *44th Annual Meeting of the Society for Neuroscience*, Washington DC. Poster presentation.

Pickett, L. A., Rosinsky, C., McCarthy, M. M., & Lenz, K. M. (2014). Mast cells contribute to early life programming of sex differences in brain and behavior. Program No:166.02. Soc Neurosci Abstr 166.02 *43rd Annual Meeting of the Society for Neuroscience*.

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McCarthy M. M., **Pickett L. A.**, VanRyzin J. W., & Lenz K. L. (2014). Microglia, mast cells and the masculinization of brain and behavior. *EMBL Conference*, Heidelberg, Germany.

Lenz, K. M., **Pickett, L. A**., Swaim H. L., & McCarthy, M. M. (2013). Sex differences in newly born microglia, mast cells and neuroimmune signaling in the developing brain. Program No:473.02. Soc Neurosci Abstr 473.02 Forty-second Annual Meeting of the Society for Neuroscience.

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Yourshaw M., Solorzano-Vargas R. S., **Pickett L. A.**, Lindberg I., Wang J., Cortina G., Baron H., Nelson S. F., & Martín M.G. (2014). Diagnosis of a Mysterious Case of Proprotein Convertase (PC1/3) Deficiency in a Patient with Malabsorptive Diarrhea with Next Generation Sequencing. *Gastroenterology.* 2014;142(5) :S–17

Abstract

Mast Cells & Microglia "organize" preoptic area neuronal circuitry during perinatal brain development for adult sexual behavior.
Lindsay A. Pickett, Doctor of Philosophy, 2022

Dissertation Directed by: Margaret M. McCarthy, Ph.D., Professor,

Department of Pharmacology

Organization of neuronal circuitry required for adult sexual behavior occurs during perinatal development and is largely carried out by resident innate immune cells, mast cells and microglia, just prior to birth and during the first week of life in the rodent, when the male brain makes estradiol from precursor androgens secreted by the testes. Mast cells degranulate in response to estradiol in the preoptic area (POA), releasing histamine, which in turn stimulates prostaglandin E2 (PGE2) production by microglia. The mechanism of PGE2-mediated differentiation of excitatory synaptic density in the preoptic area (POA) around birth is known and results in male sexual behavior in adulthood. Aberrant mast cell activation masculinizes POA spine density and sexual behavior of females and demasculinizes these measures in males. Despite its dependence on gonadal hormones for differentiation, the sexually dimorphic nucleus (SDN) of the POA does not begin differentiating until brain hormone levels are no longer different between the sexes, on postnatal day 5 (PN5). In the SDN, males and females are born with the same number of neurons. However, neurons selectively die off in females, whereas in males they are thought to be protected by the production of estradiol at birth. The SDN is located within the central medial preoptic nucleus (cMPN), where we

discovered females have more phagocytic microglia than males during the first postnatal week, a phenomenon that peaked on postnatal day 8 (PN8). Inhibition of microglial phagocytosis by intracerebral injections of an antibody to CD11b (complement receptor 3, CR3) or mast cell degranulator, c48/80, from PN5-7 reduced microglial phagocytosis and increased the volume of the SDN in both sexes on PN8, demonstrating that microglia are engaging in phagoptosis (engulfment of stressed, but viable cells) to shape the size of the SDN. Females treated neonatally with the CD11b (CR3) antibody blockade lost their typical sexual preference for male odor in adulthood. This discovery challenges the dogma that estradiol prevents neuronal apoptosis in the male SDN and reveals novel hormone and neuroimmune mechanisms that regulate phagocytic and neuroprotective cascades during normal brain development.

Mast Cells & Microglia “organize” preoptic area neuronal circuitry during perinatal brain development for adult sexual behavior.

by
Lindsay A. Pickett

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2022

Dedication

This dissertation is dedicated to Dr. Mike Bowers for his one-of-a-kind style of mentorship, education, and inspiration. From the very start of my studies in the McCarthy lab, he believed in me, made sure I knew it and relentlessly tried to convince me of the same. The neuroscience community has lost one of its brightest stars with his passing this year, but his spirit surely lives on in every student that he met, mentored and inevitably, inspired.

And to my brother, who for much of my earlier life, devoted countless hours, and loads of physical and emotional energy in opposition to Mike's theory. He sure would be grinning ear to ear with glee to see what I did with everything he gave me. Oh, and I may not have had the guts, determination, and general ninja skills, without his trial-by-fire training, which all prepared me to take on this Ph.D., this life.

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To my closest companions since college who picked me up from the airport here in Baltimore after my year teaching abroad and no place to call home and said "Good news- tomorrow morning you have a job interview at 10 and in the evening, we will take you to see your new apartment. You're staying, whether you like it or not!"

To my husband for coming to the U.S. for a "year-long" research position that ultimately became a second residency in Surgery, and a permanent position in my life as my partner and father of our two young boys. I thank my boys for being my most important experiments in graduate school. Becoming a parent has changed me for the better.

Thank you to my brother, whose life was short but, was lived to the fullest. He helped make me strong, independent, and brave. I thank him for it all- the good, the bad and the ugly. He taught me life is too short for "what ifs" so better to set your aim high and give it all you got, even when what you aim to achieve seems "impossible".

I am forever thankful for the loving support, confidence, and "extra push" that my parents have always provided, even when from a distance. My mother pushes me to be my best and do what's right, even if it's the more difficult thing to do-which it always is. I thank my father, a Vietnam Airforce veteran, for his silly & sordid sarcasm and never-ending, fountain of phrases to put life's problems in perspective:

"Don't sweat the small stuff and don't forget-it's all small stuff.
Hell, nobody is shooting at us, am I right?"

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List of Abbreviations

5-HT	Serotonin
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AVPV	anteroventral periventricular
BBB	blood brain barrier
BMMCs	bone marrow-derived mast cells
BNSTp	principal nucleus of the bed nucleus of the stria terminalis
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
CALB	calbindin
cAMP	cyclic adenosine monophosphate
CCasp3	cleaved caspase 3
CCK	cholecystokinin
Cckar	cholecystokinin A receptor
CD11b/CR3A	cluster of differentiation molecule 11b/complement receptor 3A
COX	cyclooxygenase
CR3/MAC-1	complement receptor 3/macrophage-1 antigen
CRF	corticotropin-releasing factor

CSF1R	colony-stimulating factor 1 receptor
CX3CR1	C-X3-C motif chemokine receptor 1
DAB	3,3'-diaminobenzidine
DAP12/DNAX	activation protein of 12 kilodaltons
DEHP	di-(2-ethylhexyl) phthalate
E	embryonic day
E2	estradiol
Egr1	early growth response protein 1
ELA	early life adversity
ELISA	enzyme-linked immunosorbent assay
GABA	γ-aminobutyric acid
GD	gestational day
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GnRH	gonadotropin-releasing hormone
HE	hematopoietic erythropoiesis
Iba1	ionized calcium-binding adapter molecule 1
ICV	intracerebroventricular

IF	immunofluorescence
IgE	immunoglobulin E
IGF-1	insulin-like growth factor 1
IHC	immunohistochemistry
IL-10	interleukin 10
INAH-3	third interstitial nucleus of the anterior hypothalamus
IP	intraperitoneal
LGN	lateral geniculate nucleus
LH	luteinizing hormone
LPS	lipopolysaccharide
MAP2	microtubule associated protein 2p
MC	mast cell
Mcpt2	mast cell protease 2
MPN	medial preoptic nucleus
MPOA	medial preoptic area
NeuN	neuronal nuclei
NPC	neural progenitor cell
OVA	ovalbumin

OVLT	vascular organ of lamina terminalis
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline containing Triton X-100
PCD	programmed cell death
PFA	paraformaldehyde
PGD2	prostaglandin D2
PGE2	prostaglandin E2
PKA	protein kinase A
PN	postnatal day
POA	preoptic area
PSA	passive systemic anaphylaxis
PVN	paraventricular nucleus of hypothalamus
qPCR	quantitative polymerase chain reaction
RS	restraint stress
SDN	sexually dimorphic nucleus
SFO	subfornical organ
SRY	sex-determining region Y
TBS	tris-buffered saline

TBS-T	tris-buffered saline containing Triton X-100
TDF	testes-determining factor
TNF	tumor necrosis factor
TNFα	tumor necrosis factor alpha
VMHvl	ventrolateral part of the ventromedial hypothalamus
♀	female (XX)
♂	male (XY)

Chapter 1: Introduction

1.1 Sexual Differentiation of the Brain

The origins and extent of behavioral differences between males (XY) and females (XX), men and women, and boys and girls, have long been a topic of interest and debate. In contrast, the idea that the brain is the principal driver of behavioral differences between males and females is quite modern, having only been accepted as a possibility following Frank Beach's argument that the critical variable determining behavioral differences between the sexes was the type of genitalia one possessed: either intromitting or receiving (Beach, 1974; Phoenix et al, 1959). This frame of reference was soon overturned with an iconic paper published by William C. Young and colleagues in 1959, using the guinea pig as a model. The study convincingly demonstrated that prenatal hormonal exposure could reverse the sexual behavior of females in adulthood (Phoenix et al, 1959). While this and other studies ended the debate about what organ was driving sex differences in behavior, it also generated a degree of myopia as the subsequent decades were dedicated to reproductive behavior and physiology. These studies lead to the widespread belief that sex differences in the brain are narrow in both their scope and significance, being limited to the control of the anterior pituitary gland, which is important for courtship, copulation, and parenting behaviors. Hormonal modulation of neural plasticity was a finding that made space for brain sex differences outside the context of reproduction when the McEwen lab discovered that dendritic spine density on hippocampal pyramidal neurons varied by almost 30% across the few days of the estrus cycle in female rats (Woolley et al., 1992). This amount of neuronal plasticity was deemed remarkable and initial reports were met with skepticism. However, an abundance

of data led to the general acceptance that hormones are powerful regulators of neuronal function outside of the diencephalon and outside the context of reproduction. Importantly, however, modulation of adult neural function or behavior by hormones is not the same as sex differences in neural function or behavior. Studies of adult functions known to be impacted by steroids in a modulatory manner that are sexually differentiated are relatively few. For instance, estradiol alters synaptic physiology and cognitive function in adult female rats (Reviewed by Taxier et al., 2020 & Hara et al., 2015) but whether these same endpoints are subject to sexual differentiation is a more complex query to probe due to the different hormonal landscape of males and females after puberty. Drawing comparisons during adulthood is tricky. Should estrous females be compared to gonadally intact males? Should both sexes be gonadectomized and hormone replaced to standardize their endocrinology? If the latter is your chosen route, which hormonal profile should gonadectomized animals be supplied with, or does one test both? The possibilities alone are intimidating. Further complexity is brought on when we consider a lifetime of experience and environment, which can vary in profound and significant ways between adult males and females. Differences due to these variables are lessened, however, when the study scope is narrowed to early development; a time when the effects of environment and experience have not had much time to accumulate.

The rodent is most frequently used as a model system for the study of sex differences, especially the rat because its neuroanatomical and behavioral sex differences are more robust than those in the mouse. An added advantage of using the rodent to study developmental sex differences, is that the rodent's brain is "premature" at birth and the first 10 days of postnatal life are comparable to the last trimester of pregnancy in

primates. The embryonic androgen surge in male rodents occurs during a "critical period" in which the male must receive adequate hormonal input for proper masculinization of brain and body. In the absence of adequate hormonal input (or receipt of that input by mutations or inadequate receptor expression) during this critical period, the brain will not be fully masculinized (resulting in females and unmasculinized males) (McCarthy et al., 2018). Interestingly, evidence also suggests that exposure to higher-than-average levels of androgen during this time can negatively affect brain masculinization such that males perform their sex-typical behaviors poorly or not at all as adults (Slob AK, 1991). Female rodents treated with exogenous testosterone or its major metabolite, estradiol, during this critical period (or just after it, during an extended "sensitive period" a week after birth, will be masculinized. In adulthood, developmentally masculinized females will perform male-typical behaviors if "activated" with an exogenous supply of male-typical hormones. Behavioral measures of masculinization include preferring the odor and presence of an estrus female over a sexually active male and animals will even mount and fail to exhibit female-typical sexual behavior in the presence of a stimulus male (Henley CL, 2009). Masculinization of the brain during this critical period is often referred to as the "organizational hypothesis" (Figure 1.1), which was posited after many of the initial studies laid the groundwork in the field of neuroendocrinology and sexual differentiation (Phoenix et al., 1959). The organization of the perinatal brain is crucial to formation of the sex-specific blueprints upon which the adult endocrine system later acts to enable sex-typical behaviors such as aggression, play, odor and partner preference, and the full complement of sexual behaviors in the presence of a willing partner, typically of the opposite sex. Sexual differentiation of the brain

occurs by a variety of mechanisms that differ by brain region and neuroanatomical or behavioral endpoints ranging from synaptic density, cell numbers, nucleus volume, morphology, etc. (McCarthy et al, 2017). These early architectural sex differences ultimately set the stage for the expression of sex-typical behavior (e.g., mating behavior) in mature animals, so that an organism's behavioral phenotype matches its gonadal sex when it comes time to mate. Sexual differentiation of both body and brain is a highly conserved process of great importance for survival and propagation of the species.

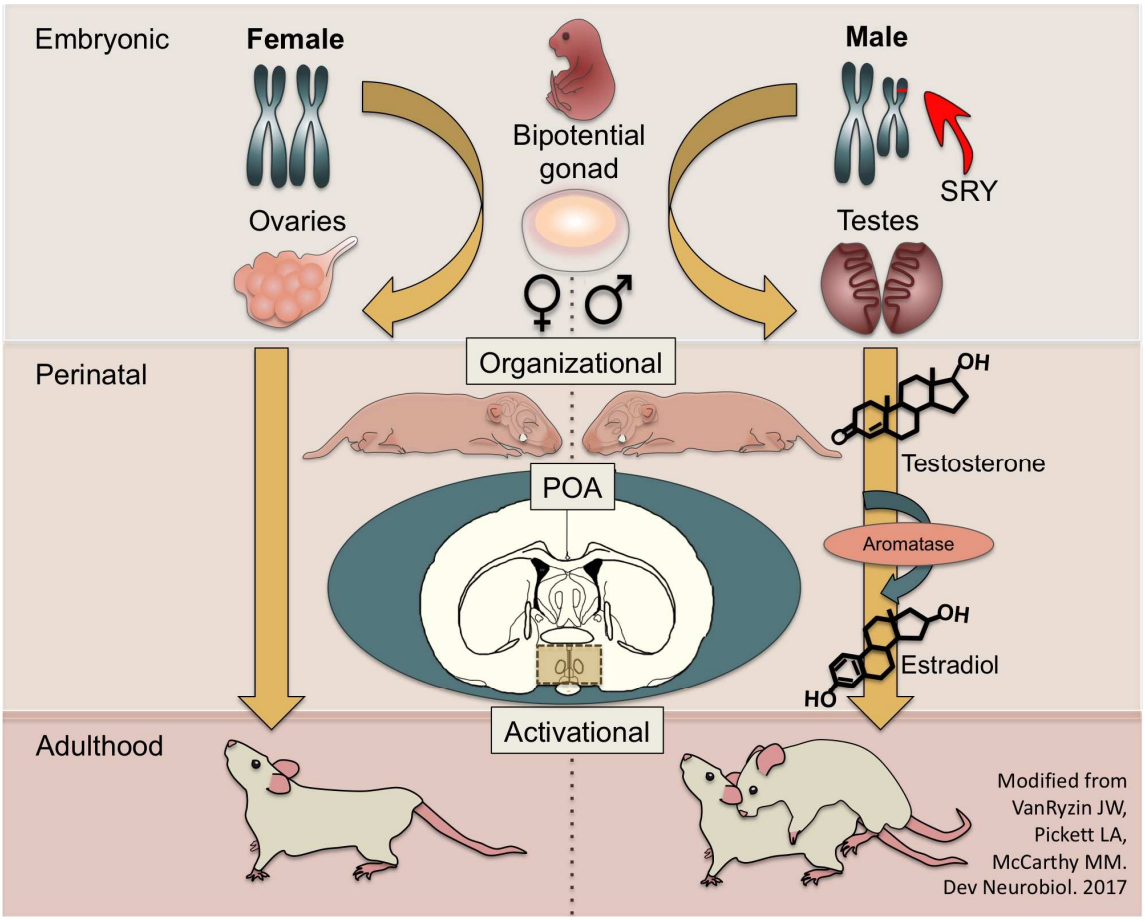


Figure 1.1. The "Organizational Hypothesis" of Brain Sexual Differentiation in the rodent. This figure illustrates rodent sexual differentiation from its initiation, when transcription of Testes-Determining Factor (TDF) from SRY on the Y chromosome occurs in males, and subsequent organizational effects of testosterone and its metabolites on the brain via local aromatase expression in regions of the brain, such as the preoptic area (POA), during perinatal development. Perinatal hormonal exposure (or lack thereof in females) aligns the brain with the genetic/gonadal sex of the rodent, which is later activated in puberty and maintained in adulthood via differential circulating hormones released from mature ovaries or testes.

1.2 Sexual Differentiation of POA and Behavior

The medial preoptic area is a region of the brain of telencephalic origin, located anterior to (but very much interconnected to, and often mistakenly treated as a part of) the hypothalamus, which is of diencephalic origin. The medial POA is positioned below the anterior commissure, above the optic chiasm and between the rostral valleys of the lateral ventricles when visualized in a coronal slice. Its borders are drawn naturally by the middle cerebral arterial branches moving up dorsally from the anterior cerebral arteries that make up the circle of Willis at the ventral surface of the brain.

This region is highly vascularized and shares neural connectivity and close proximity to the organum vasculosum (OVLT) and the subfornical organ (SFO), which are sensory circumventricular organ systems in which the blood-brain barrier is relatively permissive to allow for rapid monitoring of blood constituents (hormones, salt, glucose) for rapid regulation of necessary primitive autonomic functions such as blood osmolality, glucose metabolism, satiety, arousal and sleep (reviewed in Johnson & Gross, 1993).

The preoptic area is a region with an impressive array of efferent and afferent projections reaching and being reached by almost every part of the brain. It is, in a sense, an "interpretation station" of the most imperative rapid autonomic nervous system functions required for basic survival. In addition to its important role in everyday arousal and meeting the organism's metabolic demands via initiation of requisite behaviors, the POA is crucial to orchestrating what are perhaps the most evolutionarily important of deeds- an organism's sexual and parental behaviors. One of the most primitive and necessary behaviors for successful propagation of an organism's species (step one) is the

detection of cues from members of the same species but of the opposite sex who are fit and equipped to execute mating and reproduction. One region of the brain, named for its volumetric sex difference, that has been correlated to sexually dimorphic detection of these cues (sexual preference), is the Sexually Dimorphic Nucleus of the POA (Hofman et al.1989; LeVay, 1991; Swaab et al.,1995, Roselli et al., 2004; Figure 1.2).

The first robust sex difference discovered in the healthy mammalian brain was aptly named the sexually dimorphic nucleus of the preoptic area (SDN-POA) (Gorski et al, 1978, Gorski et al, 1980) and is one of, if not the most, extensively studied sex differences in the brain. It is located under the anterior commissure, above the optic chiasm, lateral to the third ventricle, and anterior to the hypothalamus (Figure 1.2). Neurogenesis in the SDN begins at embryonic day (E) 14 and ends on E18. The sensitive period during which the size of the SDN is influenced by gonadal steroids is from E18 to postnatal day 4 (PN4) (Jacobson and Gorski, 1981; Orikasa et al, 2010; Rhees et al, 1990a, Rhees et al, 1990b). Although first described as a densely packed bundle of neurons revealed with a simple Nissl stain (Gorski et al, 1978, Gorski et al, 1980), the SDN has since been more clearly defined by a subset of cells that are immunopositive for the expression of Calbindin-D28k (a calcium-binding protein) expressed in GABAergic (γ-aminobutyric acid- expressing neurons) neurons has been implicated in neuroprotection from excitotoxicity (Brager 1999). It is thus now also referred to as the CALB-SDN (Kato et al, 2012; Sickel and McCarthy, 2000), and is used to identify the SDN region in the mouse brain, where identification by a Nissl stain is more difficult. Sex differences in the SDN (as defined by Nissl stain) and the CALB-SDN (as defined by Calbindin-D28k immunodetection) are of the same magnitude and both arise after PN4.

During early development, the SDN of males and females have similar numbers of neurons. However, due to greater numbers of apoptotic cells undergoing DNA fragmentation in females between PN6 and PN9, this region becomes markedly smaller in females (Davis et al, 1996). Other studies support the role of apoptosis in sexual differentiation of the SDN by demonstrating that the male SDN, which maintains its size, has higher expression of anti-apoptotic Bcl-2 (at the protein but not mRNA level), while the female SDN shows higher expression of pro-apoptotic Bax from PN5–PN7 relative to males. Apoptosis via caspase-3 activation occurs at higher rates in the female SDN and this corresponds with the decrease in size of the female SDN. Sex differences in pro-apoptotic Bax and Bcl-2 levels are abolished in response to treatment of females with estradiol, but the mechanism by which estradiol modulates Bcl-2 and Bax in the SDN is not yet clear (Tsukahara et al, 2008, Tsukahara et al, 2006). Interestingly, when sex differences in the CALB-SDN were investigated in a Bax knockout mouse, there were no significant increases in the size of the CALB-SDN as compared to their wild type counterparts, suggesting that Bax expression is not the primary mechanism by which cells are dying off in females (Gilmore et al, 2012). A role for estradiol upregulation of calbindin and calretinin expression in males has been considered but not clearly demonstrated. Elevated levels of these calcium-binding proteins may protect cells from toxicity that could be the result of excessive neuronal excitation in males compared to females, during the androgen surge (Brager et al, 1999). Although testosterone treatment can upregulate calbindin and calretinin expression in the hypothalamus (Brager et al., 2000; Watson et al, 1998), this has not been demonstrated specifically in the SDN. Thus, despite much speculation over mechanisms by which estradiol regulates the volume of

the SDN, no consensus has been reached and the origins of this iconic sex difference have remained a mystery. Following the discovery of the SDN in the rodent, analogous structures were reported in the ferret (Baum et al, 1996; Park et al, 1996), sheep (Roselli et al, 2004), and primates, including humans (Hofman et al.1989; LeVay, 1991; Swaab et al.,1995). This sexually dimorphic nucleus has since been more extensively studied and found to be significantly larger in males compared to females in at least 9 other species, including humans (Hofman and Swaab, 1989).

Although the regulation of cell death in the female MPN has been studied most rigorously in the first week of life of the rodent, some earlier studies have suggested there may be a role for circulating hormones in regulation or maintenance of this nucleus throughout the lifespan. In an adult rat, gonadectomy reduces the size of the SDN, and this reduction is not seen when testosterone is replaced post-gonadectomy (Commins and Yahr, 1984a; Bloch and Gorski, 1988). In addition, there is evidence to suggest that during puberty the male rat SDN and medial amygdala (Me) experiences a greater expansion of newly born cells than females, while females gain more newly born cells in the AVPV, which is typically larger in females than males (Ahmed et al, 2008). Although Ahmed et al. initially reported that cells born during puberty were mostly neurons in the AVPV (NeuN+) and mostly astrocytes in the Me (GFAP+), the cells of the SDN did not contain appreciable numbers of either mature neurons (NeuN) or astrocytes (GFAP) that were also immunopositive for BrdU at the timepoints surveyed (Ahmed et al., 2008). A follow up study surveyed the integration of cells born during puberty into these regions at a later point in adulthood, in the Syrian hamster. They found that equal numbers of neurons and astrocytes were born in both the MPOA/SDN and in the Me. At the time

these regions were surveyed, approximately 20% of pubertally born cells were neurons and astrocytes in the MPOA/SDN and 30% in the Me, so majority of the cells born in both regions during puberty that becoming functionally integrated into limbic and hypothalamic circuits have yet to be identified with a definitive mature cell type marker (Mohr, M. A., & Sisk, C. L. (2013).

In humans, the sex difference in size of the cognate region of the SDN (INAH-3 of (Allen et al. 1989) does not appear until the fourth year of life when cells begin to die off in genetic (XX) females. The nucleus is about twice the size in males ($0.2mm^3$) as females ($0.1mm^3$), with double the number of cells (Swaab & Fliers, 1985; Hofman & Swaab, 1989). Cell numbers remain stable in males until they reach their 50^{th} year of life. At this age, cell numbers in the SDN begin to diminish at a rate of an estimated 3% per year for 1 decade, after which cell numbers are stable again. A significant wave of cell death occurs after the 50^{th} year mark in the SDN of females as well, followed by another decade and a half of stability. In females, the greatest loss of cells of the SDN occurs between the ages of 75-85 years, where it is estimated that cells are lost at a rate of 4.8% per year (Hofman & Swaab, 1989). Based on these findings in humans, it was postulated that the nucleus therefore may not only rely on hormonal input during early development but perhaps throughout the lifespan (Swaab & Fliers, 1985; Hofman & Swaab, 1989). It is equally plausible that sexual experience and changes in these behaviors may play a role in, or are reflected in the size of, this nucleus (Sonntag et al, 1987, Vermuelen et al, 1990).

To understand how volume of the SDN varies with sexual experience and sexual identity vs. genetic sex alone, studies have been conducted in humans to correlate SDN

volume with individuals of variable sexual orientation and sexual preferences. Although most participants in these studies were male, the size of the SDN seems to vary not only with genetic sex, but also perceived sexual orientation and partner preference. Homosexual males and males who identify as female and/or have undergone sexual reassignment have an SDN that is more comparable in size to that of females than heterosexual males. Two females (n=2!) who identify as male, one who has and one who has not undergone sexual reassignment, both have an SDN closer in size to heterosexual males than heterosexual females. Due to the extremely small sample size of the female group in this study, additional well-powered studies are necessary to understand how the size of the SDN varies with sexual orientation/preferences in women (LeVay S., 1991) (Byne et al., 2001) (Garcia-Falgueras & Swaab, 2008). In addition to studies conducted in homosexual male humans, the size of the SDN positively correlates with a sexual preference for females in the domesticated ram. A subpopulation of the domesticated ram (a natural occurrence observed in ~6-10% of range-bred population) shows a robust and reliable preference for mounting and "mating" with other rams, even in the presence of an ewe in estrus. This sexual preference for males over females correlates with a significantly smaller SDN and lower mRNA levels of aromatase in this region than those measured in heterosexual male rams (Roselli et al., 2004).

Moreover, several more brain regions were found to be larger in one sex, with the majority being male-dominant. Second to the SDN, the most intensely studied volumetric sex difference is that of the anteroventral periventricular (AVPV) nucleus which, like the SDN, is a collection of cells that are distinguished by Nissl. However, unlike the SDN, there are more cells in the female AVPV nucleus than in the male nucleus (Simerly et al,

1985). Also, unlike the SDN, there is a clear functional role for the AVPV nucleus in reproductive physiology. Neurons in the AVPV nucleus project directly to gonadotropin-releasing hormone (GnRH) neurons that control the release of luteinizing hormone (LH) from the pituitary, which is under distinct control in males and females (Gu et al., 1997). Males have a continuous pulsatile pattern of release, while females have a cyclic pattern marked by a large surge prior to ovulation (Simerly, 2002). A combination of GABAergic and dopaminergic neurons makes up this nucleus, with evidence suggesting that the relative survival of each type is mediated by distinct mechanisms (Krishnan et al, 2009; Waters and Simerly, 2009; Zup et al, 2003). Moreover, the principal nucleus of the bed nucleus of the stria terminalis is also different in volume between the sexes due to cell death in females, and there may be yet another distinct volume regulation mechanism here involving epigenetic programming (Murray et al, 2009). Nonetheless, it is hard to say with complete confidence that there are multiple unique mechanisms as each incidence has been discovered and studied by a different group and most studies have been conducted in the mouse, a model in which sex differences are less robust than the rat. Thus, no systematic comparisons have been made.

The hormonal modulation of dendritic plasticity demonstrated in hippocampal pyramidal neurons sparked interest in the pursuit of sex differences in dendritic morphology and synaptic patterning, and, indeed, several differences were found (Amateau and McCarthy, 2002a; Mong et al, 2001; Schwarz et al, 2008). When considering the sources of variability in synaptic patterning, the obvious candidates to consider are the neurotransmitters that traffic at those same synapses. Indeed, the notion that direct hormonal modulation of neurotransmitters and/or their cognate receptors was

the source of sex differences in the brain and behavior preceded the discovery of many neuroanatomical sex differences. The number of studies exploring virtually every neurotransmitter or neuromodulator as a candidate target for hormonally mediated sexual differentiation is too great to review but can largely be summarized as demonstrating that too much or too little of any transmitter system is sufficient to disrupt normal sexual differentiation, but none of them can substitute for the hormonal exposure. In other words, none of the transmitter systems examined were both sufficient AND necessary. While never clearly articulated, the process of brain sexual differentiation, as viewed by those who study it, is thought to be so fundamental to reproductive fitness that it is likely organized in a highly redundant manner to prevent the loss of one system from derailing or redirecting the entire process.

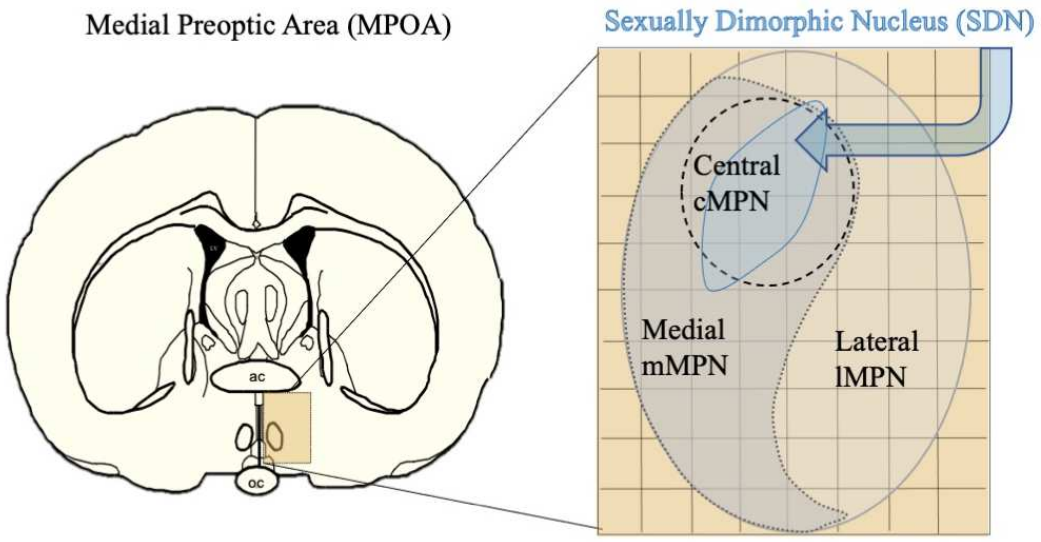


Figure 1.2 The Sexually Dimorphic Nucleus (SDN) of the MPOA/MPN
The medial preoptic area (mPOA) or MPN, contains three subdivisions. The SDN is largely but not completely overlapping with the central medial preoptic nucleus (cMPN), within the medial portion of the medial preoptic nucleus (mMPN) of the medial preoptic area (MPOA) or medial preoptic nucleus (MPN) of the preoptic area (POA).

The conclusion that sexual differentiation of the brain is multifactorial is essentially where the field stood until our laboratory made the surprising discovery in the early 2000's that prostaglandin, not neurotransmitters, were the primary target for hormonally mediated sexual differentiation of at least one system. This system, the masculinization of the synaptic patterning of the medial POA, leads to the expression of stereotypical adult male sexual behavior (Amateau et al., 2004). The McCarthy group, prior to my arrival, determined that estradiol derived from the aromatization of testosterone upregulates expression of the cyclooxygenase (COX) genes, COX-1 and COX-2, leading to increased production of the prostaglandin PGE2. Normally associated with inflammation and fever, PGE2 is a membrane-derived, rapid-acting signaling molecule which binds to G-protein coupled receptors linked to adenylate cyclase activity (Regan et al., 2003). The subsequent production of cyclic adenosine monophosphate (cAMP) by adenylate cyclase within dendritic spines activates protein kinase A (PKA) (Wright et al., 2009), leading to phosphorylation of the GluR2 subunit of glutamate α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, thereby inducing increased AMPA receptor trafficking to the membrane (Lenz et al., 2011). In a manner not completely understood, this process induces the formation and stabilization of dendritic spine synapses (Lenz et al., 2010). The resulting density of spine synapses per unit of dendrite is twice as great in males, is stable throughout life, and is highly correlated with the expression of adult male copulatory behavior measures such as frequency of and latency to mount a receptive female (Wright et al, 2008). Most importantly, the lab found that injection of female pups with PGE2 into the brain during the sensitive period was capable of fully masculinizing the synaptic pattern and adult

copulatory behavior in the absence of any neonatal hormone treatment (Amateau et al., 2004). This was the first report of a neurochemical other than steroids (testosterone or estradiol) that was both necessary and sufficient for masculinization of a neuroanatomical or behavioral endpoint. The effect of PGE2 was surprisingly specific, having no impact on the volume of the SDN, synaptic patterning in the hippocampus, amygdala or mediobasal hypothalamus, and no effect on female sexual behavior, maternal behavior, or emotionality (Todd et al, 2005). In addition to the effects of PGE2 being specific to neurons of the POA, an effect on the morphology of the local astrocytes was also detected. Males have more highly stellate astrocytes in the POA, characterized by more numerous and more branched processes than those of females (Amateau et al., 2002b). Treatment of female neonates with PGE2 shifted the morphology of their astrocytes towards that of males and we have long speculated that the astrocytes are the source of the glutamate that activates the AMPA receptors trafficking to the synaptic membrane (Wright et al, 2010). Thus, we argued, there is a two-cell system inherent in sex differentiation that requires crosstalk between neurons and astrocytes. But there was one observation that remained puzzling, the incredible finding that a single injection of PGE2, administered to a newborn female rat pup, was fully effective at masculinizing POA synaptic patterning and sexual behavior (Wright et al, 2008). This surprising observation prompted us to consider the potential for a positively reinforcing system that was initiated by this single exposure to elevated PGE2 and the most likely candidate for that was a third cell type, the microglia.

1.3 Role of Microglia in Sexual Differentiation of the Developing Brain

Microglia are unfortunately named because rather than being related to glia of the nervous system, the astrocytes and Schwann Cells, they originate from outside the brain. Unlike true glia, which have common precursors with neurons, microglia are the primary innate immune cells of the brain and are modified macrophages that originate peripherally from the yolk sac (Figure 1.3). They arrive in the brain via migration in early embryonic development, where they take up permanent residence (Ginhoux et al, 2013). Traditionally thought of as resting sentinels of the brain, microglia were believed to function primarily in the context of immune challenge or insult (Repovic and Benveniste, 2002; Streit, 2000; Taylor and Sansing, 2013). Microglia both respond to and produce prostaglandins in response to an insult, along with other inflammatory and anti-inflammatory cytokines and growth factors (Streit, 2000). It was this aspect of microglial function that presented itself as a potential mediator of a positively reinforcing effect of PGE2 and we found this to indeed be the case.

Microglia are highly variable in their morphology, but this variability conveniently provides a window into their functional status as morphology correlates with function. Ranging from a rounded, amoeboid-like appearance to highly ramified and stellate (Fig. 1.3), microglia morphology parallels a functional shift from “activated” to what was once called “quiescent,” but is now more appropriately referred to as “surveying.” The latter change is considering the high degree of motility of ramified microglia, which extend and retract their processes on a continual basis and, in this way, monitor a stable of nearby neurons (Derecki et al, 2013; Nimmerjahn et al, 2005). Microglia are tiled relatively evenly throughout the brain, leading to the belief that all

neuronal populations are constantly being monitored, and perhaps influenced, by local microglia. Moreover, if an injury does occur the local sentinels are the first to respond, followed by recruitment of other microglia and rapid microglial proliferation (Neumann et al, 2009; Taylor and Sansing, 2013).

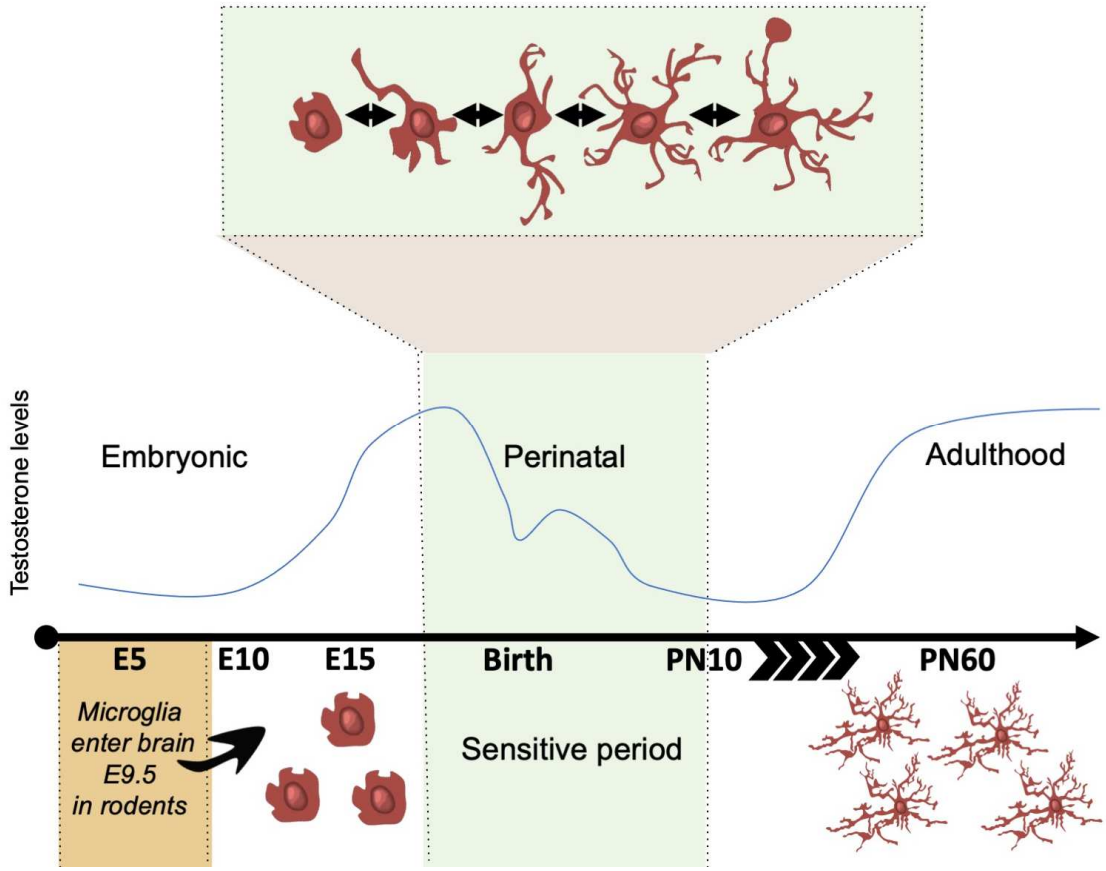


Figure 1.3. Microglial morphologies across the lifespan.
Microglia originate in the yolk sac and migrate into the brain during embryogenesis. These cells are capable of morphological switching during and after insult at all stages of life. Under normal conditions, however, microglia exhibit an especially dynamic array of morphologies during the critical period for sexual differentiation of the brain. These morphologies range from (left to right, above) amoeboid, stout, transitioning, ramified, and phagocytic. The critical period for sexual differentiation of the brain is defined by the surge in testicular hormones and their metabolites experienced by males perinatally. Sex differences in microglial morphologies during the critical period have been identified in many regions of the brain; however, these differences often disappear (or are even reversed) at the close of this critical period.

What is of interest in our system of sexual differentiation is that there is no injury, yet we found that the microglia in the male POA are more likely to exhibit an amoeboid or rounded morphology and produce more PGE2 than those in the female (Lenz et al, 2013). Moreover, when females are treated with either a masculinizing dose of estradiol or PGE2, their microglia take on an activated morphology and increase local PGE2 production, i.e., a positively reinforcing response. This additional PGE2 production is essential for both the establishment of the masculine synaptic pattern and adult sexual behavior. Thus, it takes at least three cell types to properly masculinize the preoptic area: neurons, astrocytes and microglia. The latter are not only of non-neuronal origin, but even originate outside the brain (Lenz and McCarthy, 2014).

Microglia are being increasingly recognized as far more dynamic and engaged in brain development than previously thought (see (Nayak et al, 2014)). These unique cells provide both supportive and deleterious functions. Microglia positively regulate cell number by promoting cell survival through trophic factor release (Sierra et al, 2010), influence synaptic plasticity and connectivity (Brown and Neher, 2014), and phagocytose apoptotic cells and debris (Neumann et al, 2009). Interestingly, the ability of microglia to engulf other cells is not only limited to dying cells, but also to viable cells in a process termed "phagoptosis" (Brown and Neher, 2012; Brown and Neher, 2014), allowing them to also regulate cell number, negatively. Targets of phagoptosis are stressed but otherwise viable cells, often recognized by the exposure of "eat me" signals on the surface of their membranes. The best characterized of these" eat me" signals is phosphatidylserine (PS), a membrane phospholipid normally maintained in the inner leaflet, but reversibly redistributed to the external cellular surface under stress conditions (Fadok et al, 1992).

Microglial phagocytosis is a critical regulator of postnatal neurogenesis (Sierra et al, 2013, Sierra et al, 2010), and impairments in phagocytic ability are thought to be involved in the progression of Rett Syndrome (Derecki et al, 2013). We have detected sex differences in the phagocytic profile of microglia in select brain regions (unpublished observation). However, the exact nature by which microglia sculpt behavior-specific circuitry has yet to be published and will be the primary topic of subsequent chapters.

1.4 Microglia Regulation of Critical Periods in Brain Development

The proverbial role of microglia during brain development is shifting from passive members of the brain's immune system to active participants that can dictate enduring outcomes. Despite these advances in understanding, little attention has been paid to one of the most critical components of early brain development- sexual differentiation. Mounting evidence suggests that the normal developmental functions microglia perform- cell number regulation and synaptic connectivity- may be involved in the sex-specific patterning of the brain during these early critical periods and may have lasting sex-dependent and sex-independent effects on behavior.

Development is an incredibly complex roller coaster of input-dependent processes that span the birth of a single cell to a fully mature and reproductively capable organism. Brain development is a long process consisting of dynamic and irreversible fate decisions coordinated in space and time. The brain is arguably the slowest organ to develop. Brain development begins prior to birth but does not reach maturity until early adulthood, long after sexual and physiological maturity has been reached. Unlike other essential organs, which must be mature at birth to support life, the brain is remarkably undeveloped and responsive to both internal and external cues. A central goal of neuroscience is to identify

and understand those developmental cues in both the healthy individual and those suffering environmental or genetic insult.

In recent years a surprising new player has been added to the list of change agents acting on the developing brain- microglia. Although of peripheral origin, these innate immune cells are active mediators of neuronal maturation. They are continuously active, surveying their environment and responding to neural activity (Nimmerjahn et al., 2005; Davalos et al., 2005). As regulators of developmental events like cell genesis, cell death, synaptogenesis, and synaptic pruning, these cells impact the developmental trajectory of the brain and ultimately, behavior.

Microglia are derived from embryonic yolk-sac macrophage precursors and enter the brain as early as embryonic day 9.5 (E9.5) in the rodent (Alliot et al 1999; Ginhoux et al 2010; Schulz et al., 2012; Kierdorf et al 2013) (Figure 1.3). Once in the brain, these cells continue to migrate, proliferate, and mature throughout development, until microglia attain their adult phenotype by the end of the third postnatal week (Ajami et al., 2007; Swinnen et al., 2013; Elmore et al., 2014; Nikodemova et al., 2015). By this time, microglia are tiled throughout the brain yet exhibit great regional variation in density and morphology. Microglia sustain their numbers through local self-renewal (Ajami et al., 2007; Lenz et al., 2013; Elmore et al., 2014; Bruttger et al., 2015; Doorn et al., 2015; Grabert et al., 2016; De Biase et al., 2017). Microglia orchestrate early developmental processes that impact behavior later in life. Sexual differentiation of the brain is a developmental process whereby physiological and behavioral phenotypes are modified to match gonadal phenotype. For instance, in mammals, animals with testis are exposed to high levels of androgens and their metabolites, which act on the brain prior to birth to

determine male-typical sexual and aggressive behaviors and physiology. Conversely, animals with ovaries develop a feminized brain phenotype that supports ovulation and reproductive behaviors associated with internal fertilization, pregnancy, and lactation (McCarthy et al., 2017). A key feature of many of the brain's developmental processes is the presence of critical periods (Hensch, 2004). A critical period is a window of time in which a normal developmental event must take place in response to internal or external stimuli. Once this window closes, the normal trajectory of development cannot be restored via the original triggering stimulus. During these periods, the brain's architecture is shaped in such a way that it will endure throughout the organism's lifespan. Sensory systems of the brain, such as the visual and auditory systems, are some of the most well known for having critical periods during development. It has only recently been discovered that microglia are essential to activity-dependent refinement of the visual system during its critical period (Stevens et al., 2007; Schafer et al., 2012). Given the varied and dynamic roles that microglia play during development, it is not surprising that they might be regulators of other critical periods.

Sexual differentiation of the brain occurs during a classic critical period defined by the onset and offset of sensitivity to gonadal steroids. The cellular mechanisms of sexual differentiation are diverse and brain region-specific, yet all function to coordinate brain development in a male-typical or female-typical manner. Evidence is rapidly accumulating that suggests immune cells may be far more important to establishing sex differences in the brain than previously thought (McCarthy et al., 2015). In the rodent, the critical period for masculinization of the brain begins around embryonic day 16–18 when the testes produce high levels of circulating testosterone. Circulating testosterone

decreases within hours after birth, closing this critical period (Weisz & Ward, 1980; Konkle & McCarthy, 2004). Testosterone and its androgenic metabolites are capable of sexually differentiating several endpoints in the brain; however, most are driven by estradiol synthesized from testosterone by the aromatase enzyme expressed in certain brain regions (McCarthy, 2008; Zuloaga et al., 2008). In female rodents, there is a sensitive period for brain sexual differentiation that extends past the critical period in males and continues through the first postnatal week. During this period, the female brain is sensitive to hormone exposure and masculinization can be induced by exogenous administration of testosterone, estradiol or PGE2. These two examples demonstrate the difference between critical and sensitive periods: in critical periods, normal developmental processes must be established during a short period or else they are lost, while during sensitive periods, the brain remains sensitive for longer periods of time to specific inputs/signals that have no effect on the brain's development prior to and after that period. Although many critical and sensitive periods during brain development have been established, the exact timing of each varies by specific region and many have yet to be nailed down with exact precision. Temporal differences in brain maturation across species is one reason. The mouse as a model species continues to dominate studies due to the ease of its genetic manipulation, even though the study of sexual differentiation is arguably easier to model and more relevant in the rat which exhibits more robust social and sexual behaviors that are reflected in the brain, by greater differences between the sexes that are more easily detected and tested when compared to the mouse (Reviewed by McCarthy et al., 2017).

Sexually differentiated endpoints are diverse, varying drastically between brain

regions. Hormone exposure may drive differential cell genesis and survival, cell death, synaptogenesis, synaptic pruning, and cellular migration. Ultimately, these processes result in lasting differences in brain region size, cellular phenotype and complexity, and synaptic connectivity between males and females (Arnold, 2009 and Forger et al., 2016). Actions of gonadal hormones during this time are collectively referred to as "organizational" and considered to be a form of early life programming, also referred to as the "organizational hypothesis" (Phoenix et al., 1959), which was summarized in Figure 1.1.

1.5 Microglia and sex differences

Microglia are highly dynamic and present in appreciable numbers throughout much of the brain perinatally. Their morphologies range from amoeboid to highly ramified and phagocytic phenotypes (characterized by the presence of processes with phagocytic "cups"); such variation is likely reflective of both their developmental maturity as well as their signaling and physical functions in given brain regions. Sex differences in microglia are not apparent until after the onset of the prenatal androgen surge (E16-18) and vary by brain region and age. This timing suggests a role for hormones in the diversification of microglia-mediated developmental processes (Schwarz et al., 2012). Two features of the cell population have been noted to differ markedly between males and females in rodent models of development- microglial number and morphology.

On the day of birth, female rats have more amoeboid and stout microglia in several brain regions, including the paraventricular nucleus of the hypothalamus (PVN), hippocampus, and amygdala, when compared to male rats. This sex difference quickly reverses, as males display more amoeboid and stout microglia than females four days later in the parietal cortex, hippocampus, and amygdala (Schwarz et al., 2012). By contrast, male rat pups have more microglia per unit area than females in the developing preoptic area (POA), and nearly twice as many amoeboid microglia as females from PN0-2 (Lenz et al., 2013). Microglia in the male POA are the major source of the high level of secreted inflammatory prostaglandin, PGE2, which is both necessary and sufficient for the masculinization of POA synaptic density and male sexual behavior in adulthood. This microglial sex difference is hormonally programmed, as treating females

with a masculinizing dose of estradiol increases both the number of microglia and their amoeboid morphology. Masculinization of microglia morphology does not occur, however, when females receiving estradiol are administered a low dose of minocycline, an inhibitor of microglial activation, in tandem (Lenz et al., 2013) (Figure 1.4).

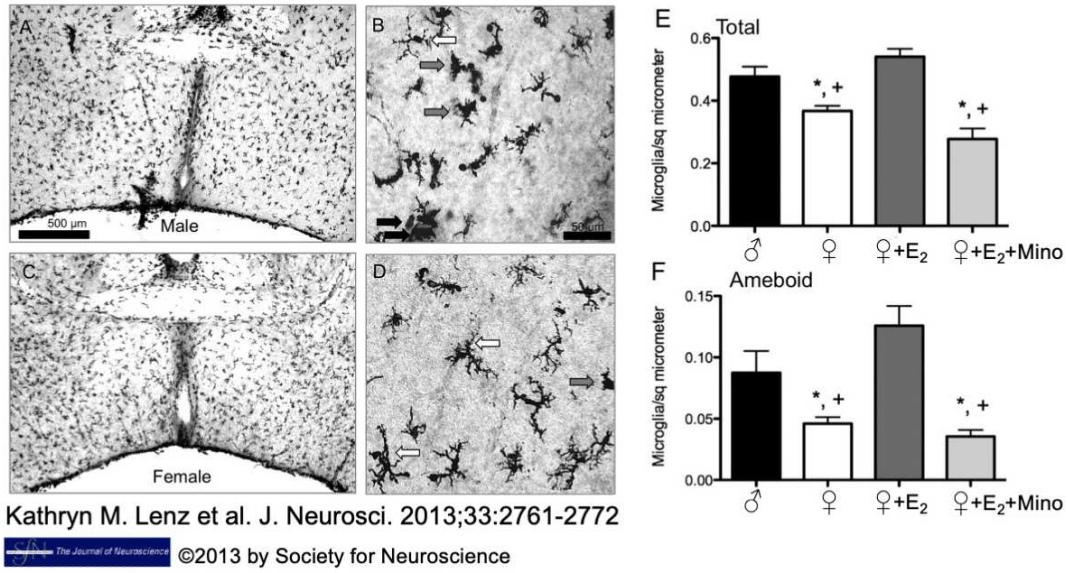


Figure 1.4. Sex differences in microglial morphology during the critical period. **A-D.** Representative photos of Iba-1 positive cells in the male **(A-B.)** and female **(C-D.)** POA, taken on PN2. **E.** Lenz et al. found similar numbers of total microglia in males and females. When masculinized by exogenous estradiol, females show similar numbers of ameboid microglia to male vehicles on postnatal day 2 (PN2) and this masculinization of microglia morphology does not occur when females are first treated with minocycline (a general tetracycline antibiotic that has been shown to inhibit microglial "activation" (Yuan et al., 2019; Ahmed et al., 2017; Scholz et al., 2015). *This figure is a modified version of Figure 1. from the following: 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), 2761–2772.

It is currently unknown whether sex differences in microglial number and morphology during development are a result of differential maturation or due to intrinsic differences in microglial function. Differences in microglial number may arise from a combination of timing of migration and/or local proliferation within a given brain region. Moreover, differences in morphology may be reflective of intrinsic functional or maturational state or driven by local cues from the developing brain region in which they reside. Amoeboid and stout morphologies are characteristic of immature microglia during development (Monier et al., 2007; Rigato et al., 2011; Swinnen et al., 2013), and in many brain regions, amoeboid microglia make up a greater proportion of the population in males than females (Schwarz et al., 2011; Lenz et al., 2013). Interestingly, in the developing POA, all microglia co-express traditional pro-inflammatory (M1) and alternative activation (M2) markers, despite males having more amoeboid microglia and higher pro-inflammatory PGE2 signaling (Lenz et al., 2013). These findings corroborate those of others who observed similar developmental co-expression of the M1 and M2 phenotypes (Crain et al., 2013; Cunningham et al., 2013), strongly suggesting that the morphologies observed in the healthy developing brain are not as easily classified by the same phenotypic markers frequently used to analyze microglia in adulthood or following insult. More research is required in this area to fully interpret and classify the relationship among microglial morphology, function, and region-specific brain development during the perinatal period.

To better understand the developmental trajectory of microglia, RNA sequencing of microglia was performed, revealed three distinct stages of development. These stages, identified as early (E10.5–E14), pre- (E14-PN9), and adult (4 weeks and older), consist

of discrete transcriptional patterns reflective of differing microglial function throughout brain development (Matcovitch-Natan et al., 2016). While only males were analyzed in this study, identification of distinct and functional periods of microglial development nevertheless opens the door for further investigation in both sexes. It remains to be determined if the perinatal transcriptional patterns are caused by the natural hormonal surge in males and whether they exist or differ in females. Such findings may shed light on early life interactions between the brain's immune system and sex, and how these factors work together to differentially influence brain development.

Alternatively, the maturation of the brain itself may exert regulatory control over microglia through changes in regional microenvironment. Longitudinal MRI confirms that regions of the brain vary in maturational trajectory in boys and girls (Geidd et al, 2004). Analogous studies have been done in rodents where other markers of maturation, such as depolarizing GABA neurotransmitter (which becomes hyperpolarizing/inhibitory in mature animals), differ between neonatal male and female rat pups (Nunez et al, 2009). It is possible that microglia in less mature brain regions are themselves less mature and could therefore differ in males and females at a particular point in development due to sex differences in the timing of maturational events in that region of interest.

1.6 Microglia Regulate Cell Number in the Developing Brain

Microglia regulate cell number during development, performing a dual role by facilitating cell proliferation and differentiation while also actively phagocytosing both dead and stressed, but viable, cells. This delicate balance of life and death is critical during early brain development to establish a healthy template from which to build on through experience in the remainder of the organism's life.

Neural progenitor cells (NPCs) are intimately linked to microglial function. NPCs coordinate colonization by microglia early in brain development, and in turn, microglia produce factors that promote proliferation and differentiation of neural progenitors (Arnò et al., 2014). In the early postnatal subventricular zone, microglia produce cytokines that act as trophic factors for normal neurogenesis and oligodendrogenesis (Shigemoto-Mogami et al., 2014). Similarly, in the developing cortex microglia support healthy neurons in layer V through production of IGF-1. Microglial inhibition or depletion increases the number of apoptotic layer V neurons, and appropriate neuronal support appears to require microglia-neuron crosstalk through the fractalkine receptor, CX3CR1 (Ueno et al., 2013). *In vitro*, the presence of microglia or microglia-conditioned media enhances proliferation of cultured NPCs, astrogenesis, and neuronal maturation (Morgan et al., 2004; Antony et al., 2011).

Much like how microglia colonize early neurogenic niches, microglial colonization patterns often chronologically match patterns of programmed cell death (PCD) at many developmental stages (Perry et al., 1985; Ashwell 1990). Microglia facilitate the removal of dead and dying cells from the developing and adult brain (Ferrer et al., 1990; Bessis et al., 2007; Sierra et al., 2010). Apoptotic neurons release several

"find me" signals, which function to increase microglial migration to the site of cell death (Ravichandran, 2011). However, while PCD does facilitate microglial colonization in some brain regions, migration is not entirely dependent on localized apoptosis as a cue (Eyo et al., 2015, Xu et al., 2016).

Instead of just passively phagocytosing dead and dying cells during development, more recent evidence suggests microglia are far more active in cell death, capable of inducing it in otherwise viable cells. In the early postnatal cerebellum, microglia induce cell death in Purkinje cells through a targeted "superoxide burst;" this process occurs during a critical period in cerebellar development in which Purkinje cells migrate to their final locations and refine their synaptic patterning (Marín-Teva et al., 2004). While most Purkinje cells targeted by microglia express markers indicative of programmed cell death (such as activated caspase-3), when microglia are depleted from cerebellar slice cultures *in vitro*, Purkinje cell survival increases, suggesting microglia also phagocytose live cells (Marín-Teva et al., 2004). Microglia in the postnatal hippocampus eliminate cells in a similar fashion, contacting caspase 3+ neurons and producing superoxide ions in a CD11b/DAP12 (DNAX activation protein of 12 kilodaltons)-dependent mechanism (Wakselman et al., 2008). Again, preventing superoxide production by genetic deficiency of DAP12 (a CD11b-associated signaling protein) or CD11b antibody blockade decreases the number of caspase 3+ cells. These data suggest that in brain regions characterized by normal programmed cell death, microglia may act independently to facilitate elimination of cells. NPCs in the cerebral cortex are also phagocytosed by microglia as a means of controlling cortical cell number. However, very few of the NPCs engulfed by microglia express markers of cell death, and when microglia are inhibited or depleted, the number

of cortical NPCs increases (Cunningham et al., 2013). Thus, microglial phagocytic function may be independent of programmed cell death in this region, providing further evidence for developmental "phagoptosis" or the phagocytosis of viable cells (Brown & Neher, 2012; 2014).

Given the evidence for microglia regulation of cell number in development, sex differences in cell number may also arise from microglia-mediated mechanisms. A recent examination of the neonatal rat hippocampus revealed females have nearly twice the number of phagocytic microglia as males (Nelson et al., 2017). This study found that prior to the onset of fetal hormone production (at E20), phagocytic morphologies did not differ between the sexes. Moreover, treating newborn female pups with a masculinizing dose of estradiol eliminated the observed sex difference. Other parameters of microglial morphology (classified as amoeboid, transitioning, or ramified) did not differ between the sexes, suggesting that phagocytic morphology occurred independent of traditional morphology or activation classifications. Further analysis found that newborn cells (marked by 5-Bromo-2′-deoxyuridine (BrdU) labeling) and Sox2+ progenitor cells were targeted by a subset of microglia for phagocytosis (Nelson et al., 2017). As the number of newborn cells in the developing hippocampus is also sexually dimorphic (Zhang et al., 2008; Bowers et al., 2010), it is tempting to speculate that microglial phagocytosis is a determining factor of newborn cell number. The relationship between newborn cells and phagocytic microglia is inverse; males have more newborn cells postnatally (Zhang et al., 2008; Bowers et al., 2010) but also have fewer phagocytic microglia (Nelson et al., 2017).

Microglia are also likely to play a role in sexual differentiation of the AVPV nucleus of the POA, although this has not yet been tested directly. The AVPV nucleus is a cluster of cells important for regulating the sex-specific LH release patterns necessary for reproduction and is approximately 2x larger in females than males, with a higher cell density (Murakami & Arai, 1989; Sumida et al., 1993). This sex difference is largely due to hormonally driven cell death, which, through inhibition of constitutively active tumor necrosis factor alpha (TNFalpha) cytokine signaling, induces higher rates of cell death in the male AVPV nucleus (Arai et al., 1996; Krishnan et al., 2009). Microglia are the major producer of TNFalpha in the brain, and trigger cell death in developing motor neurons in a TNFalpha-dependent manner (Sedel et al., 2004). As such, microglia are potential drivers of the sexual differentiation of the AVPV nucleus.

1.7 Microglia and Synaptic Development, Maturation, and Connectivity

Microglia are found in higher densities in discrete developing axonal tracts and axon outgrowth is highly dependent upon microglia. Microglia both promote axonal outgrowth and phagocytose aberrant axons to facilitate a proper balance of projections (Squarzoni et al., 2014). Microglial depletion (caused by genetic deletion of transcription factor Pu.1 or antibody

Microglial interactions with synapses are more complex, being largely driven by neuronal activity that is assessed through an array of receptors tuned to signals associated with synaptic firing (Pockock & Kettenmann, 2007). Neurotransmission induces microglial motility and process outgrowth toward the active synapse, resulting in brief and rapid contacts with the synapse (Davalos et al., 2005; Nimmerjahn et al., 2005;

Fontainhas et al., 2011; Li et al., 2012; Dissing-Olesen et al., 2014). It is estimated that microglia survey their local environment in approximately one hour (Wake et al., 2009).

Microglia actively engulf weaker synaptic inputs to facilitate the appropriate segregation of eye-specific retinal inputs to the LGN during the critical period for maturation of the visual system. Disrupting this process, pharmacologically or genetically, results in aberrant connectivity (Stevens et al., 2007; Schafer et al., 2012). In the visual cortex, engulfment is preferential towards transient and smaller spines, and is modulated by alterations in sensory experience (Tremblay et al., 2010). Similar findings occur *in vitro*, where microglial depletion from hippocampal (mixed or slice) cultures influences the number of excitatory synapses, respectively (Ji et al., 2013). These findings are likely mediated by contact-dependent synaptic pruning, as preventing physical interaction with hippocampal neurons *in vitro* increases the number of dendritic spines and functional synapses through the production of interleukin-10 (Lim et al., 2013). Microglial contact with dendrites in the developing somatosensory cortex induces cytoskeletal rearrangement and filopodia formation during periods of high synaptogenesis (Miyamoto et al., 2016). These findings suggest that the initial development and further refinement of synaptic connectivity likely depends on a microglia-mediated balance between eliminating weak or unnecessary synapses and promoting the growth of new ones.

1.8 Lasting Impacts of Microglial Function in Development

The diverse array of microglial functions- control of cell number and differentiation, synaptogenesis, and pruning- work in coordination to facilitate development and maturation of neural circuits. Alterations to these processes, at the molecular, cellular, or circuit level, are likely to have significant and lasting impacts on both brain and behavior. Studies examining long-term effects of early microglia function often use two approaches- genetic deletions (or mutation) of microglia-specific genes and microglia depletion (Frost & Schafer, 2016; Paolicelli & Ferretti, 2017). Deletion or mutation of specific genes offers the ability to study the native function of a particular protein; however, these models are often constitutive mutants. Consequently, lasting effects on parameters such as behavior become difficult to associate with the deficient microglia function rather than an accumulated effect from a lifetime of deficiency. Nonetheless, genetic mutants provide some of the most comprehensive and compelling evidence to suggest that early microglial dysfunction impacts synaptic maturation and later life behavior.

Genetic deletion of CX3CR1 (CX3CR1 KO), which in the brain is expressed by microglia and macrophages, delays colonization of the rodent barrel cortex that normally occurs during a critical period for the maturation of thalamocortical projections. As a result, glutamatergic synapse maturation is delayed (Hoshiko et al., 2012). CX3CR1 KO mice also have fewer microglia in the hippocampus postnatally, resulting in an abundance of immature synapses and delayed maturation of synaptic structure and function (Paolicelli et al., 2011). In mature animals, these deficits manifest as

impairments in long-term potentiation, learning-dependent memory, social interactions, and increased self-grooming behaviors (Rogers et al., 2011; Zhan et al., 2014).

Synaptic deficiencies and alterations in spine numbers are observed in other genetic mutant models (Roumier et al. 2004; Kim et al., 2016) and are common phenotypes associated with alterations in microglial function. Such deficiencies are corroborated by studies depleting microglia to examine their role in modulating circuit function and behavior. Conditional genetic microglia depletion in mature animals induces learning-dependent memory deficits, likely through impairments in synaptic transmission and spine remodeling (Parkhurst et al., 2013). Other behavioral impairments occur following pharmacological microglia depletion using inhibitors of the colony stimulating factor 1 receptor (CSF-1R). CSF-1R is necessary for microglia viability, and systemic treatment with its inhibitors achieves nearly complete microglia depletion, with no lasting microglia impairment following repopulation upon cessation of the inhibitor treatment (Elmore et al., 2014; 2015). Using this approach, adult mice depleted of microglia display deficits in spatial memory and abnormal social behavior (Torres et al., 2016); however, the behavioral effects of microglia depletion are reversed after microglia repopulate the brain (Elmore et al., 2015; Torres et al., 2016). Such a transient impact of microglial depletion on behavior underscores the highly dynamic role microglia play. It seems that microglia not only participate in synaptic refinement during critical periods of development, but also dynamically modulate social and learning-dependent behaviors by altering synaptic remodeling.

While the above studies underscore the importance of microglia in experience-dependent modulation of synapses and behavior in mature animals, the functional

outcomes of developmental microglia actions have received far less attention until recently. It now seems that microglia have two very different, but complementary roles in the developing and mature animal. At maturity, microglia facilitate experience-dependent synaptic remodeling that has profound impacts on learning-dependent and social behaviors. Behavioral abnormalities observed in these experimental conditions are limited to the period of dysfunction or depletion, that is, they do not persist once microglia function is restored. In development, however, microglia are far more important for facilitating the organization of brain architecture, with significant and lasting implications for behavioral changes that persist into adulthood.

The study of selective microglia depletion during discrete epochs of development has allowed for an understanding of early life microglial function and how these cells ultimately shape the animal's behavior later in life. One approach for temporary depletion of microglia is bilateral intracerebral injections of the dominant negative ATP analog, clodronate, packaged into liposomes for ready uptake by phagocytosing cells (Buiting & Van Rooijen, 1994). This approach facilitates the study of microglia with more precise temporal control as it allows a rapid and transient depletion of the microglia population.

Liposomal clodronate treatment of male and female pups on PN0, 2, and 4 reduces microglial numbers by 50–80% within 24 hours, with a full repopulation by PN10 (VanRyzin et al., 2016). Temporary microglial depletion during development produces significant immediate and long-lasting changes to various aspects of behavior in both sexes. Within days after depletion, treated pups display impaired nest seeking behavior and reduced numbers of ultrasonic vocalizations, two ethologically relevant early pro-social behaviors. By the end of the second postnatal week, once the microglia

repopulate, clodronate-treated animals are hyper-locomotive compared to controls. In juveniles, early developmental depletion produces drastic reductions in innate fear and anxiety-like behaviors, social behavior, and working memory. When all these behavioral alterations are considered together, transient postnatal microglia depletion induces two primary phenotypes- behavioral disinhibition and hyper-locomotion- which persist throughout life (VanRyzin et al., 2016). Results from our laboratory were corroborated by an additional study from a different laboratory, which found similar behavioral deficits following neonatal microglial depletion that persist well into adulthood. In that study, both male and female adult rats displayed reduced anxiety-like and despair behavior when tested in the open field, elevated plus maze, and forced swim test (Nelson & Lenz, 2017). Together, these data provide intriguing evidence implicating microglia in early life programming of various affective behaviors. Future research is needed to dissect the microglial contribution to establishing these behavioral circuits during early postnatal life. Their contributions during development are expected to vary by region, timing and perhaps by sex, as we and others have observed during our studies of microglial morphology during postnatal life (Lenz & McCarthy, 2015).

While a study of juvenile animals in our laboratory indicated sex-independent functions of microglia during sensitive periods in development, a neonatal microglia depletion study demonstrated sex-dependent reproductive behaviors in adults requires microglia in males but not females. Postnatal microglial depletion drastically reduced the expression of adult male sex behavior, impairing both motivation and execution; females, however, were completely unaffected and displayed normal proceptive and receptive behaviors, indicating divergence in microglia's roles in the formation of behavioral

circuitry (VanRyzin et al., 2016). Male-typical sex behavior requires microglia for appropriate development. Insults (reductions in microglia number or alterations to microglia signaling) drastically alter this trajectory (Lenz et al., 2013; VanRyzin et al., 2016). Yet the neural underpinnings of female sex behavior appear to develop independent of microglia during this early period of depletion. The remarkable divergence in microglia involvement could provide a valuable vantage point for identifying novel developmental processes and pathways which differ between the sexes.

Recent advances in our understanding of how microglia regulate brain development have shed light on several normal physiological processes that occur in a time- and brain region-dependent manner. As we continue to investigate the molecular and cellular interactions governing these processes, and how these developmental functions eventually influence brain architecture and behavior, it is essential to consider sex as a factor in the study of microglia and development. Similarly, studies on sex differences in brain and behavior would likely benefit from the inclusion of microglia as a key factor.

How exactly might microglia mediate sensitive periods and sculpt sex differences in development? Many studies focus on processes that appear to be specific and intrinsic to non-microglial cells, and for good reason, as microglia make up only 5–15% of the total cell population in the adult human brain (Pelvig et al., 2008; Lyck et al., 2009). Neurons, astrocytes, and even oligodendrocytes make up most of the brain; however, despite their small number and size, microglia have asserted themselves as regulators of sensitive periods in development.

Most sexually differentiated processes, such as neurogenesis and programmed cell death, have thus far been studied without consideration of microglia. We now know that microglia differ markedly between the sexes during development and play a larger role in these processes than originally thought. If we are to better understand developmental processes in the brain, particularly sexual differentiation, a greater focus must be placed on understanding sex differences in the microglial colonization and morphology from a functional perspective. Doing so is likely to uncover new and potentially sexually dimorphic functions of these cells. Microglia themselves may be sexually dimorphic- that is, the very function of microglia may be different between the sexes, and they may perform different roles during developmental sensitive periods. In these cases, microglial function may be the result of chromosome composition (XY vs XX in mammals) or of developmental exposure to gonadal hormones. Gonadal hormones may act directly on microglia or modify the local environment to elicit sex-dependent microglial responses. Differentiating between these two possibilities is paramount to understanding the generation of sex differences and how these processes, and others, may go awry. An example of this is in the developing POA, where microglia in the male brain respond to a hormonally initiated cascade of events which results in the subsequent masculinization of the POA (Lenz et al. 2013). Both male and female microglia are similar in their ability to participate in this cascade, as masculinization can be induced in females or disrupted in males (Lenz et al. 2013, VanRyzin et al. 2016). Only after careful consideration of this signaling cascade can we begin to see that microglia are the executioners, not the source, of this phenomenon.

Alternatively, microglia may have completely differing roles in development between the sexes. These cells may perform one function in the developing female brain and perform a completely different function in the developing male brain to achieve a common endpoint. Indeed, such a precedent exists in adult animals; microglia mediate mechanical pain hypersensitivity in male mice, whereas female mice use T lymphocytes to mediate the same process (Sorge et al., 2015).

As the study of microglia and sensitive periods continues to advance, it is important to remember that each of these developmental processes may be affected by exogenous insult, resulting in lasting changes in the brain. Early life stress, inflammation or immune challenge, and even environmental insult can significantly alter microglial functions during development (for reviews, see Bilbo & Schwarz, 2009; 2012; Paolicelli & Ferretti, 2017). There is growing appreciation that these early insults not only disrupt early microglial function but may also impart lasting changes to microglial activity throughout life. Enduring microglial alterations may be, in part, epigenetic in nature (Garden, 2013; Netea et al., 2016). Epigenetic alterations to the microglia may program how these cells interact with the brain later in life and have significant consequences for behavior. For example, neonatal handling of rat pups induces long-term increases in expression of the anti-inflammatory cytokine, interleukin-10 (IL-10), by decreasing microglia-specific IL-10 gene methylation. The increased anti-inflammatory signaling promotes resilience to the reinstatement of morphine-induced conditioned place preference (Schwarz et al., 2011) and induces robust down regulation of many pro-inflammatory genes in the nucleus accumbens in adulthood (Lacagnina et al., 2017).

The X chromosome represents another convergence between immune function and epigenetic regulation, as it contains many immune-related genes (Fish 2008; Bianchi et al., 2012). This makes X-chromosome inactivation essential to appropriate immune function in females. To this point, inhibiting DNA methyltransferase activity in the developing female POA results in a significant enrichment in immune-related genes (Nugent et al., 2015). These data suggest that epigenetic regulation by methylation serves to dampen immune function in the developing female as a natural part of sexual differentiation.

The last decade has been enormously exciting in the arena of microglia investigation. Tremendous strides have been made in elucidating fundamental principles of these mysterious cells, alongside startling and unexpected discoveries, many of which are summarized in Figure 1.5. The potential for treatments and interventions specific to microglia will offer new and effective therapies is growing in probability but it is clear there is still much to be learned about these small, infrequent, but oh so powerful cells.

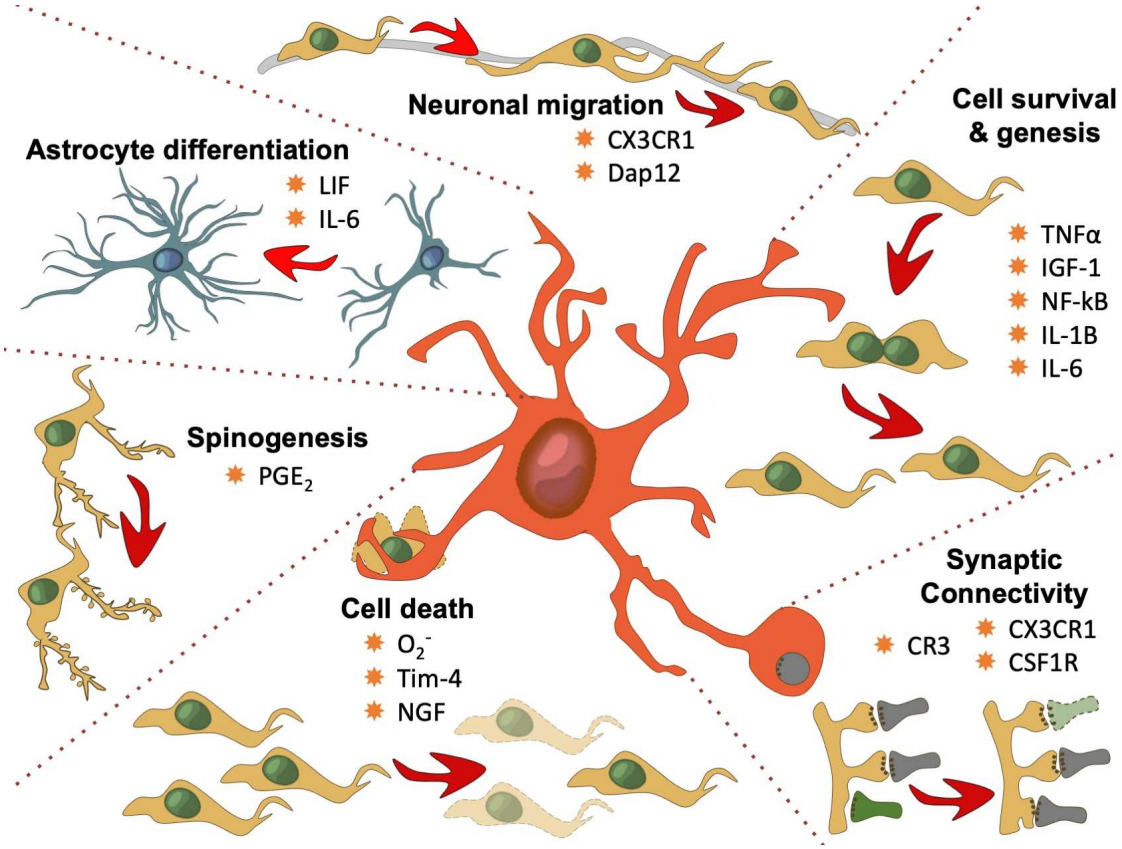


Figure 1.5. Microglia are crucial to a variety of processes during brain development. All these processes are important for sexual differentiation of different regions of the brain, but few have been studied with respect to the involvement of microglia. Abbreviations: CR3, complement receptor 3; DAP12, DNAX-activation protein 12; Insulin-like growth factor 1; IL, interleukin; LIF, leukemia inhibitory factor; NF-kB, nuclear factor-kappa B; NGF, nerve growth factor; Tim-4, T cell, immunoglobulin, mucin 4. This figure was made by the author of this dissertation (LAP), and was previously published in the following: VanRyzin, J. W., Pickett, L. A., & McCarthy, M. M. (2018). Microglia: Driving critical periods and sexual differentiation of the brain. Developmental neurobiology, 78(6), 580–592.

1.9 Mast Cells in the Developing Brain

An additional cell type that is present in even fewer numbers than microglia but has enormous implications for sexual differentiation of the brain has been identified, the mast cell. Mast cells are innate immune cells that are best known for their role in allergy and anaphylaxis, where their response to a perceived threat can range from an itchy, runny nose to dermal irritation or "wheal" formation to a life-threatening condition requiring epinephrine administration and/or hospitalization (Krystel-Whittemore, 2015). These powerful cells are most densely populated at barrier interfaces throughout the body (e.g., mucosal membranes of nasal passages, ears, eyes, intestinal lining, dermis- any membrane in contact with foreign or non-self-antigens from exterior sources). Upon recognition of noxious stimuli, these cells degranulate, releasing histamine, heparin, proteases, and a vast array of other inflammatory mediators in an autocrine, paracrine, or systemic response. In addition to these preformed mediators stored in their granules, they also synthesize and release lipid mediators, growth factors and neurotransmitters on demand, and partake in "piecemeal" degranulation where some but not all their granules are released in a continuous fashion. The variety of mediators and "nutrients" the mast cells make and respond to makes them candidate support cells for members of their developing tissue niche. Due to their cellular heterogeneity and intimate relationship with the tissue niche in which they reside, the mast cell has been difficult to study. If you've met one mast cell, you've met one mast cell. MCs have the capability to make and receive signals from more lipid and protein mediators than any other cell type in the body and are the most "unique" when compared to all other immune cells, even during development (Krystel-Whittemore et al., 2016).

MCs are of dual hematopoietic origin. A first wave of MCs originates from the Haematopoiesis Erythropoiesis (HE) of the yolk sac (just like microglia!) and is gradually diluted by a second wave of definitive MCs that, once mature, are radiation-resistant, long-lived cells that have a unique set of transcriptional and morphological characteristics that depend on their tissue of residence (Gentek et al., 2018). The study of mast cells in the context of neuroinflammation has yielded evidence of their role in maintenance of neurogenesis, neurodegeneration, and blood-brain barrier (BBB) permeability (Hendriksen, 2017; Lenz & Pickett et al., 2018).

Although MC colonization of the brain has yet to be studied, it is known that they are present in the brain by as early as E15 (unpublished observations) and unlike microglia, their location is limited to highly specific regions such as leptomeninges and perivascular and periventricular spaces. They are most numerous in the subventricular membrane between the lateral ventricles and the thalamus in the first week of life in the rat and are also present in appreciable numbers (that vary by sex) in the parenchyma of the POA and meninges located just below it, at the very base of the brain (Lenz & Pickett et al., 2018).

Very few studies of mast cells in the developing mammalian brain exist, and although much speculation about their potential roles during development have been made, our study is the first examining their role in sexual differentiation of brain and behavior, as summarized in Chapter 3 of this dissertation. More recent studies, majority of which have been led by Adam Moeser's group, aimed at understanding the lifelong consequences of androgenization on mast cell transcription and phenotype have shown that mast cells of the peritoneal cavity, mesentery and bone marrow of males are not less

responsive but make and release smaller stores of mediators than females. When testosterone secretion was inhibited during the critical period for sexual differentiation, however, male mast cells behaved like female mast cells. Evidence from a series of studies demonstrates that sex differences in mast cell phenotype are dependent on early hormonal exposure, and not differential circulating hormones in adulthood. (Acker et al., 2017, Loewendorf et al., 2016, Lovell et al 2012; Simpson et al., 2008; Gonzalez-Perez et al., 2010; Ortona et al., 2016). These studies are bolstered by epidemiological studies demonstrating a female-bias in prevalence and severity of symptoms in disorders of, or relating to, the immune system and/or associated with chronic inflammation, even prior to puberty when many are additionally exacerbated (Acker et al., 2017, Loewendorf et al., 2016, Lovell et al 2012; Simpson et al., 2008; Gonzalez-Perez et al., 2010; Ortona et al., 2016). Combined, the newest literature on mast cell biology really highlights the importance of studying early sexual differentiation, as it sets up crucial features, such as differences in our basic immune defense mechanisms with invaluable insight for understanding and treating disorders which may have a very simple explanation for their higher prevalence in one sex, once all the confounds of studying humans and sex differences in adulthood are reduced. A patient's mast cell transcriptome could even perhaps be used as gage for levels of hormonal exposure during gonadal development, if the same is true in humans.

1.10 Conclusion and Hypothesis

Future studies into the role of mast cell to microglia signaling during the first weeks of postnatal life continue and as we discover better pharmacological agents and tools for manipulating these cells without off-target effects (like those of the mast cell degranulator, compound 48/80, which also has excitatory effects on neurons (Heppner, 1992; Eglezos, 1992)). Additionally, as we now have access to techniques to better visualize their location and population expansion during embryonic and postnatal period, such as using CLARITY to clear rat embryos and visualize mast cells to understand exactly when and how they enter the brain, how they maintain and expand their population during development, and then reduce this population substantially for the remainder of life.

The work presented here aims to test the central hypothesis: ***Mast cells and microglia are required for sexual differentiation of the POA and sexual behaviors in adulthood.***

To test this hypothesis, the following chapters will address these sub-hypotheses and their predictions:

Sub-hypothesis 1: Microglial phagoptosis of neurons is required for sexual differentiation of the sexually dimorphic nucleus and for its behavioral correlate in adulthood, sexual odor preference.

Aim 1.1: To test the prediction that phagocytosis by microglia will be greater in female cMPN as compared to lateral and medial MPN during the first week of neonatal life, with greatest levels prior to and during sexual differentiation of the SDN.

Prediction 1: Phagocytic activity will be greater in the female cMPN prior

to/during sexual differentiation of SDN as compared to the lateral and medial MPN.

Prediction 2: Greater phagocytosis will be observed in females than males.

Aim 1.2: To test the hypothesis that the targets of microglial phagocytosis in the female SDN are neurons.

Prediction 1: Target cells within the cups of phagocytic microglia will be of neuronal origin, as neurons are the reported cell type undergoing cell death in the female SDN.

Aim 1.3: To test the hypothesis that microglial phagocytosis of neurons (is the primary method of higher levels of cell death in the female SDN during postnatal development.

Prediction 1: Microglial phagocytic activity will be lower following administration of the phagocytosis blocking antibody, anti-CR3.

Prediction 2: Female SDN volume will be increased following antibody blockade of microglial phagocytic receptors during peak phagocytic activity of microglia.

Aim 1.4: To test the predictions that antibody blockade of microglial phagocytosis during neonatal sexual differentiation of the SDN will masculinize female SDN volume, activation and its behavioral correlate, sexual odor preference, in adulthood.

Prediction 1: Neonatal antibody blockade of microglial phagocytosis in females will masculinize sexual odor preferences in adulthood.

Prediction 2: Neonatal blockade of microglial phagocytosis during SDN sexual differentiation will promote survival of neurons normally lost in females and

masculinize SDN volume.

Prediction 2: Neonatal antibody blockade of phagocytosis in females will result in blunted immediate early gene expression in adult cMPN in response to exposure to sexually active male odor.

Sub-hypothesis 2: Mast cell signaling via degranulation is necessary and sufficient for sexual differentiation of the POA and sexual behavior in adulthood.

Aim 2.1: Test the hypothesis that numbers of mast cells will be greater in males than females during the critical period for sexual differentiation due to high levels of estradiol (a known mast cell degranulation agent) in the brains of males and not females just after birth.

Prediction 1: Mast cells will be present in greater numbers in the developing male POA during the critical period for sexual differentiation, postnatal days 0-4.

Prediction 2: Male POA mast cells will be undergoing proliferation (dividing) at higher rates, as evidenced by BrdU incorporation just after birth.

Prediction 2: Evidence of mast cell degranulation will be more frequently observed in the developing male versus female POA.

Aim 2.2: To test the predictions that estradiol administration to neonatal females, during the critical period for sexual differentiation, will masculinize neonatal mast cell number and degranulation state.

Prediction 1: Greater mast cell numbers and frequency of degranulation will be observed in estradiol-treated females as compared to control females.

Aim 2.3: To test the predictions that degranulation of mast cells in females will masculinize neonatal POA, as evidenced by a greater population of amoeboid microglia and greater density of dendritic spines as compared to control females.

Prediction 1: The mast cell-specific pharmacological degranulation agent c48/80 will increase the observed frequency of degranulated mast cells in the POA.

Prediction 2: Mast cell-specific pharmacological degranulation via c48/80 will increase the observed frequency of amoeboid microglia in the neonatal POA.

Prediction 3: Mast cell-specific pharmacological degranulation via c48/80 will increase the spine density of neonatal female POA neurites to typical male levels.

Aim 2.3: To test the hypothesis that degranulation of female mast cells neonatally is sufficient to masculinize measures of male-typical sexual behavior in adulthood, supporting their organizational role in normal male sexual development when activated by exogenous testosterone replacement in adulthood.

Prediction 1: Neonatal mast cell-specific pharmacological degranulation via c48/80 will increase the observed frequency of male-typical sexual behaviors in adult females equipped with exogenous testosterone in adulthood when compared to vehicle-treated, testosterone replaced control females.

Aim 2.4: To test the hypothesis that neonatal organizational mast cell degranulation is necessary for male-typical sexual differentiation in adulthood.

Prediction 1: Males, but not females, treated in utero with mast cell stabilizer, Ketotifen prior to and during the critical period for sexual differentiation will

show decreased mast cell degranulation and reduced levels of male-typical amoeboid microglia in the POA just after birth.

Prediction 2: Males, but not females, treated with Ketotifen prior to and during the critical period for sexual differentiation will show reduced POA spine densities.

Prediction 3: Males treated with Ketotifen prior to and during the critical period for sexual differentiation will exhibit deficiencies in behavioral measures of male-typical sexual behavior in adulthood.

Aim 2.5: To test the hypothesis that maternal allergic challenge effects mast cell degranulation and therefore sexual differentiation of microglia, synaptic density and sexual behavior of the offspring exposed in utero.

Prediction 1: Female offspring of allergically challenged, sensitized dams will show increased mast cell degranulation and increased levels of "male-typical" amoeboid microglia in the POA, just after birth.

Prediction 2: Increased mast cell degranulation in Female offspring of challenged sensitized dams will lead to male-typical spine densities in the POA.

Prediction 3: Female offspring of challenged, sensitized dams will demonstrate increased male-typical sexual behavior in adulthood when activated in adulthood by exogenous testosterone replacement.

Aim 2.6: To test the predictions that mast cell degranulation with c48/80 (delivered intracerebroventricularly), prior to and during neonatal sexual differentiation of the SDN will decrease microglial phagocytosis and masculinize female SDN volume.

Prediction 2: Mast cell-specific pharmacological degranulation via c48/80 will decrease the observed frequency of microglial phagocytosis in the cMPN.

Prediction 3: Mast cell-specific pharmacological degranulation via c48/80 will masculinize SDN volume.

Chapter 2: Methods

2.1 Experimental Subjects

Adult Sprague-Dawley rat breeders were obtained from Charles River Laboratories and maintained on a 12:12h reverse light/dark cycle with *ad libitum* food and water. Animals were bred in our facility, and pregnant females were allowed to deliver naturally, with the day of birth being designated as postnatal day 0 (PN0). On PN0, pups were sexed, treated, and culled to no more than 14 pups per dam. Treatment groups and sexes were balanced across litters. All animal procedures were performed in accordance with the Animal Care and Use Committee's regulations at the University of Maryland School of Medicine. Litters of animals used in each experiment were sourced from 2-3 dams, counterbalanced for sex and treatment across litters.

2.2 Animal Treatments

All procedures were performed in accordance with the Animal Care and Use Committee's regulations at the University of Maryland School of Medicine. Bilateral intracerebral infusions were performed under cryoanesthesia. Pups received bilateral ICV (intracerebroventricular) infusions of 2 µl (1 µl/hemisphere) Minocycline hydrochloride (Sigma-Aldrich M9511; dose: 0.2µg/2 µl total), Compound 48/80 (Sigma-Aldrich #C2313; dose: 1µg/2 µl total) or a 50:50 mixture of 0.5 µg/µl H1 and 0.5 µg/µl H4 receptor antagonists (cetirizine and A943931; Tocris Bioscience) over 60 seconds once per day on postnatal days 0-1. Phagocytosis blocking agents, Anti-CD11b antibody (0.5 mg/ml; OX-42 clone; Bio-Rad Cat#MCA275GA, RRID:AB_566455), anti-F4/80 antibody (0.2

mg/ml; Santa Cruz Cat#sc-52664, RRID:AB_629466), Compound 48/80 (Sigma-Aldrich; dose: 4 µg/2 µl total) or phosphate-buffered saline vehicle were delivered over 60 seconds once per day on postnatal days 4-7 (or 5-8 for behavioral endpoints) using a 23-gauge Hamilton syringe mounted to a stereotaxic manipulator placed 1mm rostral to Bregma, 1mm lateral from midline. Hormonal manipulations were administered on postnatal days 0-1 via subcutaneous injections of either estradiol benzoate (10 µg, Sigma) dissolved in 100 µl of sesame oil or sesame oil vehicle. The dose of compound 48/80 used was determined based on previously published studies (Nautiyal et al., 2012) and our own validation studies (presented herein) showing its effectiveness at inducing mast cell degranulation. To label proliferating or recently divided cells, BrdU (50 mg/kg; Sigma) was dissolved in saline and delivered intraperitoneally (IP) in a volume of 0.1 ml per pup per day from PN0-2. For the gestational allergic challenge experiment, prior to pregnancy, adult females assigned randomly to the experimental group were sensitized with a subcutaneous injection of 1 mg ovalbumin (OVA grade V, Sigma) prepared at 4 mg/ml in pyrogen-free 0.9% saline and precipitated at a 1:1 ratio with $Al(OH)_3$ (Thermo Scientific) according to the manufacturer's instructions. After two weeks, a second 1 mg ovalbumin-Alum adjuvant injection was given. Control females were injected with saline at the same two timepoints to control for experimental handling and stress effects. One week later, all females were paired with males for breeding and the day of detection of sperm was assigned gestational day 0 (GD0). At GD15, pregnant rats were challenged intranasally with 1% ovalbumin in saline (experimental group) or saline vehicle (control group) (volume: 50 µl per nare), which was placed on each nare under light isoflurane anesthesia and inhaled upon regaining consciousness. At 30 min following challenge, maternal blood

was collected to assay for total serum Immunoglobulin E (IgE), using an IgE Rat ELISA kit (Abcam cat#157736) and serum samples run in triplicate. Females were paired in groups of two until GD15 and then housed individually. After birth, animals were sacrificed via perfusion to analyze brain-resident mast cells, microglia, or neuronal morphology at specified time points, or were weaned at PN22 into sex-specific groups of three containing both OVA-challenged and vehicle-exposed offspring for behavioral testing.

2.3 Histology and Immunohistochemistry (IHC/IFC)

For all *in vivo* histology experiments, animals were anesthetized with Fatal Plus (Vortech Pharmaceuticals) and transcardially perfused with phosphate-buffered saline (PBS; 0.1M, pH 7.4), followed by ice cold 4% paraformaldehyde (PFA; 4% in PBS, pH 7.4). Brains were removed and post-fixed for 48-72 hours in 4% PFA at 4 degrees C, then removed to a 30% sucrose solution at 4 degrees C until fully permeated. Brains were sectioned on a cryostat (Leica CM2050S) at a thickness of 45 μm and directly mounted onto silane-coated, charged slides. Mast cells were visualized using staining with acidic toluidine blue (0.5% in 60% ethanol, pH 2.0) as detailed in Nautiyal et al. (2012), sequential acidic Alcian blue (1% in 0.7 N HCl), and safranin O (1% in 0.125 N HCL, all stains from Sigma-Aldrich) or IHC for mast cell-specific markers as detailed below. Histological stains were performed on separate tissue sections. For immunohistochemistry (IHC) and immunofluorescent histochemistry (IFC), brain sections were rinsed twice with PBS, permeabilized with 0.3% H_2O_2 in 50% methanol, blocked with 5–10% bovine serum albumin (BSA) or normal goat serum in PBS

containing 0.4% Triton X-100, and incubated with primary antisera for 24–120 h at 4°C. For *in vitro* experiments, coverslips were washed with PBS twice and incubated with primary antisera in PBS supplemented with 10% BSA and 0.4% Triton X-100 for 24h at 4°C. Thereafter, sections or coverslips were extensively washed and either incubated with appropriate fluorescently tagged secondary antibodies for 2 h (Alexa Fluor 488, 555, 594, or 633, Life Technologies, 1:250) for IF or processed with biotinylated secondary antibodies (Vector Laboratories), avidin-biotin complex (Vector Laboratories), and reacted with 3,3′-Diaminobenzidine (DAB) with or without nickel (DAB or Ni-DAB; Sigma-Aldrich) in 0.125 M sodium acetate to visualize chromogen for IHC. For quantification of DNA material in phagocytic cups, sections were incubated in NucRed Dead 647 (Thermo Fisher Scientific) for 15 min after secondary antibody labeling at room temperature in the dark. For visualization, but not quantification, of nuclear material in cups, sections were incubated in Hoescht nuclear stain for 20 min (Invitrogen, catalog #MP-10338, 1:5000). Stained sections were cover-slipped with DPX mounting media or VectaShield Hard Set (Vector Laboratories). Primary antisera used were incubated for 24 h, unless specified, as follows: rabbit anti-NeuN (1:1000; Abcam catalog #ab177487), rabbit anti-cleaved caspase-3 (Asp175) (1:500; Cell Signaling Technology catalog #9661), rabbit anti-Egr1 (zif268) (1:1000; Cell Signaling Technology catalog #4153), mouse anti-CD11b (1:500; OX-42 clone; Abcam catalog #ab1211), Microtubule-Associated Protein-2 (MAP-2) (1:1000, Sigma-Aldrich, catalog #M1406), estrogen receptor alpha (ER-α) for 120 h at 4°C (1:1000, Millipore, catalog #06–935), 5-HT (1:1000, Abcam, catalog #sc32292); histamine (1:1000, Abcam, catalog #ab43870), Iba1 (1:1000, Wako, catalog #019–19741) or (1:1000, Novus Biologics, catalog #NB100–

1028), CD11b (1:5000, Abcam, catalog #ab75476), the IgE receptor FCεR1 (1:1000, Abcam, catalog #ab33568), rat mast cell protease 2 (RMCPII) (1:10,000, Moredun Scientific, catalog #MS-RM4), BrdU (1:250, BD Biosciences, catalog #347580), histamine receptor 1 (1:5000, Millipore, catalog #AB5652P), histamine receptor 4 (1:5000, Abcam, catalog #ab97997), and GFAP (1:5000, Sigma-Aldrich, catalog #G3893). Microglia were quantified using DAB IHC, and proliferating microglia were quantified using sequential DAB IHC against Iba1 and Ni-DAB against BrdU. Colocalization was confirmed in three dimensions using confocal microscopy on IF-stained tissue. Mast cell phenotype, mast cell estrogen receptors, and proliferating mast cells were assessed using IF and imaged with confocal microscopy. For colocalization, primary ER-α and RMCPII antibodies were labeled with secondary antibody conjugated to Alexa Fluor-594 and Alexa Fluor-488, respectively. For golgi-cox staining, whole brains from P5 pups or P80–90 adults were placed in 15 ml of Golgi-Cox solutions A and B (FD Neurotech) for 10 days, then solution C (sucrose; FD Neurotech) for 1–1.5 weeks, cut into coronal sections 100 μm thick using a Leica vibratome, and staining embedded using solutions D + E following the FD Neurotech protocol. Tissue was cleared with ascending ethanol, defatted with xylenes, and cover-slipped using Permount.

2.4 Image Acquisition of Mast Cells, Microglia, and Microglial Cup Content

Confocal fluorescence images were acquired via a Zeiss LSM 710 microscope equipped with 488, 561, and 633 lasers and a 20x (1.0 NA) water-immersion and 100x (1.46 NA) oil-immersion objective using Zeiss Zen software. Brightfield images were

captured on a Nikon Eclipse E600 with a 20x objective. Three-dimensional rendering was performed on confocal z-stacks taken with 2 μm z-intervals using a 100x objective and reconstructed in Imaris (Bitplane) as described in Schafer et al., 2014. Brightfield images were acquired via Nikon Eclipse E600 microscope and MBF Bioscience CX9000 camera.

2.5 Quantification of Phagocytic Microglia

Microglial cell counts were performed using Neurolucida (MBF Bioscience) on a computer interfaced with a Nikon Eclipse E600 microscope and MBF Bioscience CX9000 camera. Medial preoptic area contours were drawn using a 4x objective and stereological grid atop sections spanning only the central SDN (45 μm thick); counts from both hemispheres were quantified and averaged for the analysis. Microglia were included in analysis based on the presence of an observable cell body and classified as phagocytic if the microglia contained an observable phagocytic cup that was distinctly identifiable from the cell body, with a diameter >1 μm.

2.6 Quantification of Phagocytic Cup Diameter and Contents

Fixed coronal sections (45 μm thick) were immunolabeled for Iba1 and NeuN or Iba1 and cleaved caspase-3 (cCasp3) and stained with NucRed, then imaged with a Zeiss 710 confocal microscope equipped with 488, 561, and 633 lasers. Single confocal images were taken with a 20x (1.0 NA) water-immersion objective through the middle of the phagocytic cup. Images were then assessed for colocalization or measured in ImageJ.

2.7 Mast cell Quantification and Degranulation State

Mast cells were identified using Toluidine blue, a dichromatic stain that specifically stains mast cell granules purple and DNA/RNA (a light blue Nissl stain) and quantified under bright field microscopy. Mast cells that were degranulated were identified by having a much less granulated appearance and observation of granules located outside the cell's membrane. Identity of mast cells was confirmed with IF/IHC using fluorescent Avidin, anti-5HT or Anti-MCPT2. Boundaries for the POA were drawn in Neurolucida and spanned four sections of POA/animal.

2.8 Morphological Cell Analysis.

For analysis of Golgi-Cox impregnated POA neurons, neurons were chosen for analysis if the cell body was in the middle 50% of the z-plane of the tissue section, multiple processes were visible, and the cell was easily distinguishable from nearby cells. Four-to-five cells per animal across three-to-four brain sections were reconstructed in three dimensions under a 100x oil objective using Neurolucida software (MBF Bioscience). Morphological parameters for each cell were computed using Neurolucida Explorer, including cell body size, total dendritic length per cell, number of dendritic segments and branch points, and total number of dendritic spines per neuron. Data presented is the average of each of these parameters across multiple neurons for each animal. For adult analyses, dendritic spines were analyzed, but single cells were not reconstructed in three dimensions. Dendritic segments chosen for analysis were

unobstructed by other Golgi-stained material and were at least 25 μm in length without a bifurcation. Only one segment was analyzed per given cell. Four-to-five dendritic segments per animal across multiple brain sections were reconstructed, and dendritic spine density was analyzed using Neurolucida Explorer software. Data were analyzed using the average dendritic spine density for each animal.

2.9 Isolation of Microglia and Mast Cells and Maintenance in Culture

Brain mast cell and microglia isolations were performed on male and female pups (PN2–PN3) using methods published previously (Krishnaswamy and Chi, 2006; Patel et al., 2013) that included a whole-brain tissue homogenization under a 15 min 0.5% trypsin (Invitrogen) and 1% DNASE digestion. After suspension in a 37%, 50%, 70% Percoll gradient and centrifugation at 1200 × *g* for 40 min, microglia were taken from the interphase between 50% and 70%. Microglia were then passaged and used within 2 weeks of isolation.

Mast cells were later isolated using Percoll separation alone by taking cells from the very bottom of the 70% phase. Plating densities and mast cell culturing conditions were based on Krishnaswamy and Chi (2006). Mast cells were plated or replated at a density of 7.5×10^4 cells per well for a 24-well plate and 3×10^5 per well for a 6-well plate in 0.5 ml or 2 ml, respectively, of DMEM/F12 50:50 medium (Cellgro) containing 10% fetal bovine serum (FBS; Fisher Scientific), 1% penicillin-streptomycin-amphotericin (Quality Biological), 1% L-glutamine (Cellgro), 100 ng/ml stem cell factor (SCF) (Peprotech), and 30 ng/ml rat IL-3 (R&D Systems). Cells were grown overnight

and replated to remove contaminating adherent cells. Thereafter, mast cells were replated every 4 d and fed with the media described above but without penicillin-streptomycin-amphotericin. Mast cells were replated 24 h before an experiment in media lacking IL-3 and SCF at a density of 2.0×10^5 cells per well for a 24-well plate and 12×10^6 per 75 cm^2 flask (Corning). The plating medium consisted of 0.5 ml or 24 ml of DMEM/F12 50:50 medium (Corning, catalog #16–405-CV) for the respective plating densities and was supplemented with 10% FBS (Fisher Scientific), colony-stimulating factor-1 (CSF, 5 ng/ml), and L-glutamine (2 mM).

Mast cell identity and purity of cultures was verified via toluidine blue staining and fluorescent IHC against serotonin (5-HT) and mast cell protease 2 (MCPT-2); microglia were verified by fluorescent IHC against CD11b. The absence of neurons and astrocytes in cultures was confirmed by fluorescent IHC against MAP2 and GFAP, respectively. For western blots, tissue was homogenized in RIPA buffer containing 1% Igepal CA630, 0.25% deoxycholic acid, 1 mM EDTA, 154 mM NaCl, and 65 mM Trizma Base, with added protease and phosphatase inhibitors (1:1000). All chemicals were obtained from Sigma unless otherwise specified. Protein supernatant was extracted after 20 minutes of centrifugation at 3000 rpm at 4 °C, and total protein concentration determined via Bradford assay (BioRad). Fifteen μg of protein was electrophoresed on an 8–16% precast SDS polyacrylamide gel (Life Technologies) and transferred onto a single polyvinyl difluoride membrane (Bio-Rad). Membranes were blocked in 50% Odyssey blocking buffer (LI-COR) in TBS or in 10% non-fat milk in Tris-buffered saline containing 0.1% Tween (TTBS) and subsequently incubated with spinophilin primary anti-serum (Millipore cat#06–842, 1:1000) in 5% milk in TTBS overnight at 4 °C.

Membranes were rinsed and incubated with HRP-conjugated secondary antibody (1:200) for two hours. A Phototype chemilluminescence system (New England Biolabs) was used to detect the immunoblots by exposing the membrane to Hyperfield ECL (GE Healthcare). Integrative grayscale pixel area densitometry captured with a CCD camera was quantified using NIH Image J software. Ponceau S staining appearing at 45 kDa was used as a loading control and immunoblot densitometry values for each lane were expressed as a percentage of Ponceau staining for the same lane.

2.10 Behavior.

For sexual behavior testing, animals were gonadectomized between PN50–54, under isoflurane anesthesia and implanted subcutaneously with a 30-mm silastic capsule (1.57 mm inner diameter, 3.18 mm outer diameter) filled with crystalline testosterone (Sigma) placed between the scapulae. This capsule length releases testosterone in a manner that mimics physiological levels of testosterone circulating in adult males and allows appropriate activational hormones for developmentally masculinized females to perform male-typical copulatory behavior. Two weeks following surgery, animals were video recorded for at least 20 min during the dark phase of the light cycle, in a Plexiglass behavioral arena in the presence of a hormonally primed receptive stimulus female under red light illumination. Behavioral data was collected and analyzed by an observer blind to the experimental treatment of each animal. Measures included number of mounts, latency to mount, frequency of ejaculation, time of each ejaculation, and latency to resume mounting after ejaculation (ejaculation measures in males only). To record the full post-ejaculatory interval for situations during which a male started a post-ejaculatory interval

with only a portion of the 20 min testing period remaining, the male was observed beyond the 20 min period until he started mounting again. These mounts outside of the 20 min period were not tallied in total mount measures. Mount rate was calculated from the total mounts divided by the 20 minutes less the total time during that 20 min the animal was in a refractory post-ejaculatory state.

For odor preference testing, animals were weaned on PN21 and housed in same sex, randomized-treatment sibling pairs. On PN60, animals were tested for 5 min in 2-chamber preference chamber containing dishes of TEK-Fresh cellulose bedding (Harland Laboratories) that was used by sexually active males or females in estrus collected the morning of testing. Animals were given a 5 min acclimation period prior to testing, then video recorded for 5 minutes. Behavior testing took place during the dark phase of the animal's light/dark cycle under red light illumination. Females were tested for preferences during estrus. Estrus cycle tracking was performed via analysis of vaginal cytology collected daily by 300 µl saline lavage. Videos were scored using AnyMaze software to determine sexual odor preferences.

2.11 Sexual Odor Information Processing in cMPN via Egr1 Expression.

In addition to volumetric analysis, PN70-80 animals used for behavioral analysis were isolated for one hour in a clean cage outside of the colony room, after which a piece of filter paper soaked in sexually active male urine was placed in the cage and the animal observed/recorded for their interest in the novel odor for 10 min. After one hour of odor exposure, animals were anesthetized and transcardially perfused; brains were harvested,

post-fixed for 24 hours in 4% PFA, and then stored in 30% sucrose until saturated. Once sucrose-saturated, brains were flash-frozen on dry ice and stored at -80°C until sectioning.

2.12 Statistical Analysis

All values are shown as the mean ± SEM. Statistical analysis was performed using GraphPad Prism; version 9. Statistical details of experiments are provided in figure legends and in the text (tests used, exact n, p value). Comparisons between two experimental groups were performed using two-tailed Welch's t-test (for independent samples). Data including multiple experimental groups were analyzed using one-way or two-way analysis of variance (ANOVA) when appropriate. In cases where data were not normally distributed, Kruskal-Wallis or Mann Whitney U tests were used. In some experiments, post-hoc pairwise t tests were calculated for specific comparisons to determine differences in means between groups. The Linear correlation was calculated using Pearson's r. with a p value of < 0.05 was used as the criteria for significance.

Chapter 3: Microglia Are More Phagocytic in the Developing Female POA

3.1 Introduction

While surveying the preoptic area for sex differences in microglial phenotype and number, I made the discovery that a higher percentage of microglia were sporting large "cups" containing nuclear material at the tips of their processes when compared to males on postnatal day 2 (Figure 3.1). Since the cups were much larger than those that would suggest evidence of synaptic "nibbling" or digestion of debris, we reasoned that those microglia in the female POA were likely removing whole cells, rather than just regulating synapse development.

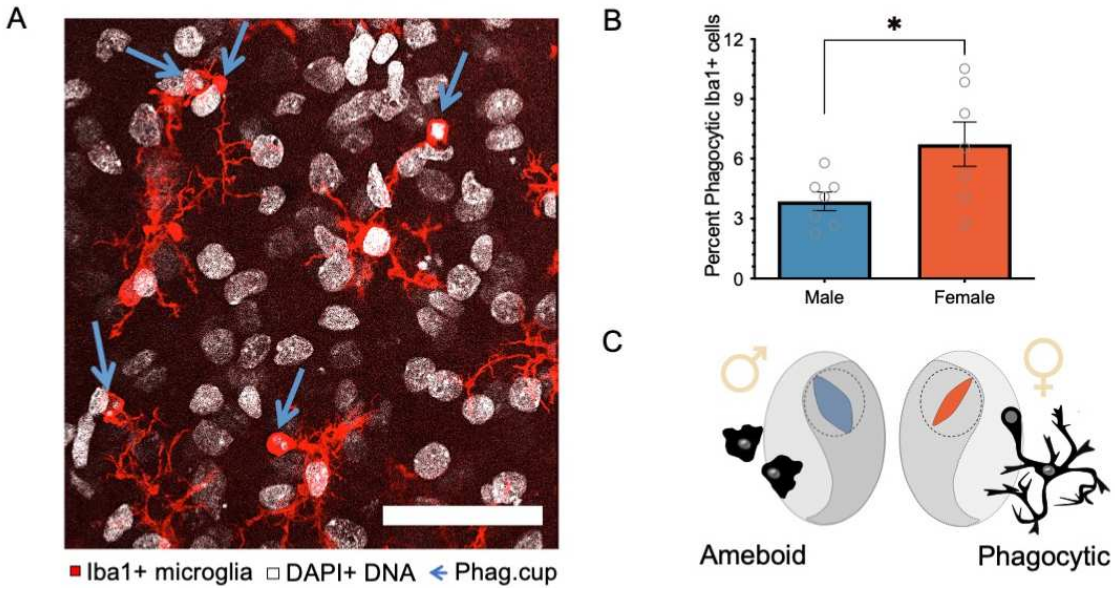


Figure 3.1: Females have a greater percentage of "surveying" phagocytic microglia in the neonatal POA. On PN2, females (♀s) have a significantly greater amount of phagocytic microglia than males (♂s) (P = 0.036 by 2-tailed, unpaired t-test). (n= 7♂s &♀s).

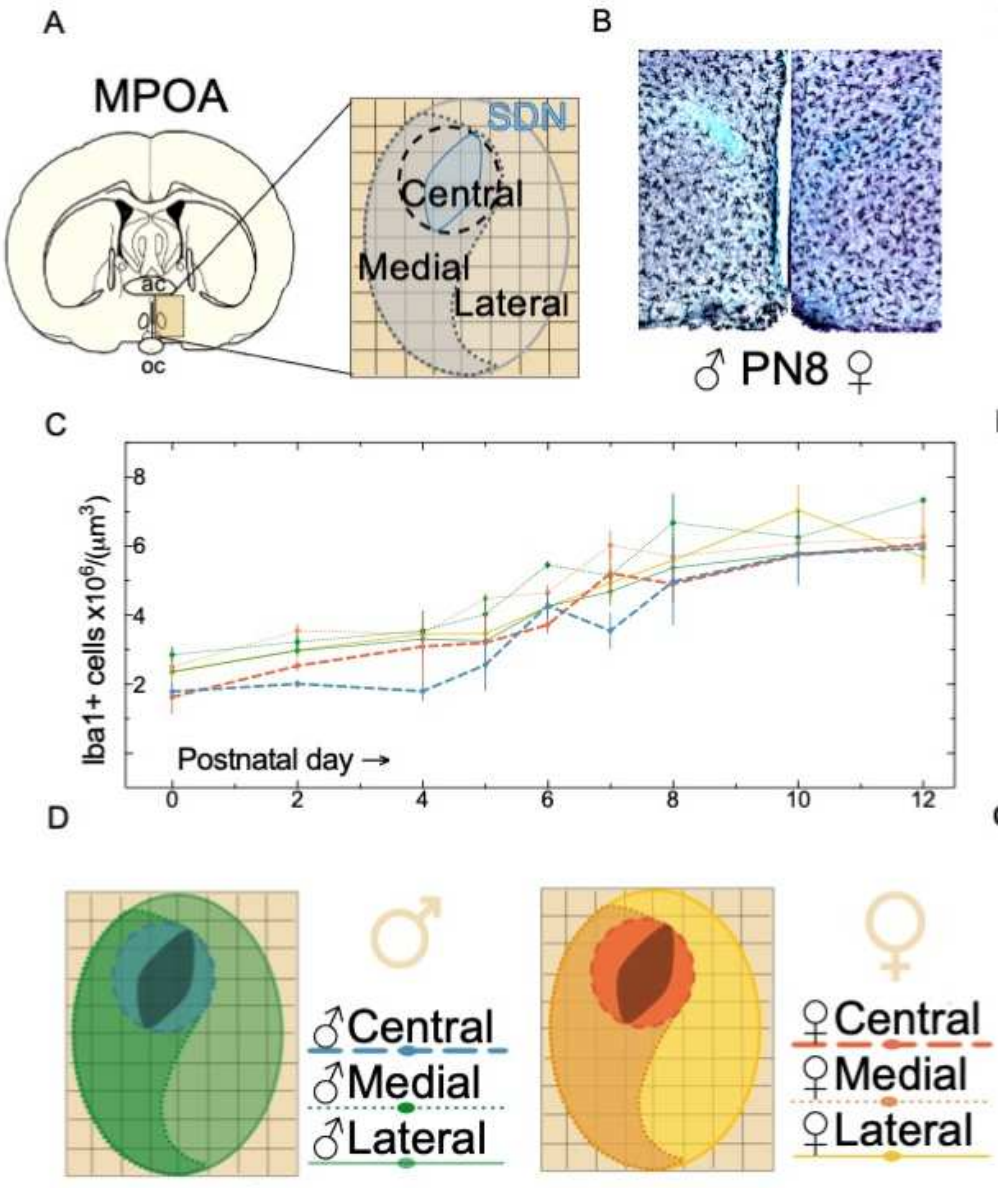
To test if the sexually dimorphic nucleus, being the largest volumetric sex difference in the brain, was driving this more phagocytic phenotype, we surveyed microglia morphology in the three subregions of the MPN over the first 12 days of life in both males and females. We predicted that we would find more phagocytic activity in the cMPN than in the lateral and the medial MPN if the SDN was the main driver of the observed sex difference in phagocytic activity. The reason for this is due to the majority of the SDN being located within the cMPN. Additionally, we reasoned that if this microglial phagocytosis was driving the sex difference, we would see more phagocytic microglia in the cMPN of females over males. We expected to see even higher levels of phagocytosis just prior to the differentiation of the SDN, around PN3-4, because PN4 is the earliest detectable reported difference in volumes between the sexes in the rat (Sickel, McCarthy, 2000).

3.2 Results

We were surprised to find that phagocytic activity began increasing in the cMPN and mMPN on PN5, with the greatest increase observed in the female cMPN. Peak phagocytic activity in the female cMPN was observed on PN8, and then decreased to the level seen in males by PN12. The sex difference in microglial phagocytic activity originally observed at PN2 when the whole POA was surveyed (Figure 3.1B), did not hold up in the time course analysis of the MPN (Fig 3.2C). The time course analysis was restricted to the MPN and only one tissue section was used, whereas in the original PN2 analysis, four sections of POA per animal were used. It is possible that there is a wave of phagocytosis happening in the POA and that, for the time course experiment, we were restricted to a very specific section- the one representing the greatest SDN volume,

coronally. If the survey had been conducted across four sections of the POA, it is possible that we would have seen some more nuanced time and region-specific phagocytic activity, like that which was observed at PN2 in the entire POA, initially (Figure 3.1). The time course was also completed with lower numbers of animals than the original PN2 survey, and this may have limited our ability to replicate the original sex difference in the whole POA. A main effect of sex was observed when all regions and time points were examined, with females having greater phagocytosis than males (Figure 3.2).

Figure 3.2. Neurons of the female SDN are removed by microglial phagocytosis with peak activity occurring at PN8. **A.** Schematic representation of 100 µm x 100 µm grid used to trace subdivisions of the MPN to count and characterize microglia from birth (PN0) to PN12. **B.** Photomicrograph of Iba1+ microglia in the MPN in both sexes at PN8. Nissl-SDN was Nissl-stained with methyl green and used as a guide for grid placement. **C.** Iba1+ microglia populate the medial preoptic nucleus across the first 12 postnatal days, with no differences between ♂s and ♀s. Main effects of time ($P<0.0001$), $F(8, 164) = 47.13$ and subdivision ($P<0.0001$; $F(8, 164) = 47.13$) were detected for each sex and subregion ($P<0.001$; $F(5, 164) = 8.26$) using 2-way ANOVA. Both ♂s and ♀s had the highest number of microglia within the mMPN subregion compared with other regions. ♀s had significantly more microglia in the mMPN than ♂'s in the cMPN ($P=0.0378$); while ♂s had significantly more microglia in the mMPN than both the cMPN ($P<0.0001$) and lMPN ($P=0.0327$) of ♀s across time. **D.** Figure legend for panel C and for panels E-G. **E.** The ♀ central cMPN has a higher density of phagocytic microglia when compared to ♂s on PN7 (**P=0.0098) and PN8 (***P<0.001), as well as when compared to the mMPN on PN8 (‘‘‘P= 0.0003) and lMPN on PN6 (^^P= 0.0019), PN7 (^^P=0.0023), and PN8 & PN10 (^^^^P<0.0001). The ♂cMPN contains a higher density of phagocytic microglia than the mMPN of ♀s on PN6 (- -P<0.01) and the lMPN on PN6 & PN7, (+P<0.05). **F.** The ♀ central cMPN has a higher percentage of phagocytic microglia when compared to ♂s on PN8 (****P<0.0001), as well as when compared to the ♂medial PN6 (‘‘‘P= 0.0014) or PN8 (‘‘‘‘ P= <0.0001), or to the lateral on PN6/8 (^^^^P<0.001), PN7 (^P=0.0112), and PN10 (^^P=0.0023). ♂s have a greater percentage of phagocytic microglia in the central vs. lateral MPN on PN6 (- - -P=0.001). **G.** A main effect of sex was detected in the central [(P=0.002), $F(1, 52) = 10.35$] and lateral (P=0.002, $F(1, 43) = 10.88$) but not medial mPOA, indicating ♀s had more phagocytic cups than ♂s in those regions. The ♀ central cMPN has a higher density of phagocytic cups when compared to ♂s on PN8 (***P<0.001) and PN10 (*P=0.0164) and within the lMPN subregion,♀s have a higher number of phagocytic cups than ♂s on PN8 (*P=0.014).


A
MPOA
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Iba1+ cells x10^6/(μm^3)
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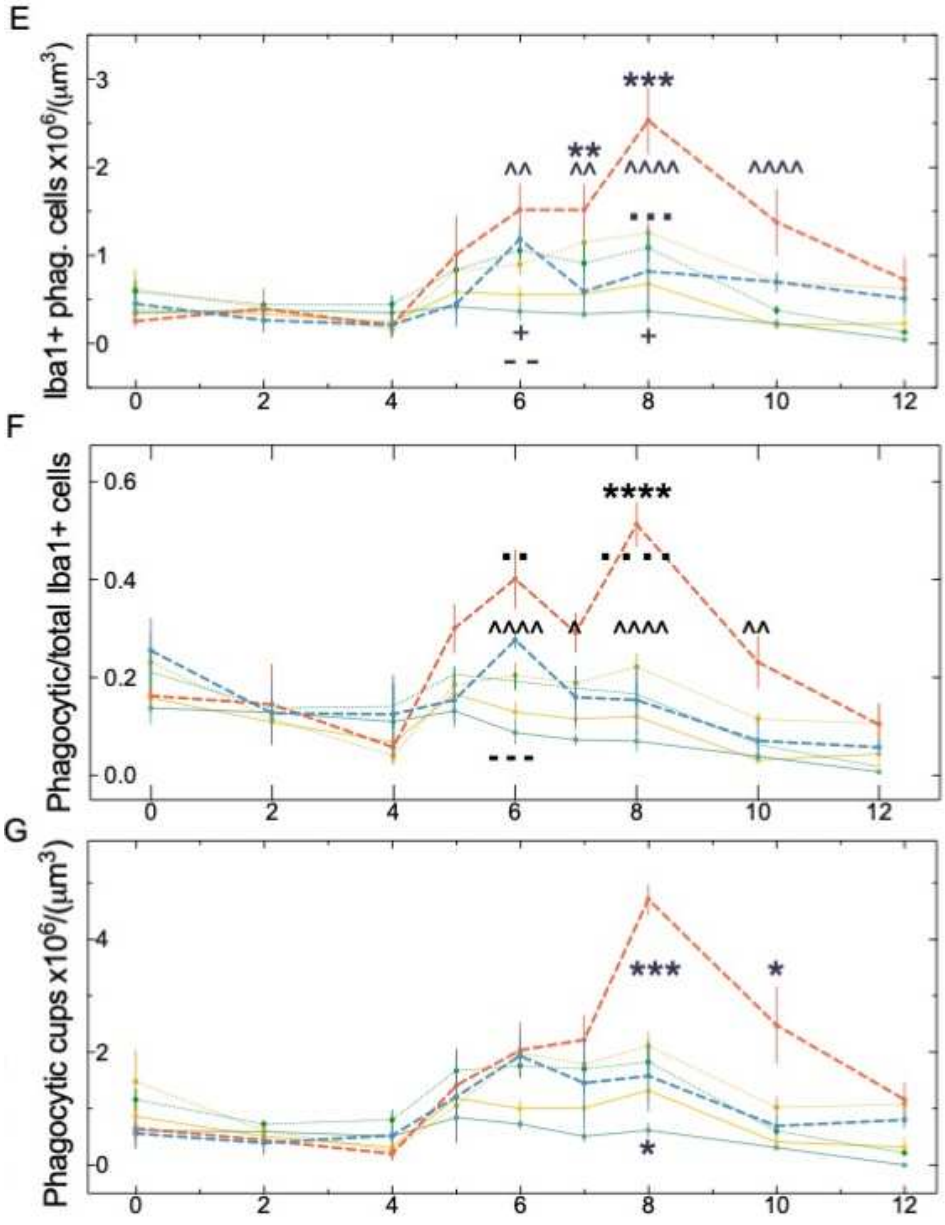

E
Iba1+ phag. cells x10^6/(μm^3)
F
Phagocytic/total Iba1+ cells
G
Phagocytic cups x10^6/(μm^3)

To determine the identity of cellular targets of microglial phagocytosis and their death factor expression status, Jonathan Van Ryzin and I teamed up, using immunohistochemistry to investigate levels of the canonical death factor, cleaved caspase-3 (cCasp3, the activate form of the protease), and nuclear neuronal antigen NeuN in the cups of four males and four females in the cMPN on PN8, during the peak of microglial phagocytosis. The cellular cup composition and diameter did not differ between males and females (Figure 3.3). Nearly all (98.21 ± 1.786%) cups surveyed contained DAPI+ nuclear material (Figure 3.3B, 96.43 ± 3.573% in males, 100% in females) and no sex difference in average cup diameter was observed (Figure 3.3A). The average cup diameter was 7.354 ± 0.209 μm in males and 6.91 ± 0.27 μm in females, with an overall cup diameter of 7.124 ± 0.21 μm when sexes were collapsed. Of the DNA-containing cups, 69.52 ±12.29% in males and 76.78 ± 9.443% in females contained NeuN+ material (Figure 3.3C) and only 26.63 ±3.30% in males and 19.24 ± 4.54% in females were immunopositive for cCasp3+ (Figure 3.3D), indicating that most of the cells engulfed in the cMPN on PN8 in both males and females were cCasp3- neurons.

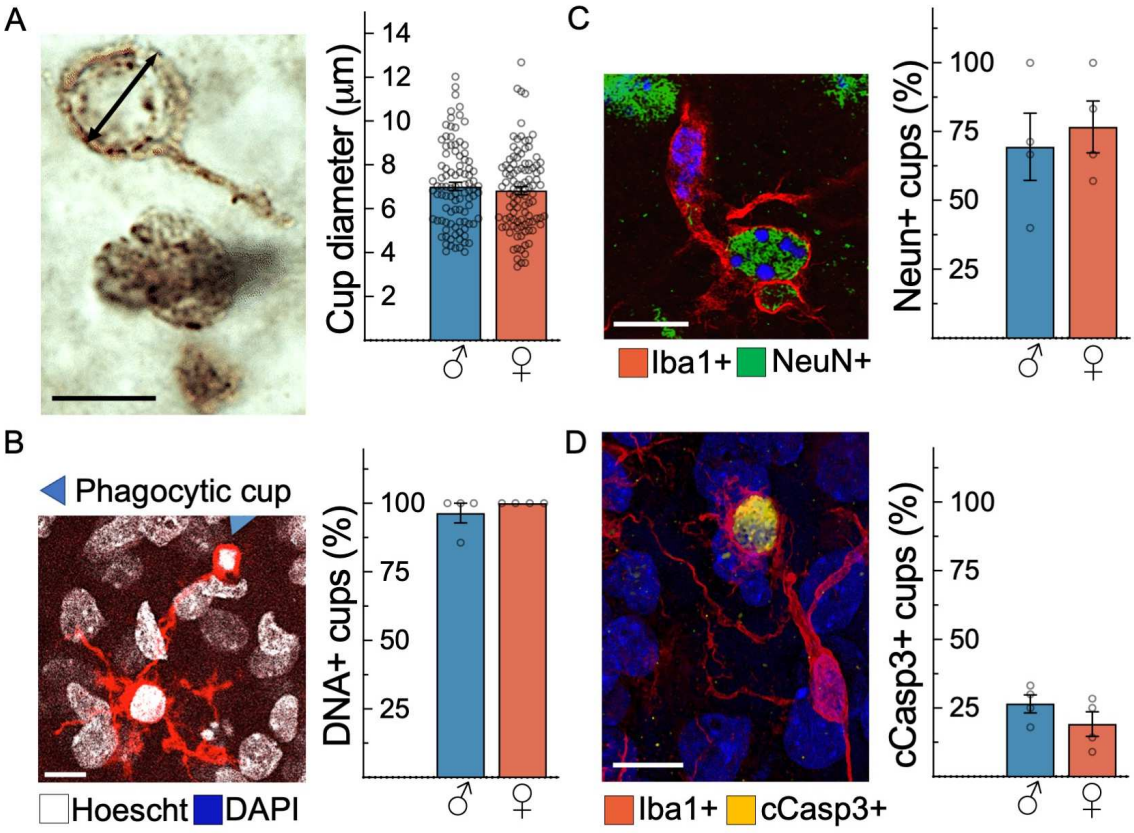


Figure 3.3 Phagocytic cups contain DNA and non-apoptotic neuronal material
A. Brightfield image of large Iba1+-DAB microglial phagocytic cup and quantification of phagocytic cup size within the cMPN. Survey of cup size in four PN8 animals (10 cups/animal) of each sex yielded no difference in cup diameter between the sexes, with an average diameter of 7.124 +/- 0.2098 µm (mean ± SEM) when sex was collapsed.
B. DAPI+ nuclear material found in majority of the phagocytic cups surveyed, with no differences between the sexes. Most of the microglial cups surveyed were DAPI+ (96.4% of ♂cups, 100% in ♀cups). **C.** Representative images of cups containing NeuN+ neuronal material, which was present in majority of the cups surveyed (69.5 +/- 12.3% in ♂s and 76.8 +/- 9.4% in ♀s). **D**. Representative images of cups containing cCasp3+ cellular material, which made up a minority (~25% of cups) were immunopositive for late apoptosis marker cCasp3 prior to engulfment (26.63 +/-3.29% in ♂s and 19.24 +/- 4.54% in ♀s. Bars represent the mean +/- SEM. Open circles represent individual data points for each animal, with cup diameter being the exception, where individual cup diameter measurements are shown. All scale bars = 10 µm.

3.3 Discussion

Microglial phagocytosis in the MPN was overall greater in females (main effect) than males, with the only significant sex difference in phagocytic microglia observed in the cMPN on PN8, when analysis was broken down by region across time. Although the timing of the sex difference and peak of phagocytic activity was much later than we had anticipated, our findings gave us a clear confirmation of our original speculation about the SDN being the major driver of higher microglial phagocytic activity at the start of the second week of life in females. The phagocytic cup contents (cellular targets) and cup diameter did not differ between the sexes, and we found that most of the microglial phagocytosis was targeting cCasp3- (i.e., non-apoptotic) neurons. As cCasp3 marks a terminal apoptosis step, these data indicate the cups are likely phagocytosing viable neurons that are being killed post-engulfment by the microglia. To determine whether microglia are the primary executioners of these cells, by engaging in phagoptosis versus cleaning up almost or already dead cells via secondary phagocytosis, we designed a series of experiments to assess the viability of these cells in the absence of phagocytic microglia.

Chapter 4: Microglial Phagocytosis Determines Sex Differences in SDN Volume

4.1 Introduction

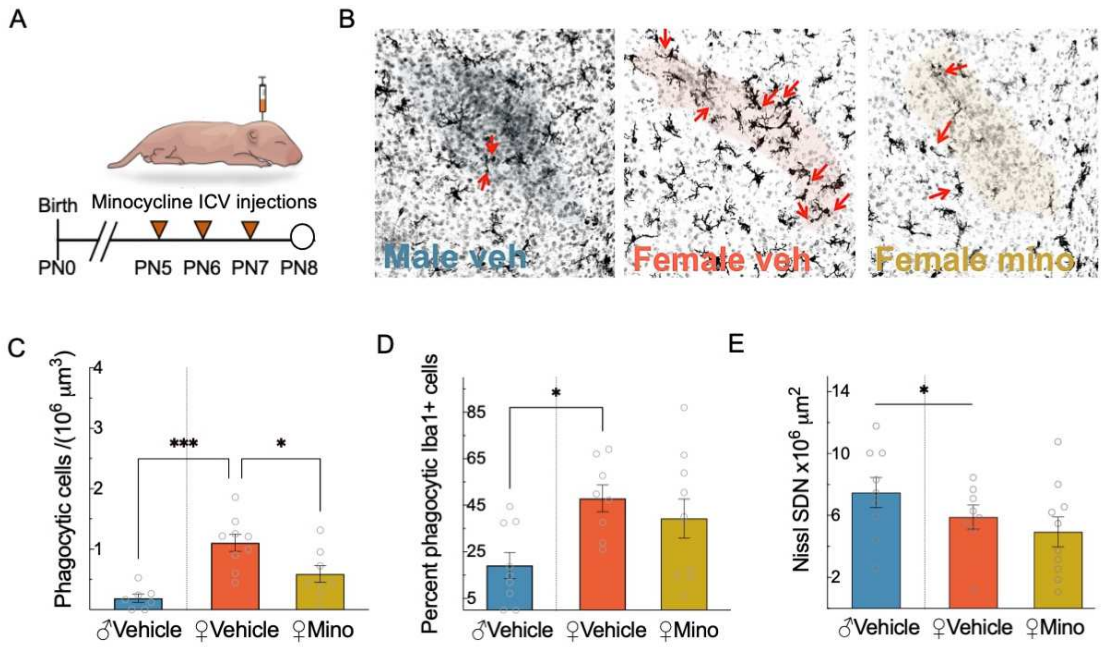
Microglia are essential partners in determining the development and modification of neural circuits as they develop. Through many studies, it is now widely established that their role during development is far beyond that of a quiescent sentinel. As modified macrophages that have the privilege of residing within the brain, microglia are highly phagocytic. These cells are capable of both "nibbling" to prune away unnecessary or unwanted synapses, as well as of "engulfing" to remove debris following cell death or injury. Phagoptosis, also known as "primary" phagocytosis, refers to phagocytosis of living cells that are viable and would otherwise survive if not actively engulfed and destroyed by microglia. Phagoptosis generally occurs when cells are distressed due to a subtoxic insult or are physiologically activated by other means, causing them to display 'eat me' signals (or lose 'don't eat me' signals) on their cell surface. Phagoptosis is also directed at precursor cells that are continuously being produced as a means for regulating population density in select regions of the brain. By inhibiting phagocytosis during a period of high phagocytic activity and tracking cellular target survival in this scenario, one can easily distinguish the phagocytic activity of microglia as either primary or secondary to the death of target cells. Secondary phagocytosis is defined as the engulfment of cells expressing the terminal apoptosis marker cCasp, which are considered either dead or past the point of no return.

4.2 Results

To determine if the neurons being engulfed by microglia would otherwise survive, we used two methods to decrease or block phagocytosis. First, we administered a low

dose of the drug minocycline during the period just before and during the highest levels of phagocytosis detected in females. Minocycline administration, ICV, from PN5-7 reduced the detection of microglial phagocytosis in females but did not significantly impact the volume of the Nissl-SDN on postnatal day 8 (Figure 4.1).

Figure 4.1 Minocycline decreases phagocytosis but does not change volume of SDN.A. Neonatal pups received a series of bilateral ICV injections of minocycline on PN5-7 (0.2µg/2µl/day) and histological measurement of SDN volume and microglial phagocytosis were assessed on PN8 using IHC for Iba1+ cells and Methyl Green for Nissl detection.**B.** Representative images of Iba1+ cells in and around the SDN, within the cMPN. A reduction in phagocytic microglia is apparent following bilateral ICV injections of minocycline, a broad-spectrum inhibitor of microglial activation. **C.** The sex difference in Iba1+ phagocytic cells per unit volume was detected on PN8 when analyzed by one way ANOVA ($F(2,21) = 11.77$, $P = 0.0004$), with Sidak's post hoc analysis for multiple comparisons showing significantly greater numbers of phagocytic cells per unit area in ♀ Vehicle vs. ♂ Vehicle, ($P = 0.0002$) and in ♀ Vehicle vs. ♀ Mino, ($P = 0.0278$). ♂ n = 7, ♀ n = 8; ♀ Mino n = 9. **D.** The percentage of phagocytic microglia was no different between the three groups, when analyzed by one-way ANOVA ($F(2, 21) = 2.630$, $P=0.0956$) with Sidak's post hoc tests: ♂Vehicle vs. ♀Vehicle, $P = 0.066$). By unpaired t-test, ♀Vehicles had a greater percentage of phagocytic microglia when compared to ♂Vehicle ($P=0.0129$, with no difference detected between ♀Vehicle and ♀Mino treatment by t-test. ♂n = 7, ♀n = 8; ♀Mino n = 9.**E.** There was no difference in Nissl-SDN volume between groups when analyzed by one-way ANOVA ($F(2, 24) = 2.006$, $P= 0.1564$); however, ♂ Vehicle had a significantly greater SDN volume vs. ♀Vehicle by unpaired t-test $P = 0.0406$). ♂ n = 9, ♀ n = 8; ♀Mino n = 10. Bars represent the mean +/- SEM. Vertical dashed line indicates separation of male and female groups. Open circles represent individual data points for each animal used in the experiment.



We next treated developing neonates with a function-blocking antibody against the CD11b component of complement receptor 3 (CR3). Upon binding CR3, this antibody prevents phagocytosis by disrupting the ability of the microglia to detect and respond to surface complement commonly considered an "eat me" signal. The anti-CR3 antibody was infused directly into the brain each day from PN5-7 (Figure 4.2A), a period just prior to the peak in microglia phagocytosis on PN8 (see Figure 3.2), and bound to microglia, as identified by morphology (Figure 4.2B). The percent of microglia engaged in phagocytosis on PN8 in the cMPN was significantly reduced in females infused with the anti-CR3 antibody but not with a control antibody (F480), when compared to vehicle-treated females (Figure 4.2C). There was no effect of either antibody on the already low percentage of phagocytic microglia in males (Figure 4.2D). A similar effect was seen for the actual number of phagocytic cups in the cMPN in females (Figure 4.2E) and neither parameter changed in response to antibody infusion in the lateral subdivision of the MPN in either sex (Figure 4.2D & E).

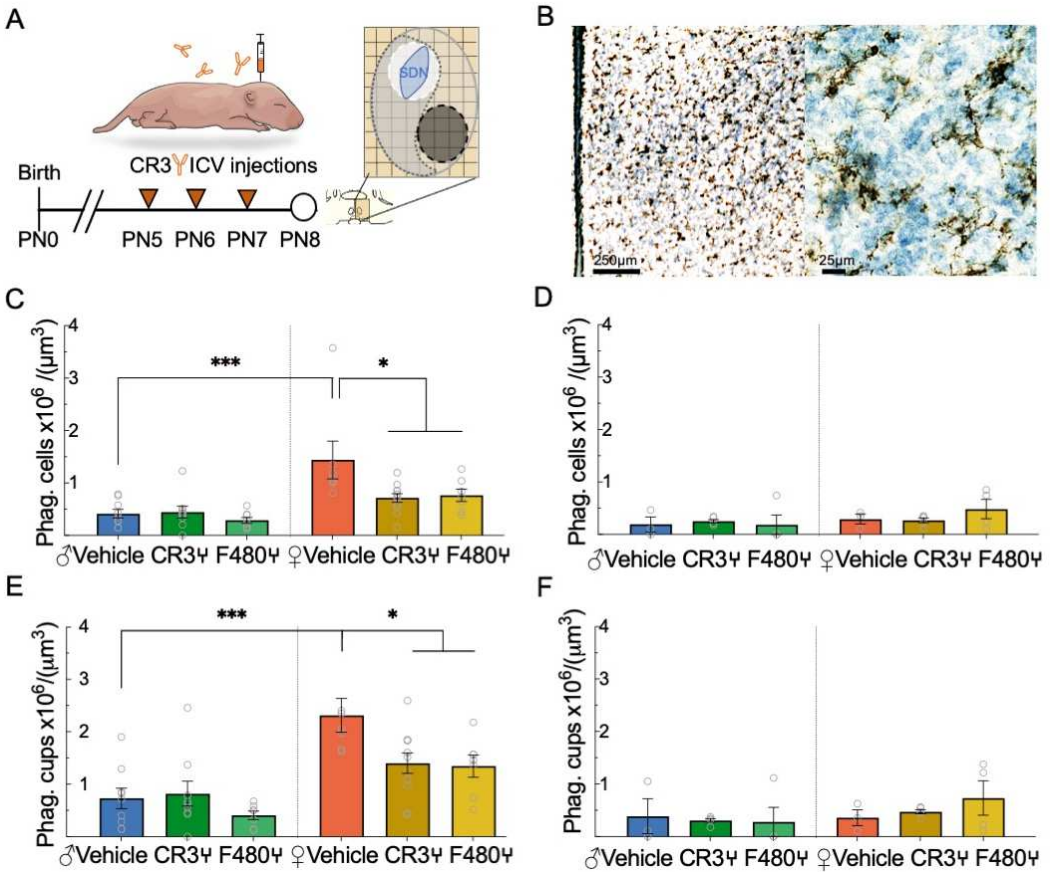
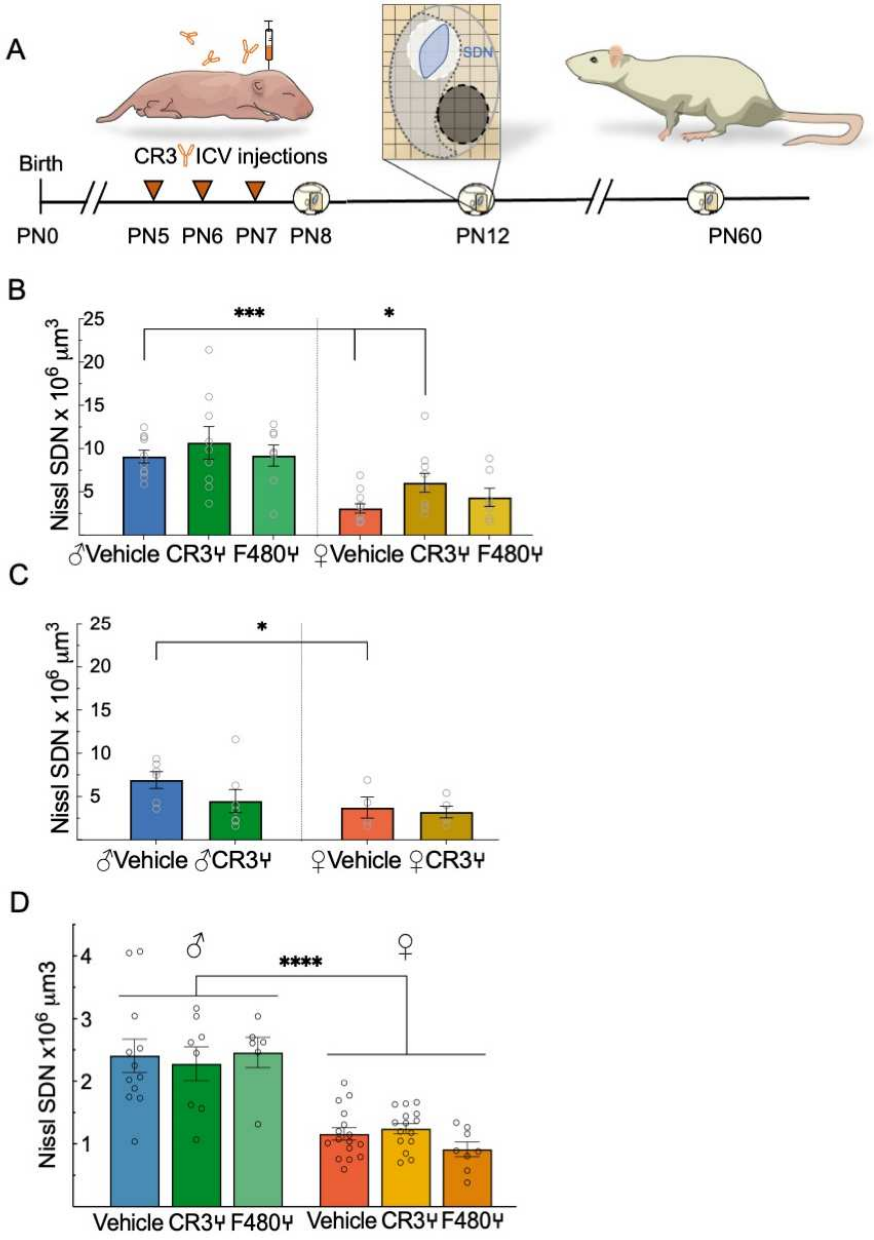


Figure 4.2. Phagocytosis is blocked on PN8 following anti-CR3 antibody blockade. **A.** Bilateral ICV injections of anti-CR3, anti-F480 antibodies or vehicle on PN5-7. Histology and Iba1+ cells were assessed on PN8 in central (C&E) and lateral (D&F) MPN. **B.** Bilateral ICV injections of 2 µg of mouse anti-CR3 antibody is shown to bind specifically to microglia/macrophages; DAB conjugated anti-mouse antibody was used to track spread of the injected IgG-CR3 (mouse) antibody. Scale bars:25 and 250µm. **C.** A main effect of sex ($F(1, 45) = 23.22$; $P<0.0001$), treatment ($F(2, 45) = 4.049$; $P = 0.0242$) and interaction of sex and treatment $F(2, 45) = 3.466$; ($P=0.0398$) was revealed when phagocytic microglia in the cMPN were analyzed by 2-way ANOVA. Number of phagocytic microglia was greater in female vs. male vehicle groups ($P = 0.0003$) and was reduced significantly by anti-CR3 ($P=0.014$). A trend towards reduction by F480 antibody treatment was detected, when groups were compared via Tukey's post hoc analysis ($P = 0.057$). **D.** Anti-CR3 and F480 antibody treatments had no effect on the already low levels of phagocytosis in the lateral MPN. **E.** A main effect of sex ($F(1, 45) = 35.07$; $P<0.0001$) and treatment ($F(2, 45) = 4.524$; $P=0.0162$) was discovered regarding number of phagocytic cups. The higher number of phagocytic cups in the female vehicle group was significantly reduced by anti-CR3 ($P=0.045$) and a trend in reduction was detected by the anti-F480 ($P = 0.060$) antibody treatment when groups were compared via Tukey's post hoc analysis. **F.** Phagocytic cup formation in the lateral MPN was not affected by either antibody treatment. Open circles represent individual data points for each animal.

We next sought to confirm that blocking microglia phagocytosis would increase the volume of the SDN. When measured on PN8, after the last antibody infusion, the volume of the SDN was significantly increased in females treated with anti-CR3 compared to either vehicle or control antibody (Fig 4.3B). However, no change in SDN volume was found 4 days later, on PN12 (Figure 4.3C), or on PN60 (Figure 4.3D).

Figure 4.3 SDN volume is significantly increased on PN8 following function-blocking anti-CR3 (Cd11b) antibody administration. A. Schematic showing neonatal ICV injections of vehicle, anti-CR3 or anti-F480 antibodies from PN5-7 for Nissl SDN volume analysis on PN8, 12, or 60. **B.** A main effect of sex was detected by 2-way ANOVA ($P<0.001$) as well as a sex difference (♂Vehicle vs. ♀ Vehicle, ($P<0.001$) via posthoc comparisons. Anti-CR3 antibody blockade increased SDN volume in PN8 ♀ groups compared via Kruskal-Wallis analysis followed by Dunn's multiple comparisons (♀Vehicle vs. ♀ CR3, $P=0.0325$). **C.** A main effect of sex was detected by 2-way ANOVA ($F(1,19) = 13.93$, $P = 0.001$) with ♂s having a greater volume when compared to ♀s (♂Vehicle vs ♀ Vehicle, $P=0.0135$) but no effect of anti-CR3 antibody treatment was detected four days following antibody treatments on PN12. **D.** A significant main effect of sex was detected on PN60 ($P<0.0001$) by 2-way ANOVA but no lasting change in SDN volume resulted from either antibody treatment. Bars represent the mean +/- SEM. Vertical dashed line indicates separation of male and female groups. Open circles represent individual data points for each animal.


A
SDN
Birth
CR3 ICV injections
PN0
PN5 PN6 PN7 PN8
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4.3 Discussion

This study is the first to report enhanced survival of the cells of the female SDN (normally destined for cell death) without the use of exogenous steroid treatment in the rat. Neonatal inhibition of phagocytosis via a series of ICV injections of anti-CR3 antibody from PN5-7 resulted in a decrease in phagocytic microglia and an increase in SDN volume in females only when evaluated on postnatal day 8. The survival of these cells and subsequent enlargement of the SDN is a neuroanatomical measure of masculinization. Thus, these results support our initial hypothesis that microglial phagocytosis is the primary driver of sexual differentiation of the SDN rather than the secondary action of microglia, as the clean-up crew for cells that had already undergone or committed themselves to canonical, self-programmed apoptosis. We found that the cells targeted were mostly negative for cCasp3 markers, also consistent with our hypothesis. The fact that female SDN enlargement was not again observed on PN12 (as assessed by SDN volume, only) may be due to several factors, one of them being that the study was underpowered, as compared to those sourced from multiple litters with larger group sizes on PN8 (which were assessed for microglial parameters, in addition to SDN volume). The second possibility: the function-blocking antibody interfered with neuro-immune landscape changes during peak phagocytic activity, developmentally delaying, rather than irreversibly interfering with, SDN sexual differentiation. If this is true, and the SDN recovers from the period of Cd11b inhibition during development, the function-blocking antibody treatment alone could have disrupted development in other subtle ways, beyond what we could measure here. We showed that phagocytosis is reduced and volume of the Nissl-SDN is increased in females at PN8, which could mean that we

prevented cell death in the model system that was the focus of this study. There could be phenotypic changes, maturation processes, or other subtle measures that are not easy to ascertain during development, especially because changes are also likely affected by later hormonal surges, for example during puberty. The most obvious way to test whether there were enduring effects of the neonatal phagocytosis blockade was to measure the female SDN volume in adulthood. Although we failed to find a volume difference at P60, the possibility that there are other changes in the adult brain resulting from the neonatal phagocytosis blockade cannot be excluded.

Chapter 5: Microglial Phagocytosis Determines Adult Sexual Odor Preferences

5.1 Introduction

A preference for females as sexual partners is a measure in rat and humans that positively correlates with volume of the SDN., or third insterstitial nucleaus of the anterior hypothalamus (InAh-3) correlates which is known to vary by sex and is a factor To understand whether or not microglial phagocytosis during the first week of life represents a "critical period" of sexual differentiation, required for sexual odor preference in adulthood, the antibody blockade approach was repeated and carried out through the peak of phagocytosis, PN8. Once weaned, animals were housed in groups of 3-4 with same-sex, weight-matched groups representing at least one member of every treatment group and randomly distributed with respect to siblinghood. Uninjected controls were substituted for vehicles to even out groups, as needed.

Between PN70-80, animals were tested for 5 min in a 3-chamber preference chamber containing a central neutral zone, as well as a dish in each of the side chambers containing fresh soiled bedding from either a sexually active male or female in estrus. Placement of odor cue was counterbalanced across treatment groups and sexes and animals were placed in the center of the neutral zone at the start of each test. Behavioral testing took place during the dark phase of the animal's light/dark cycle under red light illumination. In addition to the odor preference test, on PN70-80, animals were isolated for one hour, exposed to sexually active male urine-soaked filter paper held in place with binder clips for one hour, and then killed. The brains were harvested within an hour post-odor exposure to access enduring effects of neonatal phagocytosis blockade on SDN volume and immediate early gene Egr1 expression in the cMPN.

5.2 Results

Functional blockade of microglial phagocytosis during PN5-8 neonatal development, achieved with anti-CR3 antibody, but not anti-F480 control antibody treatment, occluded sex-typical odor preference behavior in gonadally intact adult females during proestrus/estrus (Figure 5.1).

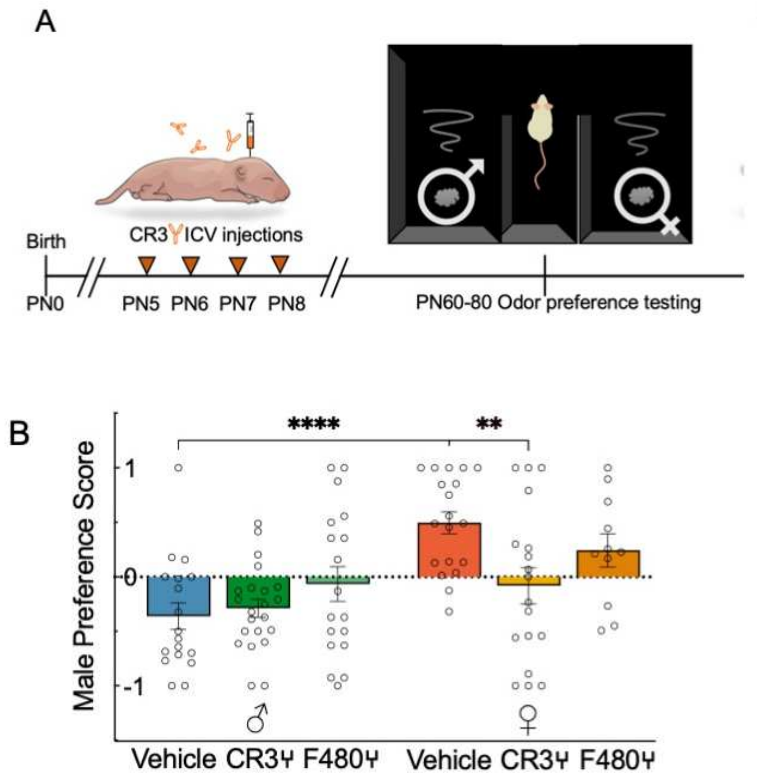


Figure 5.1 Neonatal phagocytosis blockade abolishes sexual partner preference. **A**. Neonatal pups received bilateral ICV injections of function blocking anti-CR3 antibody on PN5-8, then underwent behavioral testing for sexual odor preferences conducted from PN60 to PN80. Preference score for sexually active male vs. proestrus female odor was calculated based on mean time spent in male vs. female odor source, divided by the total time spent investigating odor source of ether sex. **B**. A main effect of sex ($F(1,100) = 17.68$, $P<0.001$) and an interaction between sex and treatment ($F(2,100) = 3.75$, $P = 0.0269$) were detected by 2-way ANOVA followed by Tukey's multiple comparisons test (♀Vehicle vs. ♀CR3Ψ $P = 0.021$); (♂ Vehicle vs. ♀ Vehicle, $P <0.0001$). Bars represent the mean +/- SEM. Vertical dashed line indicates separation of male and female groups. Open circles represent individual data points for each animal.

Neonatal blockade of microglial phagocytosis, despite having increased the volume of the SDN as measured directly after the treatment, neonatally, produced no lasting detectable volumetric differences in adulthood. However, functionally blocking phagocytosis significantly reduced immediate early gene expression in the SDN in response to acute male sexual odor exposure. Finally, the number of cells expressing Egr1, a marker for immediate early gene activation per unit area, showed a significant positive correlation with preference for male odor when all animals were included in the analysis ($P=0.02$).

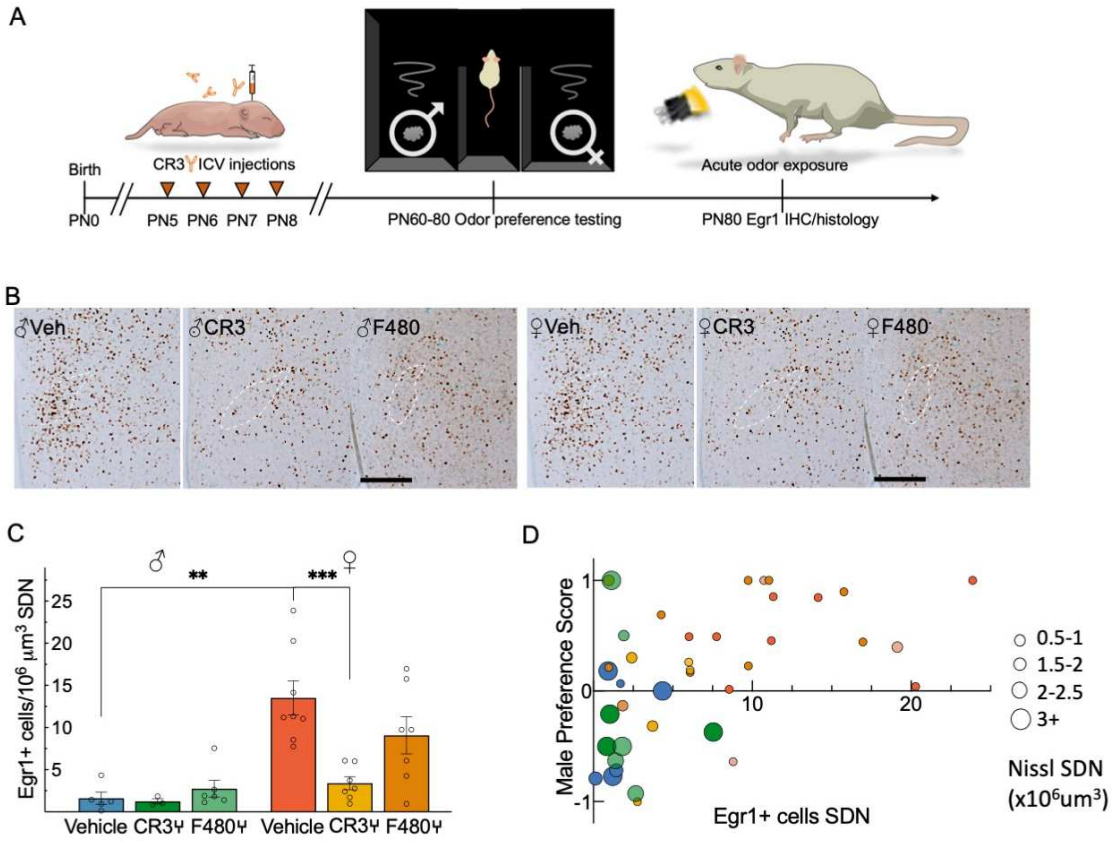


Figure 5.2 Neonatal phagocytosis blockade significantly reduces Egr1 expression in the Nissl-SDN of females in response to male urine. A. Neonatal pups received bilateral ICV injections of function blocking anti-CR3 antibody on PN5-8 and underwent behavioral testing for sexual odor preferences from PN60 to PN80. On or after PN80, animals were given an acute exposure to male urine prior to sacrificing for histological measurements of SDN volume and Egr1 expression in the cMPN. **B.** Representative images of Egr1+ cells in the cMPN/SDN in PN80 adult in response to acute male odor. **C.** A Sex difference in Egr1+ expression in the SDN following acute male urine exposure ($F(1,30) = 22.55$ $P<0.0001$), was reduced in neonatal anti-CR3 phagocytosis blockaded females when compared to vehicles (♀V vs.♀CR3 ($P<0.01$)) **D.** A positive correlation was found between Egr1+ cells per unit area in response to acute male odor stimulus ($P=0.02$) and male preference score. Bars represent the mean +/- SEM. Open circles represent individual data points for each animal.

Next, I ran an experiment to determine if mast cell activation in females by pharmacological activation of mast cells via c48/80, during the same neonatal period (PN5-7) had any effect on microglial phagocytosis or SDN volume., since the number of mast cells in the male POA peaks on PN7, just prior to the period in which females but not males have high microglial phagocytosis in the central MPN. I administered c48/80 via bilateral ICV injections from PN5-7 and then measured the SDN volume and the percentage of phagocytic microglia in the cMPN on PN8. The c48/80 treatment significantly reduced microglial phagoptosis and increased the volume of the SDN on PN8 (Figure 5.3), with a similar effect size measure as the anti-CR3 antibody blockade experiments in Figures 4.2 and 4.3.

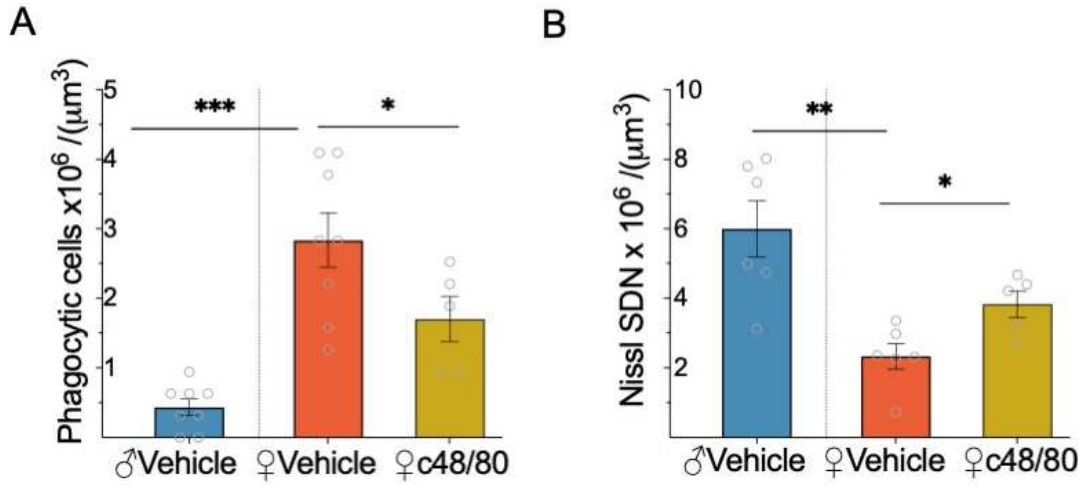


Figure 5.3 Mast cell activation from PN5-7 decreased the number of phagocytic microglia in the cMPN and increased the volume of the SDN in PN8 females.
A. Phagocytic microglia per unit volume in the female cMPN were reduced following mast cell activator c48/80 administration from PN5-7 ($F(2,18) = 18.2$, $P<0.001$). **B**, the volume of the SDN was increased when compared to females receiving vehicle treatment ($F(2,14) = 10.63$, $P=0.0016$). Males had significantly fewer phagocytic microglia than females ($P<0.0001$) and a significantly greater SDN volume than females ($P<0.001$), both by one-way ANOVA followed by Sidak's post hoc comparisons. Bars represent the mean +/- SEM. Open circles represent individual data points for each animal.

5.3 Discussion

This study is the first to report success in increasing the survival of the cells of the female SDN (normally destined for cell death) without the use of exogenous steroid treatment in the rat. Neonatal inhibition of phagocytosis via ICV injection of anti-CR3 antibody from PN5-7 resulted in a decrease in phagocytic microglia and an increase in SDN volume in females only when evaluated at postnatal day 8, and disruption of sex-typic responses to sexual odor in adulthood. This combination of findings was surprising because of the existing dogma that suggests the volume of the SDN is important to sexual preferences. The results of our study suggest that size isn't all that matters,]. Although the volume of the SDN assessed in adulthood suggests that the volume returns to normal, the brief retention of cells in the SDN, just after phagocytosis was blocked obviously still had a significant effect on activity of this nucleus in response to sexual odors. We and others have shown that the SDN is involved in the interpretation of sexual odor cues, and the change in activity of this "interpretation station" (that we have demonstrated here) is crucial for attraction to odor from the (reproductively capable) opposite sex during peak periods of receptivity (Proestrus/Estrus), for females. The change in early gene expression in response to acute male sexual odor that was observed in the SDN of females treated with anti-CR3 antibody, may be the result of several factors. The delayed phagocytosis of stressed but viable cells may have resulted in excessive inflammatory signaling in this region, that may have caused the death of other cells that are not usually targeted for removal. The decreased response of the SDN may be due to changes in synaptic connectivity or number of contacts to other regions of the brain that are necessary for this behavior, such as the principal nucleus of the bed nucleus of the stria

terminalis (BNSTp) and anteroventral periventricular (AVPV) nucleus, regions with cell death episodes occurring on a similar timeline to those seen in the SDN, just slightly before. Since adult females that were neonatally blockaded with anti-CR3 antibody were cycling regularly and no difference in this measure was detected amongst groups, this is unlikely but is still a possibility. One caveat to the antibody blockade approach is that it was administered to the lateral ventricles and thus may have affected other sexually dimorphic brain regions relying on similar mechanisms of microglial phagocytosis in addition to the SDN. These other regions may also be important for interpretation of odor cues of the opposite sex, especially the BNSTp AB (aromatase-expressing) neurons that were recently identified as a crucial component for sexual partner recognition in the male mouse (Bayless et al. 2019).

The cMPN contains mostly GABAergic fibers that are immunoreactive to calbindin (CALB), corticotropin-releasing factor (CRF), dopamine beta-hydroxylase, and neuropeptide Y (NPY). It also contains cell bodies immunoreactive to thyrotropin-releasing hormone (TRH) and both fibers and cell bodies containing cholecystokinin (CCK) (Simerly, 1985). Dopamine beta-hydroxylase is important for the conversion of dopamine to norepinephrine (also known as noradrenaline) CRF promotes glucocorticoid release. Norepinephrine and glucocorticoid steroids both promote the "fight or flight" response of the sympathetic nervous system. All the neurotransmitters, neuropeptides and neurosteroids mentioned here are important to facilitate social behaviors and are all candidates to be examined further.

Activation of cell bodies as indicated by cFos expression within the cMPN is sexually dimorphic. Increased activation of cells occurs in the female cMPN in response

to intact male sexual odor cues, while little to no response to the same stimuli is observed in males (Pierman & Bakker 2008). Although cell type identification of activated cells in this region has not yet been carried out, cholecystokinin A receptor (Cckar)-expressing (as well as PR-expressing) neurons of the ventromedial hypothalamus (VMHvl) are important for sexual receptivity in females, such that knockout models of these receptor types of results in diminution of proceptive behaviors in rodents, although the source of CCK supplying this region has yet to be identified, it may be from the SDN (Bloch GJ 1987).

In another study, PN11/12 pups administered CCK_A and CCK_B receptor antagonists demonstrated an increased preference for maternal odor cues, suggesting that CCK is important for development of relevant odor sensory stimuli early in life (Shayit & Weller, 2000). Antagonists to CCK_B but not CCK_A receptors delivered to newborn sheep also increased their preference for their dam over unfamiliar ewes when tested 24 or 48 hours later, implicating CCK_B receptors in innate rather than learned behavioral responses to maternal sensory stimuli (Nowak et al. 1997). The role of CCK in olfactory social recognition was studied with receptor subtype specific agonists and antagonists in adult male rats. It was concluded that the CCK_A receptor subtype is important for facilitating olfactory recognition of littermates while the CCK_B receptor subtype is important for inhibiting this discriminative behavior. (Lemaire et al.' 1992, 1994). Taken together with our current study, there is substantial evidence indicating that CCK is important for development of innate as well as learned preferences for olfactory sensory stimuli and the atypical survival of neurons of this type in either sex in the present study may have played a role in the diminished detection of sex-typical innate olfactory sensory

stimuli.

The increased volume observed after mast cell activation via c48/80 during the period of highest phagocytosis in females, is an interesting result that warrants further study. Since mast cell numbers in the POA continue to increase until PN7 and drop off, soon after, it's likely they could be participating in sexual differentiation processes that are delayed, as compared to the majority, which are only present during periods of differential brain hormone levels (the critical period for masculinization). Due to the off-target excitatory effects of the compound 48/80, it is hard to draw conclusions on the observed results without additional information and better means of causing degranulation without exciting neurons. Although I worked on alternative methods for activating mast cells, after several months of transfecting rat basophilic leukemia cells (RBLCs, often used as a model for mast cell degranulation assays) with a plasmid carrying genes necessary for channel rhodopsin-protein expression, I finally had a clone that expressed a non-functional channel rhodopsin, as I had most likely selected for this feature by using GFP as a tag, which when stimulated during sorting, also may have killed off clones that had functional rhodopsin expressed, due to hyperexcitability. This avenue and others regarding the role of mast cells in the SDN are currently pending investigation by the newer graduate students in the laboratory and will hopefully be a line of research that yields many interesting findings soon.

Chapter 6: The Role of Mast Cells in Masculinization of the POA & Sex Behavior

6.1 Introduction

Mast cells are innate immune cells that are best known for their role in allergy and anaphylaxis, where their response to a perceived threat can range from an itchy, runny nose to dermal irritation or "wheal" formation to a life-threatening condition requiring epinephrine administration and/or hospitalization (Krystel-Whittemore 2015). These powerful cells are most densely populated at barrier interfaces (mucosal membranes of nasal passages, ears, eyes, intestinal lining, dermis-any membrane in contact with foreign or non-self-antigens from exterior sources. Upon recognition of noxious stimuli, these cells degranulate, releasing histamine, heparin, proteases, and a vast array of other inflammatory mediators in an autocrine, paracrine, or systemic response. In addition to these preformed mediators stored in their granules, they also synthesize and release lipid mediators, growth factors, and neurotransmitters on demand and partake in "piecemeal" degranulation where some, but not all, their granules are released. The variety of mediators and "nutrients" the mast cells make and respond to makes them candidate support cells for members of their tissue niche, as it develops. Due to their cellular heterogeneity and intimate relationship with the tissue niche in which they reside, the mast cell has been difficult to study. If you've met one mast cell, you've met one mast cell. MCs have the capability to make and receive signals from more lipid and protein mediators than any other cell in the body and are the most "unique" when compared to all other immune cells, even during development.

MCs are of dual hematopoietic origin. A first wave of MCs originates from the HE of the yolk sac (just like microglia!) and is gradually diluted by a second wave of definitive MCs that once mature, are radio-resistant, long-lived cells that have a unique set of transcriptional and morphological characteristics that depend on their tissue of residence (Gentek et al., 2018). The study of mast cells in the context of neuroinflammation has yielded evidence of their role in maintenance of neurogenesis, neurodegeneration, and blood-brain barrier (BBB) permeability (Hendriksen, 2017, Lenz & Pickett et al 2018).

Although embryonic MC colonization of the mammalian brain has yet to be studied, it is known that they are present in the brain by as early as E15 (unpublished observations) and, unlike microglia, their location is limited to highly specific regions such as leptomeninges and perivascular and periventricular spaces. In the neonatal rat, mast cells are most numerous in the velum interpositum, an invagination of pia mater which is located, from rostral to caudal, between the subfornical organ to the spelium of the corpus callosum, separating the hippocampus from the thalamus (De Leucio et al., 2021). During the first week of life in the rat they are also present in appreciable numbers (that vary by sex) in the parenchyma of the POA and meninges located just below it, at the very base of the brain (Lenz & Pickett et al 2018).

The mechanism by which PGE2 stimulates dendritic spine synapse formation in the POA is known and involves EP2 and 4 receptors, activation of protein kinase A, and AMPA-type glutamate receptors (Wright and McCarthy, 2009; Lenz et al., 2011). Excitatory afferent input to the POA from the medial amygdala integrates olfactory stimuli from sexually receptive females (Baum and Everitt, 1992). Increased

glutamatergic transmission in the POA is a prerequisite for male mating behavior (Dominguez et al., 2006). Therefore, the increased density of excitatory dendritic spine synapses on POA neurons in males ensures that proper connections are established and maintained until adulthood, where under the influence of pubertal hormones, the masculinization process is crystallized and sexual behaviors in line with gonadal assignment and hormonal milieu are established for success in the end goal of most species (evolutionarily): reproduction. A study of differential gene expression in the POA just after birth conducted in our lab prior to my arrival revealed several immune-relevant genes of interest, one being mast cell protease 2 (Mcpt2), a mast-cell specific gene that was expressed in males only. This discovery opened a new avenue regarding important cellular players involved in the masculinization of the POA. The following chapter will cover the results we found when we sought to determine the role of mast cells in masculinization of the preoptic area and sexual behavior in adulthood. Based on our results, we have evidence that mast cells are more numerous in the male POA and are stimulated by estradiol (possibly via their estrogen receptors (ERα)), mast cell degranulation and the release of histamine into the male POA during the critical period for sexual differentiation. Histamine binds to H1- and H4-type histamine receptors on microglia and stimulates microglia to release PGE2. Microglia-derived PGE2 stimulates the masculinization of dendritic spine patterning on POA neurons in early life that enables male-typical copulatory behavior in adulthood.

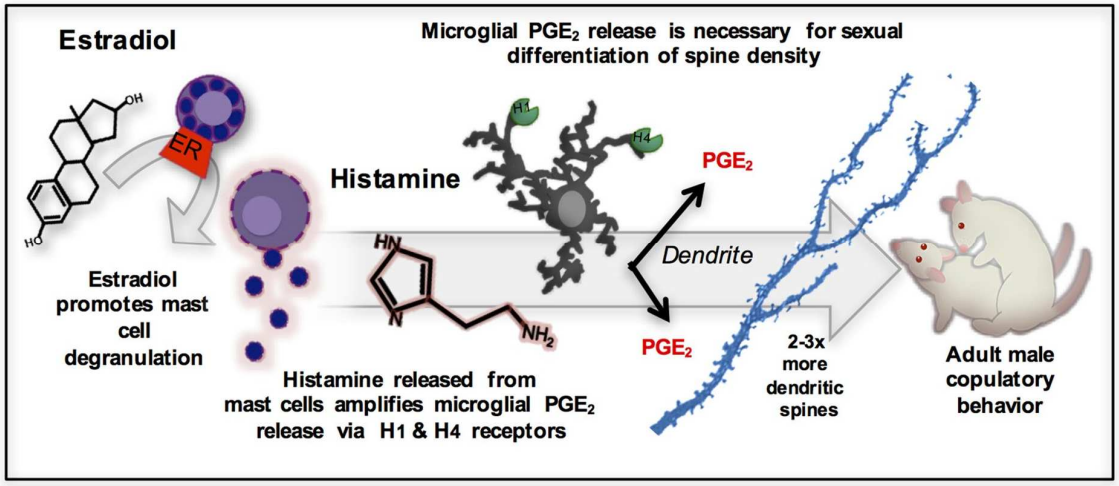
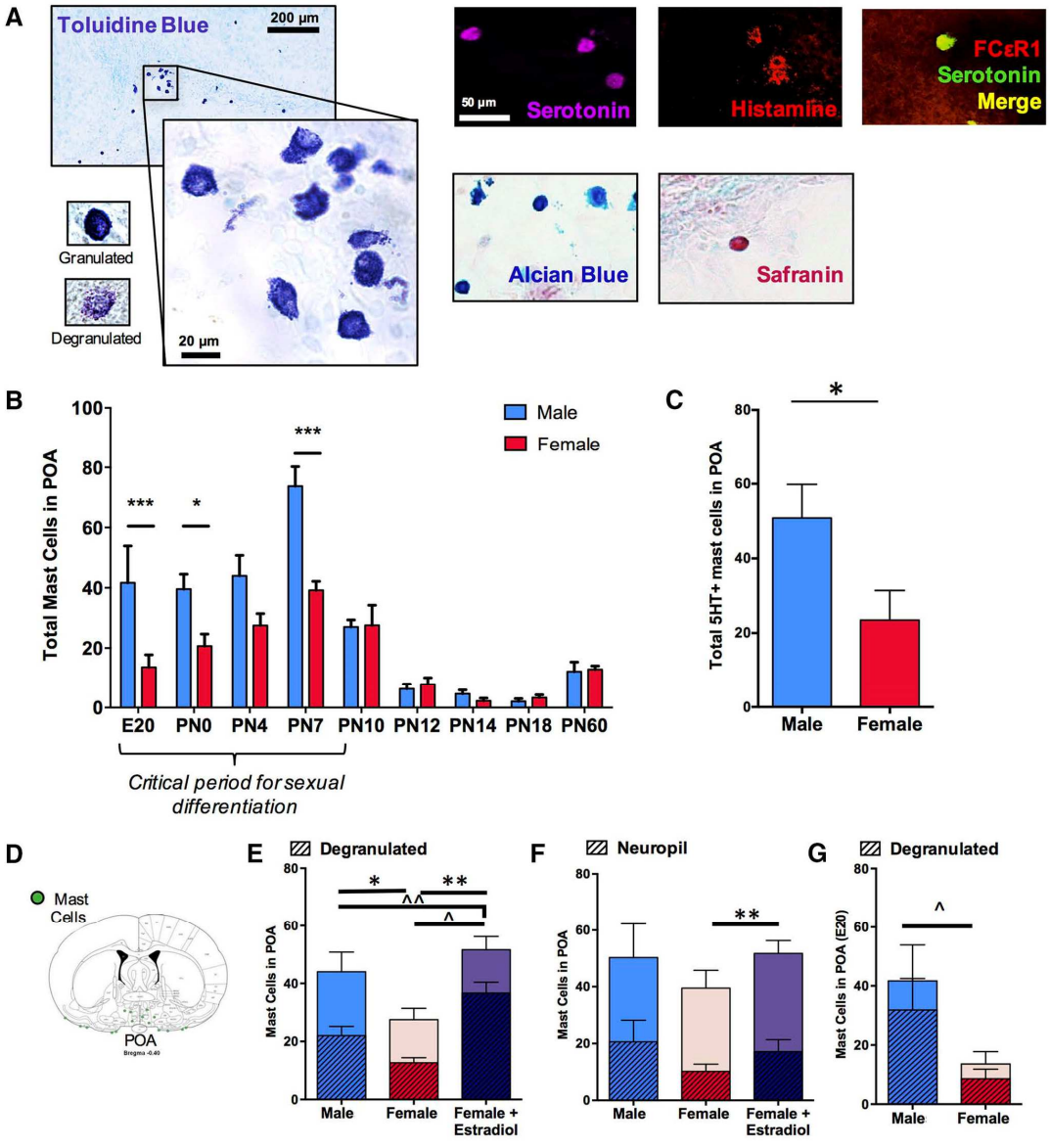


Figure 6.0 Proposed mechanism for mast cell-mediated masculinization of the POA and Sexual Behavior Together, the data that will be detailed in the following chapter suggest that estradiol acts on estrogen receptor (ER) on mast cells to stimulate mast cell degranulation and the release of histamine into the male POA during the critical period for sexual differentiation. Histamine binds to H1- and H4-type histamine receptors on microglia and stimulates microglia to release PGE2. Microglia-derived PGE2 stimulates the masculinization of dendritic spine patterning on POA neurons in early life that enables male-typical copulatory behavior in adulthood. *Author Contributions for published body of work discussed in the following chapter: Author contributions: K.M.L. wrote the first draft of the paper; K.M.L., L.A.P., C.L.W., and M.M.M. edited the paper; K.M.L., L.A.P., C.L.W., and M.M.M. designed research; K.M.L., L.A.P., C.L.W., K.T.D., and A.J. performed research; K.M.L., L.A.P., C.L.W., and A.J. analyzed data. *K.M.L. and L.A.P. contributed equally to this work.

6.2 Results

My first goal when I joined the McCarthy lab was to use quantitative polymerase chain reaction (qPCR) to validate the gene expression results from the initial gene array analysis undertaken by fellow lab members. When we were unable to validate the sex difference in Mcpt2 expression via qPCR, we began our analysis of mast cell number and degranulation state in the POA just after birth, because seeing is believing. Brain sections containing the entire POA were stained with toluidine blue and analyzed for total mast cell number and degranulation status. This study revealed that there were more mast cells located in the POA of males than females from E20 to PN7, after which numbers in the POA dropped off abruptly in both sexes (Figure 6.1B). In addition, we found that they were degranulating, but only during the critical period for masculinization (Figure 6.1E-G). Due to the location within the preoptic area and timing of the mast cells sex differences we observed, we next sought to determine if mast cell numbers were modulated by estradiol, the product of testosterone's turn-over by local aromatase expression in the POA. Females treated with estradiol just after birth showed higher mast cell numbers and degranulation (Figure 6.1E-G), consistent with our prediction.

Figure 6.1 Sex differences in mast cells in the POA during the critical period for sexual differentiation are organized by estradiol.
A. Mast cells in the POA neuropil and leptomeninges on P0 stained with toluidine blue (left). Mast cells in the POA stained positive for Alcian blue and immunolabeled for serotonin (5-HT), histamine, and the IgE receptor FCER1, indicating a mucosal phenotype. Occasional safranin-positive mast cells (bottom right), indicative of a connective tissue phenotype, were detected in the meninges near the cortex, but not in the POA neuropil. **B.** Males had more mast cells than females on E20, P0, and P7, the critical period for sexual differentiation following staining for toluidine blue. Mast cell numbers significantly increased until P7 and decreased thereafter in both sexes, with no further sex differences. **C.** Counts of 5-HT-positive mast cells in the POA on P2 corroborated toluidine blue staining counts in B, with males having significantly more mast cells than females.
D. Schematic of mast cell location in the forebrain (green circles). **E.** Mast cells in the POA are situated in both the neuropil and leptomeninges, and an appreciably large population resides in the velum interpositum, adjacent to the hippocampus. F. Treatment of females on P0-P1 with a masculinizing dose of estradiol (100 μg/ 0.1cc sesame oil) on P0–P1, increased the total number of POA mast cells (full bar graph) and the percentage of degranulated mast cells measured on PN2 (shaded portion of bar graph shows number of degranulated cells). **F.** Total number of mast cells in the neuropil (shaded bars) versus the meninges on P4 is greater in males and females treated with a masculinizing dose of estradiol.**G.** On E20, during the testicular androgen surge in males, males had a higher percentage of degranulated mast cells than females (shaded bars depict number of degranulated cells). Data presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ for full bar graphs; ^$p < 0.05$ for percentage total shaded bar graphs, ^^$p < 0.01$ for percentage total shaded bar graphs. Group sizes: B: E20:♂n = 6, ♀n = 5; P0: ♂n = 8, ♀n = 7; P4 ♂n = 6, ♀n = 7; P7: ♂n = 7, ♀= 5; P10: ♂ n = 7, ♀= 5; P12: ♂n = 10, ♀n = 10; P14: ♂n = 6, ♀= 8; P18: ♂n = 7, ♀n = 8; P60: n = 6, ♀n = 6; C: ♂n = 6; ♀n = 6; E: ♂n = 6, ♀n = 7, E2 ♀n = 7. F: ♂n = 10, ♀n = 10, E2♀n = 7. G: ♂n = 6, ♀ n = 5.


A
Toluidine Blue
200 µm
Granulated
Degranulated
20 µm
50 µm
Serotonin
Histamine
FCεR1
Serotonin
Merge
Alcian Blue
Safranin
B
Total Mast Cells in POA
Male
Female
***
*
***
E20
PN0
PN4
PN7
PN10
PN12
PN14
PN18
PN60
Critical period for sexual differentiation
C
Total 5HT+ mast cells in POA
*
Male
Female
D
Mast Cells
POA
E
Degranulated
Mast Cells in POA
Male
Female
Female + Estradiol
F
Neuropil
Mast Cells in POA
**
G
Degranulated
Mast Cells in POA (E20)
^

To understand if the higher numbers of mast cells within the male POA were due to local proliferation vs. migration, we studied the proliferation of mast cells and microglia within the POA by administering (IP) bromodeoxyuridine (BrdU), a thymidine analog, on PN0 and PN1. We measured incorporation of BrdU into recently divided microglia cells by immunohistochemistry for BrdU in nickel-dab and Iba1 with brown-dab. We used IF IHC for the detection of BrdU incorporated into newly divided serotonin (5-HT)-positive mast cells (Figure 6.2). Labeling of proliferating cells (green, BrdU-labeled), microglia (red, Iba1-labeled), mast cells (purple, 5-HT-labeled), and merged images (right) in the POA of the newborn animal on P0 (dotted white lines indicate third ventricle and leptomeninges). There was no evidence of mast cell proliferation in the neonatal POA. **B**. Males had more microglia than females in the POA on P2. Although there were no mast cells that colabeled with BrdU in any group, Iba1-positive microglia colocalized with BrdU in the POA on P2, indicating microglial proliferation, and males have higher numbers of proliferating microglia than females. **C**. Representative image of IHC to double-label microglia (Iba1 stained with DAB; brown) and proliferating cells (BrdU stained with Ni-DAB; black) in the POA on P2 (3V =third ventricle). **D**. Representative images and *Z*-stack of immunofluorescently stained BrdU-positive (green) and Iba1-positive (red) cells showing colocalization. Counts of proliferating microglia were performed on DAB-stained tissue as seen in **C** and colocalization was confirmed via confocal imaging.

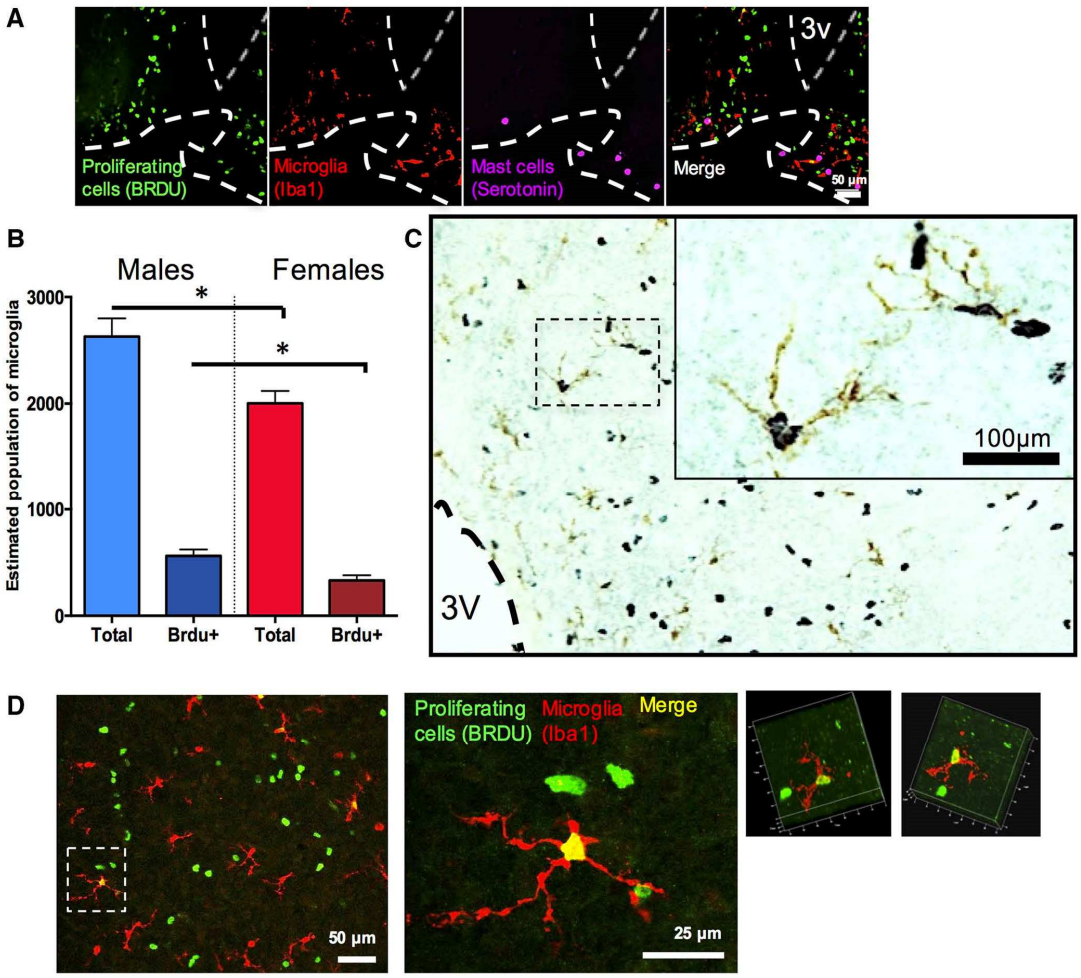


Figure 6.2 Microglia, not mast cells, are proliferating more in the developing ♂POA. **A**. Labeling of proliferating cells (green, BrdU-labeled), microglia (red, Iba1-labeled), mast cells (purple, 5-HT-labeled), and merged images (right) in the POA of the newborn animal on P0 (dotted white lines indicate third ventricle and leptomeninges). There was no evidence of mast cell proliferation in the neonatal POA. **B**. Males had more microglia than females in the POA on P2. Although there were no mast cells that colabeled with BrdU in any group, Iba1-positive microglia colocalized with BrdU in the POA on P2, indicating microglial proliferation, and males have higher numbers of proliferating microglia than females. **C**. Representative image of IHC to double-label microglia (Iba1 stained with DAB; brown) and proliferating cells (BrdU stained with Ni-DAB; black) in the POA on P2 (3V =third ventricle). **D**. Representative images and *Z*-stack of immunofluorescently stained BrdU-positive (green) and Iba1-positive (red) cells showing colocalization. Counts of proliferating microglia were performed on DAB-stained tissue as seen in **C** and colocalization was confirmed via confocal imaging (**D**). *$p < 0.05$. Group sizes: **A**: n = 4/group. **B:** ♂n = 11; ♀n = 8). Data presented as mean ± SEM. *p < 0.05.

Since it is already known that estradiol increases degranulation *in vivo*, and that although microglia are required for masculinization of the POA, they do not express estrogen receptors, we speculated that mast cells were the "initiators" of the masculinization cascade. The mast cells could accomplish this by degranulating to communicate to microglia that a stimulus had been received, signaling that it was time to ramp up prostaglandin synthesis for execution of the masculinization of neuronal circuitry that are a part of that "new" male blueprint moving forward. We used IHC to confirm estrogen receptor expression on mast cells, which we found in ~50% of the mast cells surveyed in the neonatal POA of males and females (Figure 6.3).

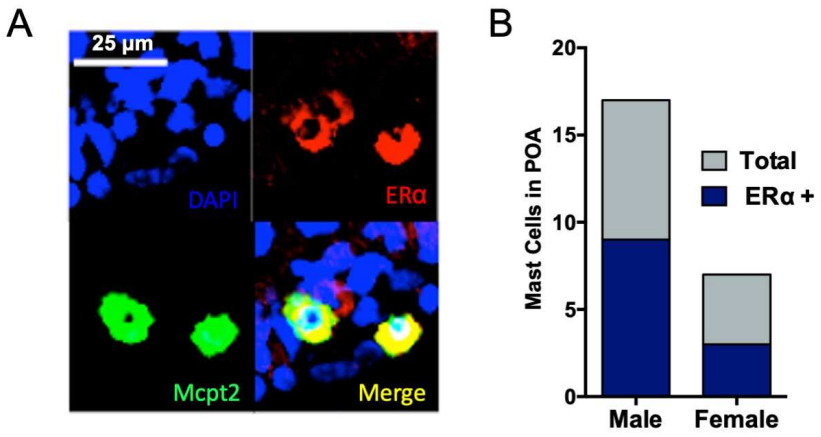


Figure 6.3 Estrogen receptor alpha is expressed by half of the mast cells expressing Mcpt2 in the POA on PN2. **A**, **B**. Approximately half of POA mast cells staining positively for mast cell protease 2 (Mcpt2) also stained positive for estrogen receptor α (ERα) in males and females. Group sizes: **B**: ♂$n = 3$, ♀$n = 3$.

With the fortuitous publication of a novel "working protocol" for neonatal brain mast cell isolation from the Vanucci group at Weill-Cornell medical college, NYC (Patel et al., 2013), I proposed that we isolate the brain mast cells to (i) study their degranulation response to estradiol as compared to the pharmacological activator, c48/80, and to (ii) then test the spinogenic effects of the mast cell mediators released on neonatal POA cultures. I took a field trip to the Vannucci lab in NYC to learn the laborious isolation protocol, which makes use of Fcer1 receptor expression for positive selection of brain mast cells from PN10-14. After about a year of very little success isolating appreciable numbers of neonatal mast cells with their lab's prep, we know now (unpublished observations) that Fcer1 is not expressed by the entire population of POA mast cells just after birth (most likely due to immaturity). Therefore, I developed my own protocol that only requires mechanical homogenization and a percoll purification step, followed by two weeks growth time in culture. We studied the response of isolated brain mast cells in culture to c48/80 and estradiol exposure, using a histamine assay as a measure of mast cell degranulation. We found that estradiol was in fact as potent a mast cell degranulating agent as c48/80 when applied to pure brain-derived mast cells *in vitro* (Figure 6.4C). Because mast cells are known to synthesize and release prostaglandin D2, we needed to rule out that the mast cells themselves were not the source of prostaglandin E2 required for spinogenesis in the POA. Using enzyme-linked immunosorbent assay (ELISA) for both PGD2 and PGE2, we studied prostaglandin release in the conditioned medium from brain mast cells in response to estradiol *in vitro*. We found that estradiol treatment did not result in a significant increase in PGE2 or PGD2 production from our pure brain mast cells (Figure 6.4E-F).

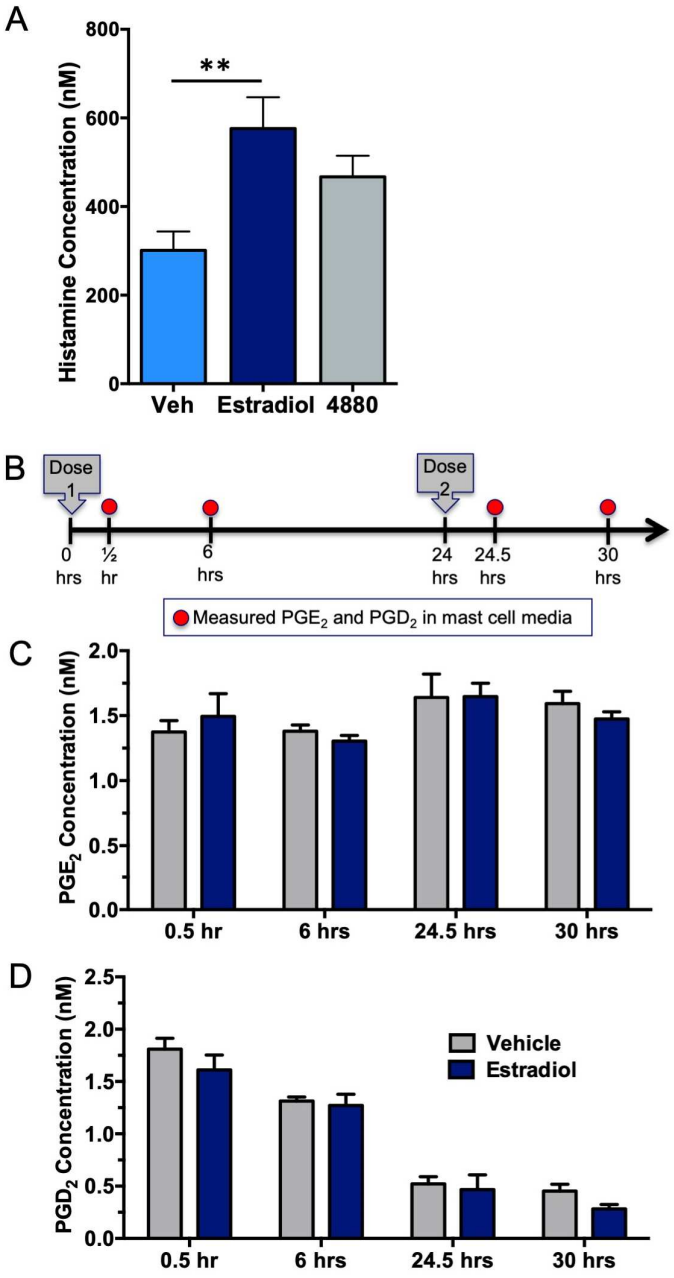


Figure 6.4 Estradiol is a powerful mast cell degranulator but does not elicit prostaglandin production by mast cells *in vitro*. **A**. Treatment of primary cultured mast cells with estradiol increased histamine release to the same extent as the mast cell secretagogue compound 48/80 **B**: Primary mast cells cultured from the brain were treated with estradiol and concentration of PGE2 (**C.)** and PGD2 (**D.**) measured in the conditioned media at 0.5, 6, 24.5, and 30 h after initial treatment did not increase at any time point. Estradiol was given a second time 24 h after initial treatment to the 24.5 and 30 h samples. ***p* < 0.01. Group sizes: **A**: *n* = 10 per group (two experiments with *n* = 5 for each); **C-D:** all groups: *n* = 3 per time point.). Data presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 for full bar graphs.

Once we confirmed that mast cells were not the source of masculinizing PGE2, we speculated that a third signaling molecule was likely initiating the masculinization cascade, released from mast cells degranulating in response to local estradiol in the POA. To test if mast mediators alone, in the absence of estradiol, could masculinizes POA cultures, as indicated by increased spine densities, we grew isolated brain mast cells in culture, degranulated them with c48/80, and then applied mast cell medium to mixed POA cultures on day *in vitro* 1 & 2 (DIV1-2). Our results confirmed that mast cell "conditioned" media from cultures degranulated with c48/80 can increase spine densities in POA cultures *in vitro* (Figure 6.5A, B). In addition, we found that mast cell medium did not elicit spine formation when microglia were removed from the POA cultures or when c48/80 alone was used to treat POA cultures (Figure 6.5C)

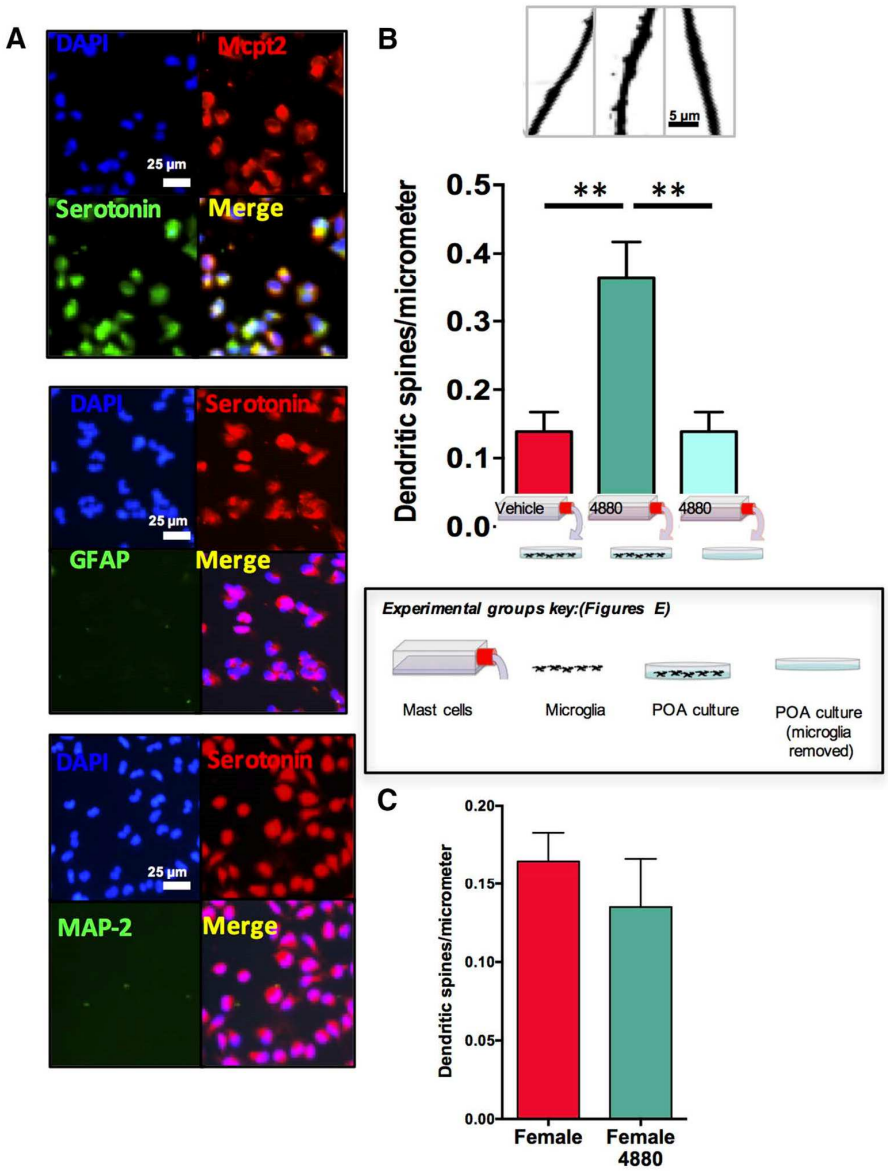
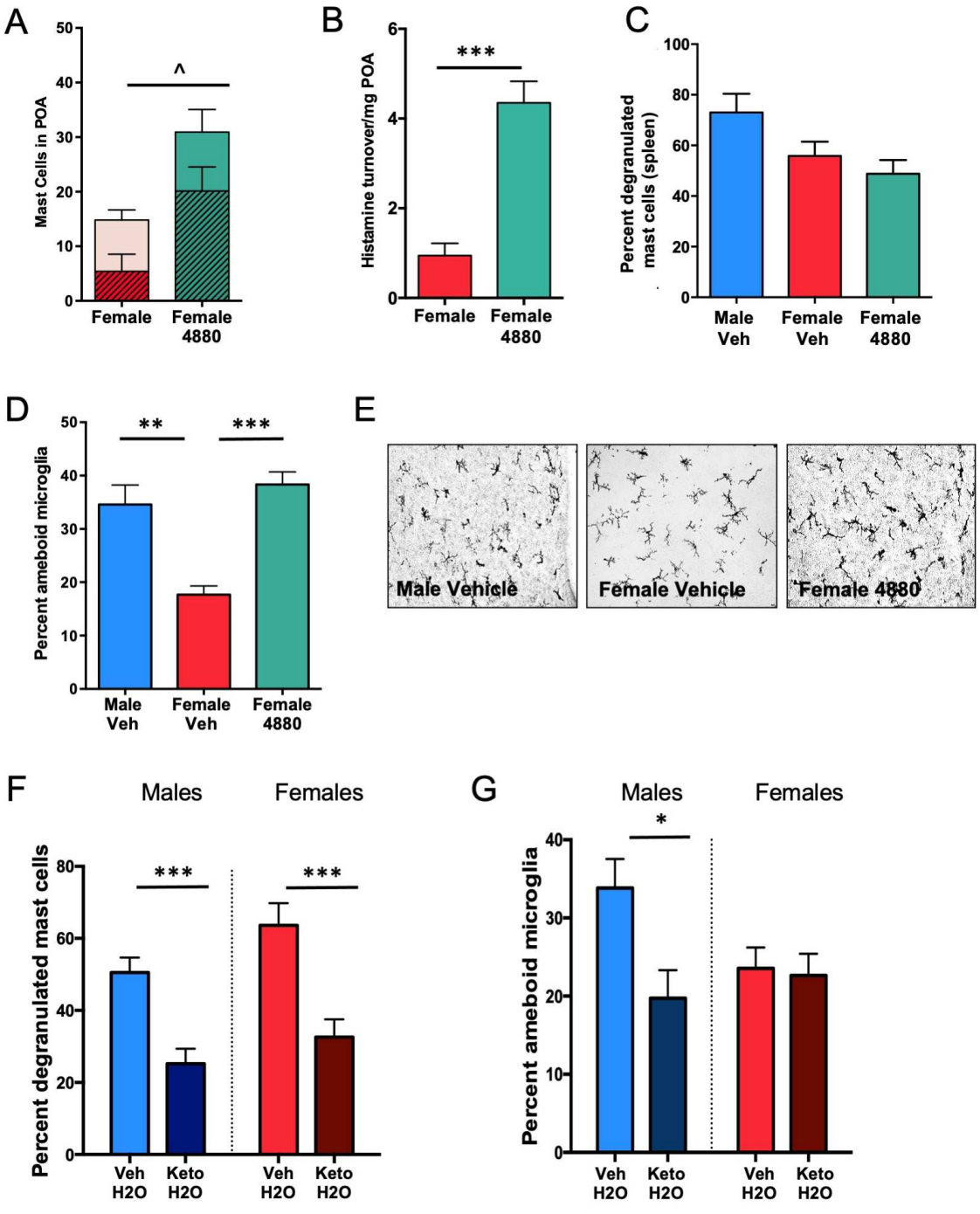


Figure 6.5 Mast cell-induced masculinization of POA dendritic spines *in vitro*.
A. Validation of pure mast cell primary cultures. All DAPI-positive cells from primary mast cell cultures stained positive for mast cell protease-2 (Mcpt2) and 5-HT (top) but did not show any staining for GFAP to mark astrocytes (middle) or MAP-2 to mark neurons (bottom). The bottom right of each panel is the merged image of costaining. **B**. Density of dendritic spine-like protrusions on female primary POA neurons increased after exposure to conditioned medium from mast cells stimulated with compound 48/80, but prior removal of the microglia prevented this increase. **C**. Compound 48/80 had no effects on the density of dendritic spines when given directly to the neurons. This contrasts with the spinogenic effects of compound 48/80 when it is first administered to mast cells and the mast cell-conditioned media is then applied to the neurons (**B**). $^{**}p < 0.01$. **B**: All groups, $n = 6$. **C**: All groups, $n = 14$). Data presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 for full bar graphs.

We next sought to determine if mast cell mediator release was necessary and sufficient for masculinization of spines and amoeboid microglia phenotype observed in the male POA *in vivo*. We first treated females with mast cell degranulator, c48/80 and males with a mast cell stabilizer, cromolyn, and then studied mast cell degranulation and microglia phenotype via toluidine blue staining and IHC for Iba1+ cells. We also measured histamine content *in vivo* after c48/80 administration on PN2 via mass spectrometry. We found that our dose of degranulator, c48/80 to females induced mast cell degranulation in the POA but not spleen (Figure 6.6A), increased free histamine on P2 (Figure 6.6B), and masculinized the microglia phenotype in the POA (Figure 6.6D-E). However, the mast cell stabilizer, cromolyn, had no effect on mast cell degranulation or free histamine in males (data not shown). We reasoned that cromolyn, the mast cell stabilizer, did not prevent degranulation of male mast cells because by the time the compound was administered on PN0-1, the androgen surge had already begun and POA mast cell degranulation due to estradiol production was well underway. Mast cells are not only capable of producing many different signaling molecules within the body, but they also respond to their own mediator levels within the extracellular space to continuously monitor and guide subsequent signaling events of the local mast cell population as well as any mast cells newly recruited to that region (Krystel-Whittemore et al., 2016). In this sense, they are the perfect cell type to interface neuroendocrine and immune processes with tight regulatory control of not only their own mediator levels in the extracellular space, but also the continuously changing hormonal or other signals within the environment after the initial mediator release to inform and guide subsequent signaling events. We speculated that it may be difficult to silence the mast cells in the POA once degranulation and mast cell recruitment has already begun.

Therefore, to inhibit mast cells prior to the androgen surge, when the cells are more likely to respond to pharmacological inhibition, we treated pregnant dams with Ketotifen, a mast cell inhibitor that freely crosses the blood brain barrier, by administering the drug daily to their mother's drinking water from E13.5 to PN7 (while nursing the first week). We then analyzed immune cell status at PN7 with a subset of pups to confirm mast cell degranulation inhibition and prevention of microglia morphology masculinization that we now know can be elicited by c48/80-induced mast cell degranulation (Figure 6.6F-G).

6.6 POA mast cells degranulate in response to c48/80 and are inhibited by ketotifen administration to their mother *in utero* and during the first week after birth.
A-B. Compound 48/80 treatment *in vivo* (intracerebroventricularly) led to significant increases in percentage of degranulated mast cells in the POA of females on P2 (full bars indicate total number of mast cells, shaded bars indicate number of degranulated mast cells (**A**), as well as increased histamine turnover in the POA as measured by LC-MS/MS (**B**). **C-D**. Treatment with compound 48/80 (4880) by ICV did not change the proportion of degranulated mast cells in the spleen **(D**.) **E**. Treatment of females with the mast cell-degranulating agent compound 48/80 increased the proportion of microglia in an amoeboid state in females to male levels.**F-G**. Treatment of maternal dams with the mast cell stabilizer ketotifen (keto) in their drinking water led to significant decreases in the percentage of degranulated mast cells in the POA of offspring of both sexes compared with vehicle treatment (**F**) and decreased the proportion of microglia in the amoeboid state in males to the level seen in females (**G**). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ^$p < 0.05$ for percentage degranulated mast cells. Group sizes: **A**: ♀Veh, $n = 4$; ♀4880 $n = 5$; **B**: all groups, $n = 5$; **C**: ♂Veh, $n = 5$; ♀Veh, $n = 3$,♀4880, $n = 4$. **D**: ♂Veh, $n = 7$; ♀Veh, $n = 5$; ♀4880 $n = 6$; **F**, **G**: ♂Veh, $n = 5$; ♂ keto, $n = 5$; ♀Veh, $n = 5$; ♀keto, $n = 6$.).
Data presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 for full bar graphs.


A
Mast Cells in POA
50
40
30
20
10
0
^
Female
Female 4880
B
Histamine turnover/mg POA
6
4
2
0
***
Female
Female 4880
C
Percent degranulated mast cells (spleen)
100
80
60
40
20
0
Male Veh
Female Veh
Female 4880
D
Percent ameboid microglia
50
40
30
20
10
0
**
***
Male Veh
Female Veh
Female 4880
E
Male Vehicle
Female Vehicle
Female 4880
F
Males
Females
Percent degranulated mast cells
80
60
40
20
0
***
***
Veh H2O
Keto H2O
Veh H2O
Keto H2O
G
Males
Females
Percent ameboid microglia
40
30
20
10
0
*
Veh H2O
Keto H2O
Veh H2O
Keto H2O

The remaining pups treated with mast cell stabilizer, ketotifen, *in utero* or compound 48/80 at birth and on PN1 (but not used for neonatal immune cell characterization Figure 6.6), were grown to PN50, gonadectomized, and male testosterone levels were established via silastic capsule implantation prior to testing their sexual behavior on PN60. We found that females treated with c48/80 neonatally took less time to mount the stimulus female (Figure 6.7A) and mounted them more frequently (Figure 6.7B). Mast cell stabilization with Ketotifen decreased mast cell degranulation status of the dam's male offspring (Figure 6.6F) and prevented microglia from assuming their male-typical amoeboid phenotype in the POA on PN7 (Figure 6.6G). As adults, Ketotifen-treated males showed deficits in sexual behavior, as demonstrated by a reduced mounting rate (Figure 6.7D) and number of ejaculations (Figure 6.7F) when paired with a stimulus female, reached their first ejaculation later, and ejaculated fewer times than vehicle (Veh) control males.

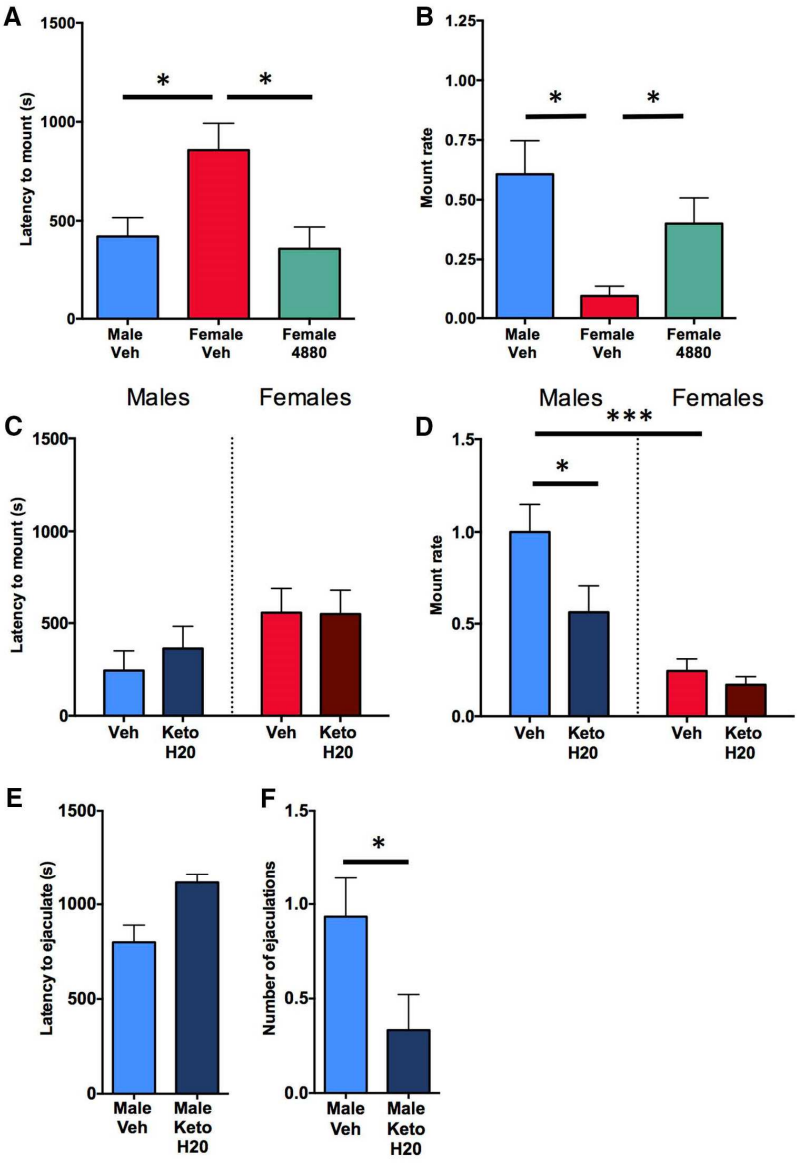


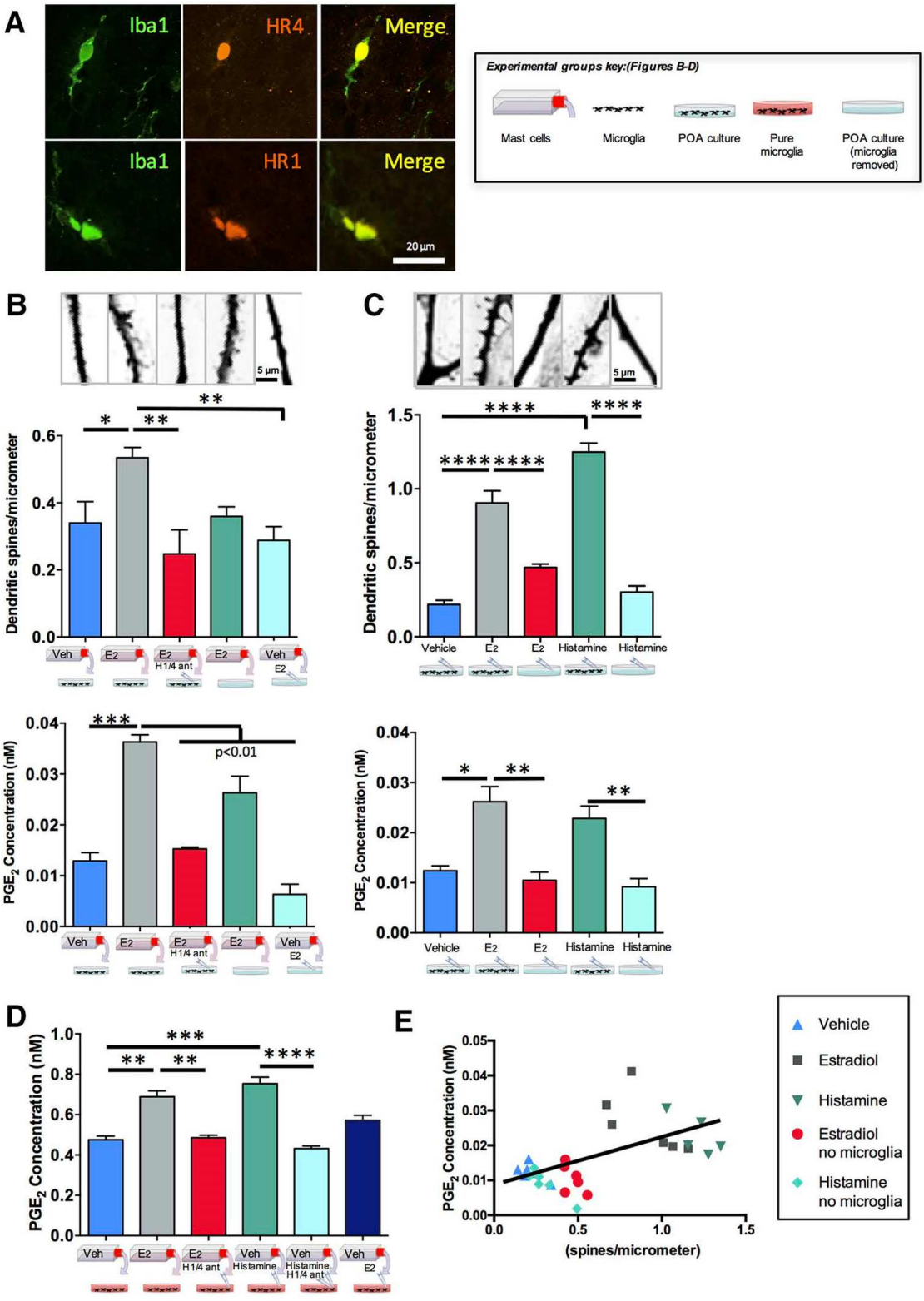
Figure 6.7 Manipulation of mast cells perinatally modifies male-typical reproductive behaviors in adulthood. A-B. Females treated postnatally with compound 48/80 (4880) and then given testosterone as adults began mounting sexually receptive females more quickly (**A.)** and mounted more frequently (**B.)** than control females. **C-F**. Although adult males treated perinatally with the mast cell stabilizer ketotifen (keto, given via the pregnant dam's drinking water between gestational day 17 and P7) did not have delayed mounting (**C.**), they mounted less frequently (**D.**), reached their first ejaculation later (**E.**), and ejaculated fewer times (**F.**) than vehicle (Veh) control males. Data are presented as mean ± SEM. #Trend $0.05 < p < 0.1$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Group sizes: **A, B:** Veh, ♂$n = 17$; ♀Veh, $n = 10$; ♀4880, $n = 11$; **C–F:** ♀Veh and ♀keto, $n = 12$; ♂Veh, $n = 15$; ♂keto, $n = 11$). Data presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 for full bar graphs.

With the knowledge that the mast cells were not making the PGE2 required for spinogenesis in the POA themselves (Figure 6.4), I searched the literature for the effects of histamine (the one mediator we already had evidence was being released from the brain mast cells in response to estradiol) on microglial activation. I found a study that described histamine receptor expression on microglia (all, H1-H4) and the specific receptors (H1 and H4) required for their "activation" and release of the proinflammatory molecules tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6) (Dong et al., (2014), Zhang et al., 2020). Although the proinflammatory mediators used as measures of microglial activation in response to histamine in this study were not the PGE2 response from microglia that we were after, we reasoned that since PGE2 is usually released in conjunction with TNF-α and IL-6 (as well as nitric oxide (NO), and reactive oxygen species (ROS)), as found in other studies (Block, M. L., & Hong, J. S. (2005), Herrera, A. J., Tomás-Camardiel, M., Venero, J. L., Cano, J., & Machado, A. (2005)), then histamine may also be the key mast cell-derived molecule that elicits PGE2 release via stimulation of H1 and H4 receptors on POA microglia.

To determine if histamine released from mast cells can activate H1 and H4 receptors that we confirmed are expressed by microglia (Figure 6.8A) to evoke prostaglandin synthesis and release, we stimulated mast cells in culture with estradiol, as done previously, and then added the conditioned media to POA cultures. Prior to application of the mast cell media to POA cultures, we also pre-treated a subset of POA cultures with H1 and H4 antagonists (10 μM cetirizine HCl (H1 receptor antagonist) and 10 μM A94391 (H4 receptor antagonist) (cat #2577 and #3753, Tocris Bioscience). We measured the levels of prostaglandin released into the conditioned medium of the POA

cultures as well as spine densities of neurites within those same cultures. In addition, we applied the same estradiol-stimulated conditioned mast cell medium and estradiol alone to cultures in which the microglia had been removed via agitation as negative controls. We that blocking H1 and H4 receptors impaired spinogenesis of neurites (Figure 6.8B, top) and prostaglandin release by the culture (Figure 6.8B, bottom, and as expected, the removal of microglia from these cultures blocked these two measures as well. Now that data suggested that H1 and H4 receptors on microglia are required for prostaglandin release and increased spine formation, to test if histamine is sufficient for prostaglandin release and spinogenesis, we treated microglia-replete and microglia-depleted POA cultures with histamine alone. We found that histamine, in the presence (but not in the absence) of microglia produced the same spinogenic effect (Figure 6.8C, top) and increased prostaglandin release (Figure 6.8C, bottom, as measured with the medium of POA cultures. In addition**,** PGE2 concentration increased in microglia cultures after exposure to histamine or conditioned medium from mast cells stimulated with estradiol, but not after pretreatment with H1 and H4 histamine receptor antagonists (Ant) Fog6.8D). Finally, dendritic spine levels positively correlated with PGE2 concentration in primary POA cultures treated with vehicle (Veh), estradiol, histamine, or in which microglia were removed from the cultures before addition of estradiol or histamine (R^2 = 0.318, $p < 0.001$).

Figure 6.8. Mechanisms of mast-cell-induced masculinization of POA dendritic spines *in vitro*. **A**. Iba1+ microglia within the POA are immunopositive for histamine receptors type 4 (top) and type 1 (bottom). **B**. Treatment with conditioned media from estradiol (E2)-stimulated mast cells increased the density of dendritic spines (top graph) and PGE2 concentration (bottom graph) and prior removal of microglia or inhibition of H1 and H4 histamine receptors prevented these increases. **C**. Administration of histamine directly onto primary POA cultures also increased the density of dendritic spines (top graph) and PGE2 concentration (bottom graph) to levels equivalent to that induced by estradiol treatment and, again, prior removal of microglia prevented these increases. **D**. PGE2 concentration increased in microglia cultures after exposure to histamine or conditioned medium from mast cells stimulated with estradiol, but not after pretreatment with H1 and H4 histamine receptor antagonists (Ant). **E**. Dendritic spine levels positively correlated with PGE2 concentration in primary POA cultures treated with vehicle (Veh), estradiol, histamine, or in which microglia were removed from the cultures before addition of estradiol or histamine ($R^2 = 0.318$, $p < 0.001$). Data are represented as mean ± SEM.*p <0.05, **p <0.01, ***p <0.001, ****p <0.0001. Group sizes: **A**: all groups, n =4; **B**: all groups, n =6; **C** and **E**: all groups, n = 6; **D**: Veh, mast cells + E2, mast cells + E2 + histamine ant, and histamine, n = 8; histamine + histamine ant, n = 7, histamine and direct E2 treatment, n = 6). Data presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 for bar graphs.


A
Iba1
HR4
Merge
Iba1
HR1
Merge
20 µm
Experimental groups key:(Figures B-D)
Mast cells
Microglia
POA culture
Pure microglia
POA culture (microglia removed)
B
5 µm
Dendritic spines/micrometer
Veh
E2
E2 H1/4 ant
E2
Veh E2
PGE2 Concentration (nM)
p<0.01
C
5 µm
Dendritic spines/micrometer
Vehicle
E2
E2
Histamine
Histamine
PGE2 Concentration (nM)
D
PGE2 Concentration (nM)
Veh
E2
E2 H1/4 ant
Veh Histamine
Veh Histamine H1/4 ant
Veh E2
E
PGE2 Concentration (nM)
(spines/micrometer)
Vehicle
Estradiol
Histamine
Estradiol no microglia
Histamine no microglia

To ensure that the results from our culture experiments were biologically relevant, we administered histamine receptor antagonists *in vivo* and measured prostaglandin E2 measured in fresh dissected, PN2 POA tissue was reduced (Figure 6.9A). Spinophilin protein, as a proxy for spine density, was then measured in the POA via western blot. Mast cell degranulation via c48/80 administered to females on PN0-1 increased the amount of spinophilin detected, but not when c48/80 was administered together with histamine H1 and H4 antagonists (Figure 6.9B).

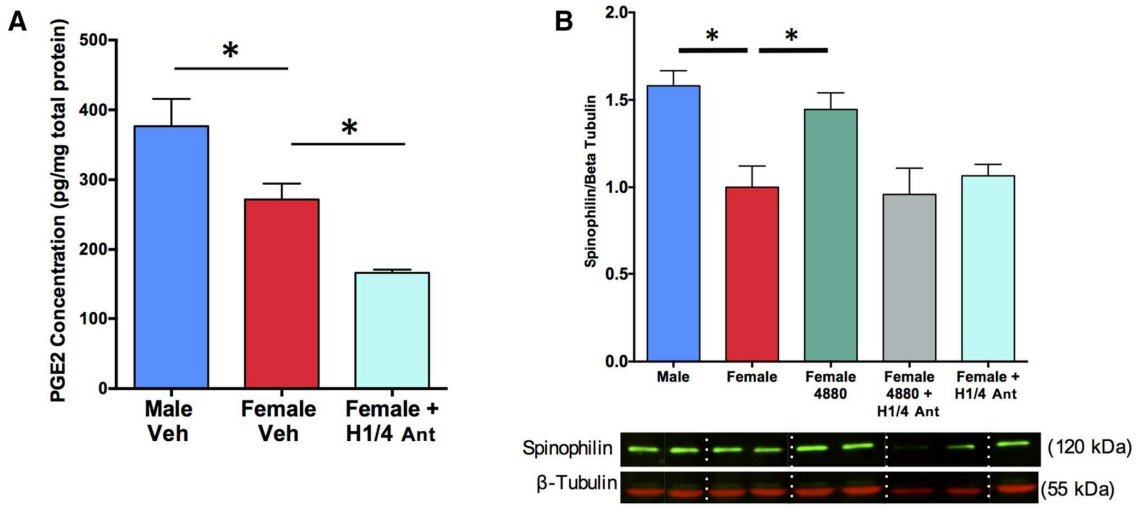


Figure 6.9. Histamine receptor antagonism reduces PGE2 production and mast cell-mediated masculinization of dendritic spine proteins, in vivo.
A. *In vivo*, the neonatal male POA showed higher PGE2 concentrations than female POA and treating females with histamine receptor 1/4 antagonists (ant) further decreased PGE2 levels compared to vehicle (Veh) treatment. **B**. Treatment of neonatal females with compound 48/80 (4880) intracerebroventricularly led to increased spinophilin content in the POA and cotreatment of females with histamine receptor 1 and 4 antagonists prevented this increase (F (4,32) = 7.16 $p < 0.001$, Tukey's HSD $p < 0.001$; Tukey's HSD for M-F and F-F4880, $p < 0.05$], indicating that histamine receptor activation is necessary for the masculinizing effects of mast cell degranulation on dendritic spines to occur *in vivo*. *$p <$ 0.05. All immunoblots are representative of treatment group. Group sizes: **A**: ♂Veh, n = 9; ♀Veh, n = 9; ♀H1/4 ant, n = 7; **B**: ♂Veh, n = 9; ♀Veh, n = 8; ♀4880, n = 7; ♀4880 + H1/4 ant, n = 7; ♀H1/4 ant, n = 6. *$p < 0.05$). Data presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 for bar graphs.

6.3 Discussion

These results are the first to demonstrate the role of brain mast cell-derived histamine on masculinization of the POA and adult sexual behavior. We found that mast cell degranulation occurs in response to estradiol (from androgen turnover by aromatase) and subsequently activates H1 and H4 receptors on microglia to amplify prostaglandin E2 production. PGE2 enhances spinogenesis and, ultimately, the behavioral correlate of these neuroanatomical measures, male sexual behavior (Figure 6.0). Aberrant mast cell activation in females, triggered by the degranulation-inducing compound 48/80, and the resulting elevated histamine, is sufficient to masculinize neuroanatomical and behavioral measures in the absence of "organizational" steroid input. Inhibition of mast cell degranulation in males prevents masculinization and results in deficient spine formation in the POA and male sexual behavior as adults.

Steroid hormones are the principal drivers of brain sexual differentiation. There has been a long-standing assumption that hormonal action in the brain is mostly upon neuronal subtypes that express estrogen receptors at high levels during the critical period for sexual differentiation. However, here we demonstrate that this process is largely dependent on the brain's innate immune cells: local mast cells and microglia.

Mast cells are present in the brains of many species, however their impact on the brain has been discounted or ignored likely due to their small numbers (Georgin-Lavialle et al., 2016). Our study shows that although present in small numbers within the POA, their activity is crucial to the developmental organization of this region and provides the male with sufficient excitatory dendritic synapses in the POA to supersede the inhibitory threshold for mating behavior. Although we focused on the mast cell mediator, histamine,

here, mast cells are capable of synthesizing and releasing a large repertoire of inflammatory mediators, depending on the microenvironment in which they reside (Silver and Curley, 2013). More research into brain mast cell mediators in addition to histamine is warranted, as these too may contribute to masculinization of the POA.

The finding that aberrant mast cell degranulation in females affects sexual behavior even with no steroidal input is fascinating and warrants further exploration. The immune system may be a node by which female sexual behavior has greater room for complexity and thus, variability, than that of the male, as is the case for human sexuality (Zucker and Bradley, 1995). The human studies, of course, come with inextricable confounding variables such as societal expectations and cultural roles, but biological origins are nonetheless possible considering our findings.

Many types of stimuli evoke degranulation of mast cells, such as allergen exposure, stress, mild inflammation, and minor injury (Wong et al., 2014) Although these seemingly innocuous events are largely ignored during pregnancy, they may affect mast cell activity and, therefore, alter the immune profile of the fetal brain and, ultimately, its development.

Sex differences in the transcriptome of mast cells in post-pubertal (PN42) mice were discovered in 2016 by Mackey et al. These transcriptional sex differences affect the mast cell's capacity to synthesize, store and release inflammatory mediators at baseline and in both FcεR1(IgE)-dependent and IgE receptor-independent degranulation responses. When mast cell degranulation responses are compared between the sexes, total numbers, and percentages of degranulated mast cells are no different between males and females, regardless of IgE receptor dependence, cytokine mRNA levels, upstream

FcεR1(IgE)-mediated P-tyrosine phosphorylation patterns, and downstream Ca^{2+} mobilization. However, females exhibit 1.8-foldhigher clinical scores for increased vascular permeability, hyperemia, swelling, and hypothermia and have almost double the serum histamine as males in response to the same stimuli *in vivo*. In culture, unstimulated female peripheral bone marrow-derived mast cells (BMMCs) contain more pre-formed histamine, tryptase, and chymotryptase than males and release approximately 2-fold or higher levels of histamine, tryptase and TNF-α when both IgE-dependent or IgE-independent degranulation is evoked. When BMMC gene expression patterns were compared via RNA sequencing, 8,233 genes were differentially expressed between male and female BMMCs. Most of the genes that differed in expression level between the sexes were upregulated in females and were genes associated with biosynthetic, cell cycle, cellular component organization, and biogenesis when evaluated for gene ontology using Protein Analysis Through Evolutionary Relationships (PANTHER) software. These findings are in line with what we expected for mast cells making and storing more pre-formed mediators, as was reported (Mackey, 2016).

To understand if the increased storage of pre-formed mediators observed in females varied with circulating hormone levels, mast cells were isolated from the peritoneal cavity of mice at different stages of the estrus cycle. No differences in histamine storage or degranulation were detected but they found higher total numbers of mast cells in the peritoneum during proestrus and estrus (when estradiol is high) as compared to Metestrus and Diestrus, suggesting that differences in mast cell mediator storage are not entirely dependent on differences in circulating gonadal hormones between males and females. In addition, although psychological restraint stress (RS)

elicited the same cortisol response in male and female mice, mast cell responses to RS were greater in females, as evidenced by higher serum histamine and intestinal permeability as compared to males. As a result, although the number, degranulation status, and sensitivity to stimuli did not differ, post-pubertal female mast cells exhibited a much more robust inflammatory response to the same stimuli as males due to increased storage and release of pre-formed inflammatory mediators. This is a difference that did not seem to depend on circulating estrogen levels and, consequently, elicited higher clinical scores for symptomology in female mice as compared to males (Mackey, 2016).

The results of this study are in accordance with clinical data in humans indicating that females are at greater risk to the incidence and severity of many inflammatory and functional bowel diseases, allergic diseases, and disorders that are closely associated with psychological stress, such as irritable bowel syndrome (IBS) (González-Pérez et al., 2010; Korterink et al., 2015; Osman et al., 2007; Ballardini et al., 2013; Klein, 2016; and Klein, 2004). It also highlights both the complexity and subtle nature of sex differences orchestrated by means that are not solely dependent on differences in circulating gonadal hormones in sexually mature adults, as the sex bias in many mast cell-associated diseases show a female bias in prepubertal children, as well (Korterink et al., 2015; Osman et al., 2007; Ballardini et al., 2013; Klein, 2016; and Klein, 2004). In fact, the Moesler group teamed up with Cynthia Jordan, a leader in the field of sex differences, to study the organizational effects of perinatal androgens on mast cell phenotype and severity of anaphylaxis in prepubertal and adult mice. In this study, they demonstrated that gonadectomy of adults did not change passive systemic anaphylaxis (PSA)-induced serum levels of histamine or hypothermic responses in either sex. They confirmed that

these responses are indeed mast cell-dependent because MC-deficient Kit $^{w\text{-}sh/W\text{-}sh}$ mice have virtually no serum histamine elevation or hypothermic response to the same stimuli. To test if perinatal androgen exposure was responsible for the reduced mast cell-mediated response to PSA in adult males, they prevented androgen production in developing males by adding anti-androgen, di-(2-ethylhexyl) phthalate (DEHP), a known environmental endocrine disruptor that reduces testosterone production by the testes (Ernst et al., 2020), to the drinking water of pregnant dams from E16.5 to PN7, and then measured serum histamine and hypothermic responses of gonadectomized offspring as young adults. Male offspring that were not exposed to normal levels of testosterone perinatally, due to DEHP treatment, had significantly greater serum histamine and hypothermic responses to PSA as compared to male controls. When female offspring were conversely exposed to high levels of testosterone (androgenized) perinatally, their serum histamine and hypothermic responses to PSA were reduced to control male levels. None of the treatments changed mast cell number or degranulation status of mesenteric mast cells. When female BMMCs from androgenized offspring were grown in culture, they showed reduced mediator content and release as compared with control females, and were no different from male controls in response to PSA. In addition, mast cell-deficient mice, when engrafted with BMMCs originating from androgenized females and compared to those engrafted with BMMCs from control males and females, showed systemic responses matching donor BMMC phenotype regardless of the sex of the mast cell-deficient host/recipient, with animals who received BMMCs from control females having the most severe response to PSA. This body of work elegantly demonstrates the enduring effects of organizational programming of mast cell phenotype by perinatal gonadal hormone exposure, which

cannot be “undone’ by differences in the microenvironment or gonadal sex of the host (recipient).

This group then went on to investigate sex-specific meningeal mast /immune cell gene expression responses in males and females exposed to early life adversity (ELA), which in humans is known to increase susceptibility to mood, gastrointestinal, cardiovascular, and metabolic diseases by inducing persistent, mild inflammation through unknown mechanisms. In their 2022 publication, they found that maternal separation from PN1-17 (3hrs/day, no heat source) and early weaning (PN17), when combined with sub-chronic variable stress (consisting of 10 min/day of tail suspension, restrained, or delivered foot shocks from PN56-58), reduced sucrose preference in females but not males when tested just after PN59-61 and compared to females that were only stressed as adults. In addition, they found increased mast cell specific protease cma1 and mouse endothelial growth factor Vegf-c gene expression in the meninges of these same ELA, early weaned, adult-stressed females. Both the female-specific anhedonia behavior and elevated meningeal mast cell gene expression detected in these females was alleviated when females were treated with the mast cell stabilizer, ketotifen, for 30 minutes prior to each 10 min bout of stress from PN56-58. When they compared dura mater meningeal mast cell histology across four different regions: parietal, interparietal, sagittal sinus, and transverse sinus, they found sex differences in mast cell activation state in the parietal region of normally handled males vs. females that were only stressed in adulthood, as evidenced by a greater proportion of their total mast cells in the degranulated state as compared to females. Greater total numbers of mast cells and proportion of mast cells with pseudopodia were found in the interparietal meninges of males and females that

experienced maternal separation and early weaning in addition to adult stress when compared to those who were only stressed as adults. Within the transverse sinus meninges, females had more activated (degranulated) mast cells as compared to males when stressed as adults, regardless of ELA/early weaning (Natalia Duque-Wilckens et al., 2022).

In summary, our study was the first to report robust mast cell sex differences early on during the sensitive period for gonadal hormone-mediated sexual differentiation. In line with our results, other groups (majority led by Adam Moeser and colleagues), have demonstrated the importance of "organizational" hormonal programming of mast cells and/or their precursors very early in development and the complex and enduring sex-specific outcomes that these intrinsic differences evoke during critical periods of development as well as when challenged by psychological or inflammatory insult later in life, as sexually mature adults (Natalia Duque-Wilckens et al., 2022 Mackey 2020, Mackey, 2016). In conclusion, there is strong support for the importance of "organizational" sex differences in mast cells that should be considered by physicians treating patients with diseases or disorders that directly or tangentially involve mast cells. Sex differences in mast cells may underlie the observed poorer outcomes in boys with neurodevelopmental disorders like autism and ADHD (Theoharides et al., 2019 and 2016) and may play a role in the more severe symptoms experienced by preadolescent as well as adult females with mast cell-related disorders and those associated with stress (González-Pérez et al., 2010; Korterink et al., 2015; Osman et al., 2007; Ballardini et al., 2013; Klein, 2016; and Klein, 2004).

Chapter 7: Prenatal Allergen exposure Perturbs Sexual Differentiation &Programs Lifelong Changes in Adult Social and Sexual Behavior

7.1 Introduction

To apply our new understanding of the mechanisms underlying sexual differentiation of the POA, which depend crucially on not just one, but two different cell types belonging to the brain's immune system during a critical period of development, to a scenario that would garner more real-world relevance, we developed a model of maternal immune activation. We chose to study the sexual development of neonatal rat offspring in the context of an indirect immune activation of their mother, *in utero*, during the same critical period of development to see if a more natural causative agent rather than pharmacological means may enact similar changes on sexual differentiation of the POA and sexual behavior as adults. We chose to use an allergen-based immune challenge due to our newfound knowledge that mast cells are so critical to proper initiation of brain masculinization. To evaluate sexual differentiation, we profiled mast cell activation and spine density formation in the neonatal POA as well as the olfactory preferences, sexual behavior, and POA spine density when the offspring reached adulthood.

7.2 Results

Prior to breeding, dams were sensitized to ovalbumin (OVA)-alum adjuvant (the allergen) via IP injections spaced one week apart. Dams received ovalbumin-alum adjuvant on Weeks 1 and 2 and were bred on Week 3. Dams were then administered an intranasal allergen (or vehicle) challenge during gestation (GD15, prior to androgen surge) after which point they were singly housed and allowed to deliver naturally. Their pups were separated into two groups 1) to assesss neonatal mast cell numbers and

degranulation status and spine densities and 2) to be grown to adulthood to test for olfactory preferences, sexual behavior, and adult spine density (Figure 7.1).

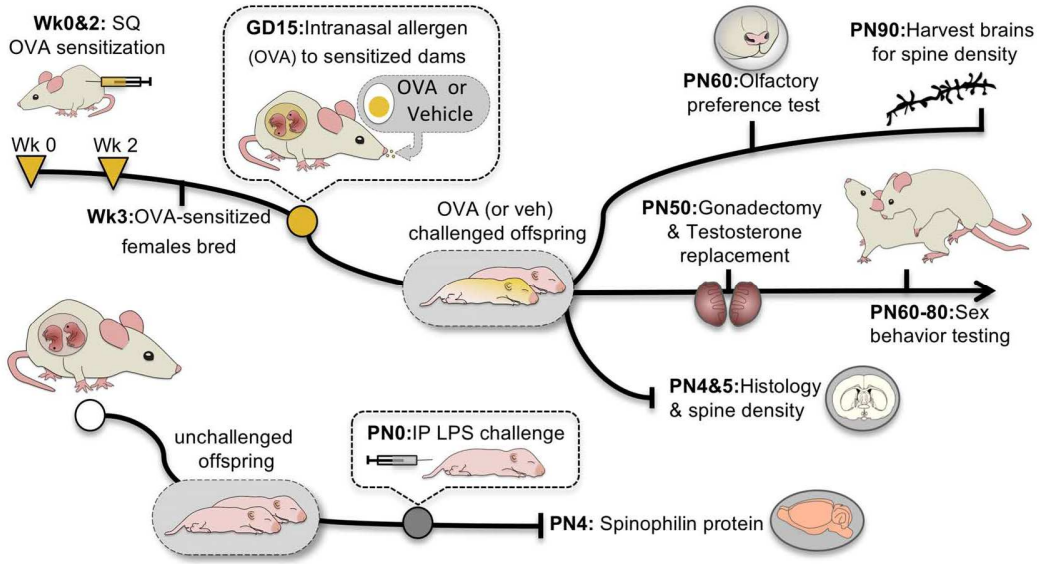
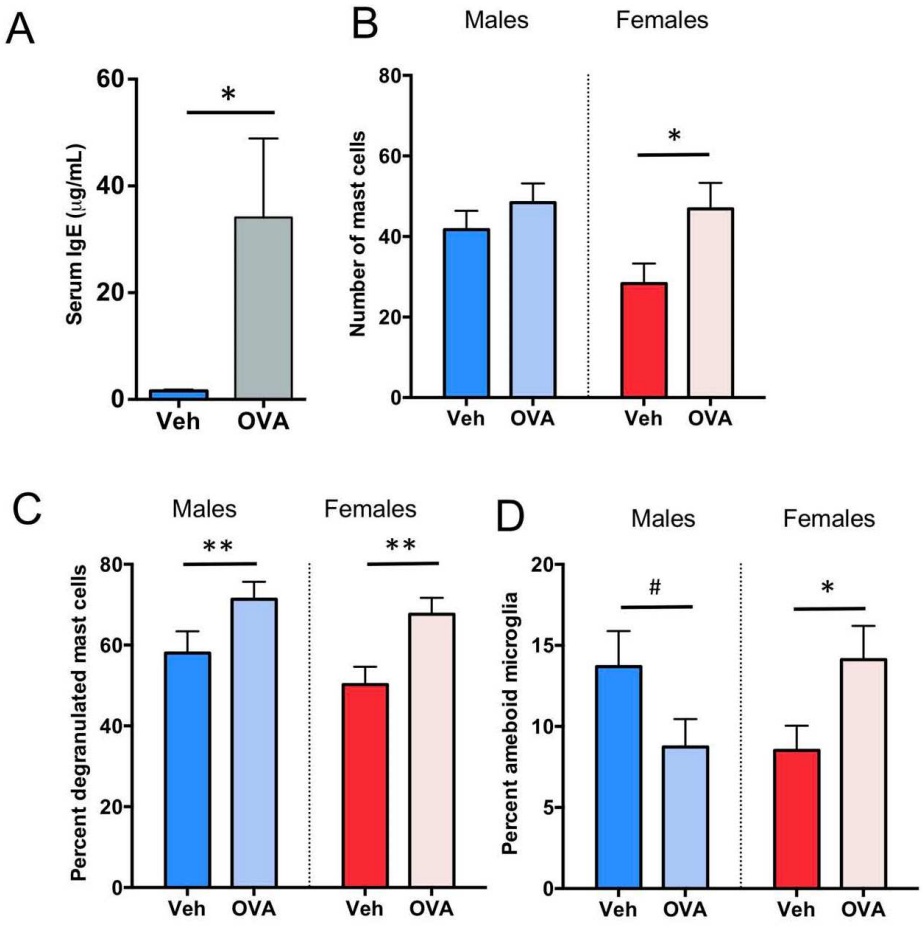


Figure 7.1 Prenatal allergic and postnatal immune challenge models. Three weeks prior to breeding, adult dams were sensitized to ovalbumin (OVA) allergen via delivery of ovalbumin-alum adjuvant subcutaneously two weeks apart; controls received vehicle injections. Sensitized and control dams were bred on Week 3 and on gestational day (GD)15 of pregnancy were challenged intranasally with OVA or vehicle under light anesthesia. Maternal blood was collected 30 minutes post-challenge and serum was isolated to assess total serum Immunoglobulin E (IgE) via ELISA. Neonatal pups from allergically-challenged or control dams were fatally anesthesized and transcardially perfused for histology/IHC on postnatal day 4 (PN4) to survey POA-resident immune cell numbers & morphology or on PN5 to assess POA spine density via golgi impregnation. Remaining pups were weaned on PN22 and grown to adulthood for behavioral testing. Adult offspring from challenged and control dams were then subdivided into two groups. The first group underwent sexual odor preference testing on PN60 and brains were collected post-test and then golgi-impregnated for adult POA spine density analysis. The second group of adult offspring from challenged and control dams was gonadectomized on PN50, implanted with a silastic capsule containing testosterone (to mimic adult male hormone levels), and "male-typical" sexual behavior was assessed between PN60-PN80 with a novel, hormonally-primed stimulus female. *Author Contributions for published body of work discussed in this chapter: K.M.L., L.A.P. and M.M.M. designed experiments. K.M.L. and M.M.M. wrote the manuscript. K.M.L. performed allergic challenge treatments, IHC, histology, western blot, immunoassays, adult surgeries, behavioral experiments, designed and made figures, and analyzed data. L.A.P. performed IHC, histology, cell counting, and designed and made figures. C.L.W. performed behavioral data analysis. A.G. performed allergic sensitization treatments, animal husbandry, behavioral testing, completed immunohistochemistry, histology, and cell counting.

To confirm that pregnant dams were sensitized and mounted a response to the OVA challenge administered on GD15, maternal serum IgE levels were measured, and Katy Lenz confirmed IgE levels were increased significantly following maternal allergic challenge (Figure 7.1A), confirming allergic response (* $p<0.05$, $n=3$). To assess the effects of the maternal allergic challenge on brain mast cells of the offspring, mast cell number and degranulation status were profiled in postnatal day 7 pups. We found that the prenatal allergic challenge increased mast cell number to near-male levels in female offspring relative to control females (Figure 7.2B) and increased the proportion of mast cells undergoing degranulation in the POA of offspring of both sexes (Figure 7.2C, **$p<0.01$).

Allergic challenge had opposite effects in male vs. female offspring regarding microglia morphology. OVA increased the proportion of POA microglia with amoeboid morphology in females (* $p<0.05$) and, although not statistically significant, a trend (# $0.05\leq p\leq 0.1$) was detected in challenged male offspring, indicating a possible decrease in the proportion of microglia with amoeboid morphology relative to unchallenged control offspring (Figure 7.2D).



7.2 Prenatal allergic challenge "masculinized" immune cell status in ♀neonatal POA. **A**. Maternal serum IgE levels increased following maternal allergic challenge with ovalbumin (OVA) on gestational day (GD)15, confirming allergic response (* $p<0.05$, n=3). **B-C**. Prenatal allergic challenge increased mast cell number to near-male levels in female offspring relative to vehicle (Veh) control females (**B.**) and increased the proportion of mast cells undergoing degranulation in the POA of offspring of both sexes (**C.**) (**$p<0.01$). ♂Veh n=10, ♂OVA n=15, ♀Veh n=15, ♀OVA n=14. **D**. Allergic challenge increased the proportion of POA microglia with amoeboid morphology in females (* $p<0.05$) whereas a trend (# $0.05 \leq p \leq 0.1$) towards a decrease was detected in challenged male offspring. ♂V n=4, ♂OVA n=7, ♀V n=8, ♀OVA n=6.
Data presented as mean + SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ for bar graphs.

Prenatal allergic challenge increased the density of dendritic spines in Golgi-Cox impregnated POA neurons of female offspring assessed on PN5 (Figure 7.3A and B), and decreased the density on male POA neurons, but had no effect on dendritic length (Figure 7.3C) or cell body area (Figure 7.3D). Prenatal allergic challenge just prior to the androgen surge, led to lifelong masculinization of dendritic spine density of females relative to vehicle females, as measured in adult POA neurons visualized with Golgi-Cox impregnation, but had no enduring effect on males (Figure 7.3E).

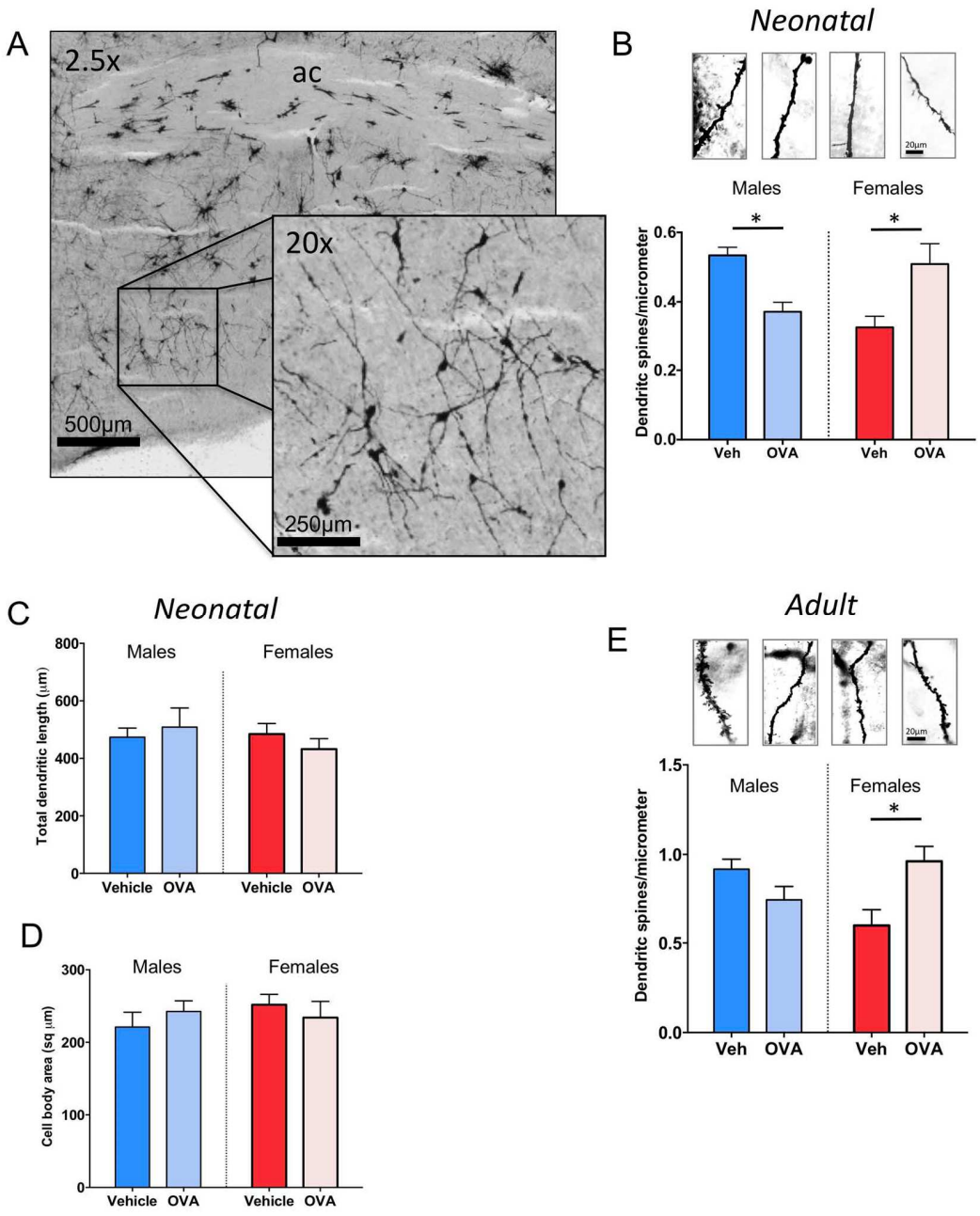


Figure 7.3. Prenatal allergic challenge masculinized POA dendritic spine density of female offspring for life and delayed masculinization in neonatal male offspring.
A-D. Prenatal allergic challenge increased the density of dendritic spines in Golgi-Cox impregnated POA neurons of female offspring on PN5 **(A-B.)** and decreased the density on male POA neurons but had no effect on dendritic length (**C.**) or cell body area (**D**). **E**. Prenatal allergic challenge led to lifelong masculinization of dendritic spine density of females relative to vehicle (Veh) females, as measured in adult POA neurons visualized with Golgi-Cox impregnation but had no enduring effect on male POA spine density.
Group sizes: Panel **B–D:** All groups n=4. **E**: ♂Veh n=4; ♀Veh n=3; ♂Veh n=4; ♀OVA n=5). Data presented as mean + SEM. *p < 0.05, **p < 0.01, ***p < 0.001 for bar graphs.

Prenatal allergen-challenged female offspring that were gonadectomized and "activated" via testosterone capsule implantation as sexually mature adults took less time to mount a stimulus female (Figure 7.4A) and mounted more frequently than unchallenged female offspring (Figure 7.4B). Unchallenged male offspring preferred female odor when olfactory preferences were tested on PN60, while unchallenged females showed no odor preferences (Figure 7.4C). Prenatal allergic challenge abolished the preference for female odor demonstrated by unchallenged male offspring and had no significant effect on odor preference as compared to unchallenged female offspring, who showed no significant preference for male or female odor (Figure 7.4C).

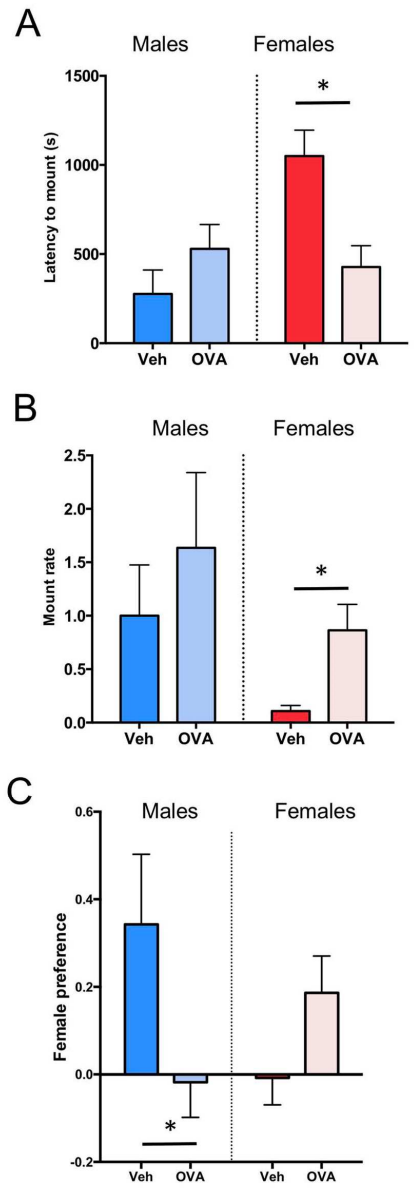


Figure 7.4. Prenatal allergic challenge masculinized sexual behavior of female offspring and reduced the preference for female odor in male offspring.
A–B. Prenatal allergen-challenged female offspring that were gonadectomized and "activated" via testosterone capsule implantation took less time to mount a stimulus female **B.** and mounted more frequently than unchallenged female offspring. ♂Veh n = 6, ♂OVA n = 9, ♀Veh n = 5, Ova ♀ n = 9. **C**. Unchallenged male offspring preferred female odor while unchallenged females showed no odor preferences. Prenatal allergic challenge abolished the preference for female odor demonstrated by unchallenged male offspring and had no significant effect on odor preference as compared to unchallenged female offspring, who showed no significant preference for male or female odor ($p = 0.2$). ♂Veh n = 7, ♂Ova n = 7, ♀Veh n = 6, Ova ♀ n = 7. Data presented as mean + SEM. *Indicates $p < 0.05$.

7.3 Discussion

Steroid hormones are the principal drivers of brain sexual differentiation. There has been a long-standing assumption that hormonal action in the brain is mostly upon neuronal subtypes that express estrogen receptors at high levels during the critical period for sexual differentiation, however, here we demonstrate that this process is largely dependent on the brains innate immune cells, local mast cells and microglia.

Mast cells have been reported in the brains of many species, however their impact on the brain discounted or likely ignored due to their small numbers (Georgin-Lavialle et al., 2016). Our study shows that although present in small numbers, within the POA, their activity is crucial to the developmental organization of this region and provides the male with sufficient excitatory dendritic synapses in the POA to supersede the inhibitory threshold for mating behavior. Although we focused on the mast cell mediator, histamine, here, mast cells are capable of synthesizing and releasing a large repertoire of inflammatory mediators, depending on the microenvironment in which they reside (Silver and Curley, 2013). More research into brain mast cell mediators in addition to histamine is warranted, as these too may contribute to masculinization of the POA.

The finding that aberrant mast cell degranulation in females affects sexual behavior without steroidal input is fascinating and warrants further exploration, as the immune system may be a node by which female sexual behavior has greater room for complexity and thus variability than that of the male, as is the case for human sexuality (Zucker and Bradley, 1995). The human studies of course come with inextricable confounding variables such as societal expectations and cultural roles, but biological origins are nonetheless possible considering our findings.

Many types of stimuli evoke degranulation of mast cells such as allergen exposure, stress, mild inflammation, and minor injury and although these seemingly innocuous events are largely ignored during pregnancy, they may affect mast cell activity and therefore alter the immune profile of the fetal brain and ultimately its development.

Sex differences have been identified in the transcriptome of mast cells in post-pubertal (PN42) mice that affect the cell's capacity to synthesize, store and release inflammatory mediators at baseline and in both FcεR1(IGE)-dependent and IGE receptor-independent degranulation responses. When mast cell degranulation responses are compared, with regard to sex, regardless of IGE receptor dependence, cytokine mRNA levels, upstream FceR1(IGE)-mediated P-tyrosine phosphorylation patterns, downstream Ca2+ mobilization, total numbers and percentages of degranulated mast cells are no different between males and females in response to both IGE-dependent (PSA) and independent (Ca2+ influx via ionophore A23187) mast cell degranulation responses are the same in both males and females, females exhibit (1.8 fold) higher clinical scores for increased vascular permeability, hyperemia, swelling and hypothermia and have almost double the serum histamine as males in response to the same stimuli, in vivo. In culture, unstimulated female peripheral bone marrow-derived mast cells (BMMCs) contain more preformed histamine, tryptase and chymotryptase than males and release approximately 2-fold or higher levels of histamine, tryptase and TNFα when both IGE-dependent or IGE-independent degranulation is evoked. When BMMC gene expression patterns were compared via RNA sequencing, 8233 genes were expressed differentially between male and female BMMCs. Majority of the genes that differed in expression level between the sexes were upregulated in females and were genes associated with biosynthetic, cell

cycle, cellular component organization and biogenesis when evaluated for gene ontology using Protein Analysis Through Evolutionary Relationships software (PANTHER), which is what we would expect for a mast cell making and storing more preformed mediators, as is the case here (Mackey, 2016). To understand if the increased storage of preformed mediators observed in females varied with circulating hormone levels, mast cells were isolated from the peritoneal cavity of mice at different stages of the estrus cycle, and no differences in histamine storage or degranulation state detected but they found higher total numbers of mast cells in the peritoneum during proestrus and estrus (when estradiol is high) as compared to Metestrus and Diestrus, suggesting that differences in mast cell mediator storage are not entirely dependent on differences in circulating gonadal hormones between males and females. In addition, although psychological restraint stress (RS) elicited the same cortisol response in male and female mice, mast cell responses to RS were greater in females, as evidenced by higher serum histamine and intestinal permeability as compared to males. So, although the number, degranulation status and sensitivity to stimuli did not differ, post-pubertal female mast cells exhibit a much more robust inflammatory response to the same stimuli as males due to increased storage and release of preformed inflammatory mediators, a difference that does not seem to depend on circulating estrogen levels and consequently elicits higher clinical scores for symptomology in female mice as compared to males (Mackey, 2016).

The results of this study are in accordance with clinical data in humans which puts females at greater risk to the incidence and severity of many inflammatory and functional bowel diseases, allergic diseases and disorders that are closely associated with psychological stress such as irritable bowel (IBS) and chronic pain syndromes. The same

enhanced immune system response that puts females at greater risk to autoimmune disorders and worse outcomes when exposed to inflammatory stimuli is countered by the greater protection from viral, bacterial, and parasitic infections it offers (Klein et al., 2004, Klein et al., 2016). Females even mount greater immune responses to vaccines, which in the short term is uncomfortable, it renders the vaccines more efficacious, than those administered to males. This series of studies also highlights both the complexity and subtle nature of sex differences orchestrated by means that are not solely dependent on differences in circulating gonadal hormones in sexually mature adults, as the sex bias in many mast cell associated diseases show a female bias in prepubertal children, as well (Remes et al., 1998, González-Pérez et al.; Abu-Arafeh et al. 2010, Ballardini et al, 2013, Korterink et al., 2015, Osman et al, 2015 & Chiaroni-Clarke et al., 2016).

In fact, this same group teamed up with Cynthia Jordan, a leader in the field of sex differences, to study the organizational effects of perinatal androgens on mast cell phenotype and severity of anaphylaxis in prepubertal and adult mice. In this study, they demonstrate that gonadectomy of adults did not change PSA-induced serum levels of histamine or hypothermic responses in either sex. They confirmed that these responses are indeed mast cell-dependent because MC-deficient Kit $^{w\text{-}sh/W\text{-}sh}$ mice have virtually no serum histamine elevation or hypothermic response to the same stimuli. To test if perinatal androgen exposure was responsible for the reduced mast cell-mediated response to PSA in adult males, they prevented androgen production in developing males by adding antiandrogen, di-(2-ethylhexyl) phthalate (DEHP), a known environmental endocrine disruptor that reduces testosterone production by the testes, to the drinking water of pregnant dams from E16.5 to PN7 and measured serum histamine and

hypothermic responses of gonadectomized offspring as young adults. Male offspring that were not exposed to normal levels of testosterone perinatally, due to DEHP treatment, had significantly greater serum histamine and hypothermic responses to PSA as compared to male controls and when female offspring were conversely exposed to high levels of testosterone (androgenized) perinatally, their serum histamine and hypothermic responses to PSA were reduced to control male levels. None of the treatments changed mast cell number or degranulation status of mesenteric mast cells. When female BMMCs from androgenized offspring were grown in culture, they showed reduced mediator content and release as compared with control females, and were no different from male controls in response to PSA. In addition, mast cell deficient mice, when engrafted with BMMCs originating from androgenized females and compared to those engrafted with BMMCs from control males and females, systemic responses matched donor BMMC phenotype regardless of the sex of the mast cell deficient host/recipient, with animals who received BMMCs from control females having the most severe response to PSA. This body of work elegantly demonstrates the enduring effects of organizational programming of mast cell phenotype by perinatal gonadal hormone exposure, which cannot be "undone' by differences in the microenvironment or gonadal sex of the host (recipient).

This group then goes on to investigate sex-specific meningeal mast /immune cell gene expression responses in males and females exposed to early life adversity (ELA), which in humans is known to increase susceptibility to mood, gastrointestinal cardiovascular and metabolic disease by inducing persistent, mild inflammation although the mechanisms underlying these changes are not known. They find that maternal

separation from PN1-17 (3hrs/day, no heat source) and early weaning (PN17), when combined with subchronic variable stress (by 10 min/day of tail suspension, restrained or delivered foot shocks from PN56-58), reduced sucrose preference in females but not males when tested just after PN59-61 and compared to females that were only stressed as adults. In addition, they find increased mast cell specific protease cma1 and mouse endothelial growth factor vegf-c gene expression in the meninges of these same ELA, early weaned, adult-stressed females. Both the female-specific anhedonia behavior and elevated meningeal mast cell gene expression detected in these females is alleviated when females are treated with mast cell stabilizer, ketotifen, 30 minutes prior to each 10 min bout of stress from PN56-58. When they compared dura mater meningeal mast cell histology across four different regions: parietal, interparietal, sagittal sinus and transverse sinus, they found sex differences in mast cell activation state in the parietal region of normally handled males vs. females that were only stressed in adulthood, as evidenced by a greater proportion of their total mast cells in the degranulated state as compared to females. Greater total numbers of mast cells and proportion of mast cells with pseudopodia were found in the interparietal meninges of males and females that experienced maternal separation and early weaning in addition to adult stress when compared to those who were only stressed as adults. Within the transverse sinus meninges, females had more activated (degranulated) mast cells as compared to males when stressed as adults, regardless of ELA/early weaning (Natalia Duque-Wilckens et al., 2022).

In summary, our study was the first to report robust mast cell sex differences early on during the sensitive period for gonadal hormone-mediated sexual differentiation, and

when combined with those previously discussed in this chapter, majority of which were led by Adam Moeser and colleagues, are the first to demonstrate the importance of "organizational" hormonal programming of mast cells and/or their precursors very early in development and the complex and enduring sex-specific outcomes that these intrinsic differences evoke both during critical periods of development as well as when challenged by psychological or inflammatory insult later in life, as sexually mature adults (Natalia Duque-Wilckens et al., 2022 Mackey 2020, Mackey, 2016).

Synthesis and Future Directions

Much of the work presented here was conducted to test the central hypothesis that microglial phagoptosis of neurons is required for sexual differentiation of the sexually dimorphic nucleus and its behavioral correlate, sexual odor preference. In our first aim, we sought to define the period of highest levels of phagocytosis in the MPN, predicting that the highest levels of phagocytosis would be in the female cMPN, where the SDN is undergoing sexual differentiation. We did find that phagocytosis by microglia was greatest in female cMPN as compared to other subdivisions of the MPN, with peak levels on PN8, when the sex difference in SDN volume is first apparent. To determine if this phagocytic activity is targeting neurons of the SDN, we performed immunohistochemical analysis of the cellular contents within the microglia cups on PN8 and found that most of the contents do appear to be neuron derived. When immunohistochemical analysis was performed for terminal apoptosis marker cleaved caspase 3, we found that most neurons engulfed by microglia were not cleaved caspase 3-positive, suggesting that they were not already committed to canonical apoptosis prior to engulfment and might survive if left unmolested by microglia. To test whether microglia are truly the drivers of cell death by engulfment, rather than clearing cells that had already undergone self-programmed apoptosis, we used a function-blocking antibody against Cd11b (CR3) to prevent microglia from contacting their target cells during the period of higher phagocytosis observed in females (PN5-7/8). The function-blocking antibody treatment lowered microglial phagocytic activity, as expected. Furthermore, volume of the SDN, when assessed right after the phagocytosis blockade on PN8, was significantly increased, indicating that without microglia engulfment, most of these neurons were able to survive.

To assess if the neurons that escaped microglial engulfment survive and confer male-typical behaviors in adulthood to the females that were administered function-blocking antibody, we repeated the neonatal antibody blockade, carrying it out through PN8, the peak in microglial phagocytosis. We found that the volume of the SDN in adulthood was unchanged, contrary to our expectations. However, we found that, as predicted, female-typical sexual odor preferences were indeed lost in adulthood. Puzzled by the unchanged SDN volume, we then repeated the neonatal treatments in another cohort of animals. After first confirming that sexual odor preference was indeed lost in the second cohort treated with function-blocking antibody, we gave all animals an acute exposure of sexually active male odor (urine) just prior to being killed. Using immediate early gene (Egr1) expression as a marker for cellular activity, we counted the number of cells in the cMPN and SDN that expressed Egr1 in response to the male odor exposure and found that this immediate early gene activation was significantly reduced in females treated with function-blocking anti-Cd11b (CR3) as neonates, whereas there was no change in vehicle or F480 control antibody-injected females. These results confirm those already discovered by others (albeit with a different genetic marker, Egr1, instead of c-Fos) that activation in the cMPN/SDN in response to sexual male odor (urine) is sexually dimorphic, with females demonstrating robust activation, and males very little (Pierman & Bakker, 2008). It is not possible to determine if the change in odor preference in antibody blockade females translates into an actual mating preference because the females in which the SDN responsivity was masculinized were not exposed to the androgens and estrogens required for masculinization of actual mating behavior, i.e. mounts and The surprising finding that the increased volume observed postnatally in

females treated with anti-CR3 was not maintained in adulthood, but activity of the cells in response to the male odor cue was reduced, may be explained by several aspects of SDN development, many of which are still poorly understood. The first interpretation of our results is that the neurons that escaped engulfment by microglia and were retained into adulthood were a specific subpopulation that survived and established synaptic connections or inputs that normally occur in males during the period when phagocytosis was blocked. Room for these surviving cells was made at the expense (loss) of cell types that would normally survive in females. A second interpretation is that the cells that escaped microglial phagoptosis survived only during the antibody blockade but once the antibody was cleared, were then removed by microglia as originally programmed. The loss in immediate early gene activation observed in the SDN could reflect developmental changes to neighboring cells during the period of extended survival of those that were destined for removal by microglial phagocytosis, perhaps due to cellular stress, competition for resources or ineffective synaptic partnering, during a critical period. A third interpretation is that all the cells, both microglia-targeted cells and untargeted neighbors, survived until puberty, when the adult SDN volume is "crystallized" by circulating gonadal hormones. During puberty, a greater number of proliferating cells, that do not express neuronal marker NeuN or astrocyte marker GFAP are present in the male vs. the female SDN (Ahmed et al., 2008). In the case of neonatally blockaded females, there was no subsequent exposure to male hormones, and thus no opportunity for male-typical cell birth to facilitate "masculinization" of SDN volume. Although the identity, purpose, and fate of the cells born in the SDN during puberty is unknown, it is possible that their presence indicates a second period of reorganization of the SDN,

offering another opportunity for masculinization or feminization in accordance with circulating gonadal hormones. For example, if complications arose during the first critical period of masculinization, resulting in a sub-masculinized or sub-feminized SDN, perhaps puberty is a time where one such individual may recover necessary male-typical or female-typical cells and synaptic connections in this region. It is equally plausible that following this newborn population of cells during puberty, there is a second period of microglial phagoptosis to again cull unnecessary cells to ensure that the correct number and type of cell is consistent with level and type of circulating gonadal hormones experienced during puberty. Finally, our results may reflect a change in cell type rather than cell number, in which case we would expect an unchanged SDN volume, composed of the same number of cells, but phenotypically different from a typical female. To understand the exact means by which the neonatal function-blocking antibody reduced immediate early gene activation in the SDN (a marker for neuronal activity) and sexual odor preference, additional studies would need to be done to assess the number and phenotype of cells that make up the SDN prior to and after the antibody blockade but also, most importantly, the number and phenotypes of cells that survive to adulthood. Although beyond the scope of the current study, cell phenotypes could be defined using RNAscope, either alone or in conjunction with an acute odor exposure, and Egr1 could be used to assess the important cell types that are activated in response to the odor cue that are not present in or are not responding in the antibody-blockaded females. Additional studies of SDN development during puberty to assess the cell type(s) born, and more importantly, their fate, are of great interest here, as well as the behavior of microglia during this pubertal period of cell genesis, to investigate whether or not there is second

period of microglial phagoptosis prior to, during, after, or all of the above, to regulate the "permanence" of those newly born cells or make room for them in the SDN. The SDN is an objectively small subnucleus, but it is nonetheless highly complex. The cMPN consists mostly of GABAergic inhibitory neurons which also express calbindin, and sometimes CRF, dopamine beta-hydroxylase, and NPY. It also contains cell bodies expressing TRH and both fibers and cell bodies containing CCK (Simmerly, 1985). Expression of somatostatin mRNA peaks in males during the postnatal period when the sex difference is materializing, but nothing is known about the significance of this differential gene expression (Orikasa et al. 2007). Many of these neuropeptides and neurotransmitters have been implicated in detection of odors of various sorts, (Shayit & Weller, 2000; Lemaire et al., 1992, 1994; Nowak et al. 1997). It will be of interest in future studies to determine whether a specific subpopulation of SDN neurons is subject to phagoptotic attack or if there are different populations being culled on a slightly different timeline (or even on different circadian schedules) that we were not able to capture here with our focus on PN8, the peak of phagocytic activity in the female cMPN.

An important caveat to the current results is that administration of blocking antibodies to the lateral ventricles may have affected other sexually dimorphic brain regions relying on similar (yet, unknown) mechanisms of microglial phagocytosis in addition to the SDN. We chose this approach both to avoid damage to the small cMPN in week-old animals and to reduce the number of injection "misses." Nonetheless, it is possible that other brain regions are involved in the behavioral response. The medial amygdala MeA, BNSTp, and ventromedial hypothalamus are sexually dimorphic nuclei that undergo extensive hormone-mediated cell death and/or differentiation on a similar,

albeit slightly earlier timeline than the SDN. These nuclei all contain neuronal populations enriched for the expression of gonadal hormone receptors for estrogen (ER), progesterone (PR), and androgen (AR), and are members of the main and accessory olfactory projection pathways while also crucial for gating social and sexual behaviors in both males and females. Aromatase-expressing neurons of the BNSTp have been identified as a crucial component for the recognition of a sexual partner in the male, but not female mouse. In fact, the same group who made this discovery interrogated differential gene expression in Esr1+ neuronal populations from all four of these sexually dimorphic regions in males and females under physiological conditions mimicking estrus and diestrus. They found by profiling gene expression across these groups and regions that Esr1+ cell types co-clustered most reliably with respect to region, instead of by experimental group, sex, or stage of estrus. Most of the Esr1+ cell types analyzed were neurons that additionally express PR and AR. By profiling cell types that showed differential gene expression between groups and targeting them specifically using a cre recombinase-dependent, inhibitory chemogenetic actuator, expressed virally, they found that only BNSTpr, Esr1+, and Tac1 (encoding neuropeptide tachykinin1, or Substance P) neurons are necessary for distinguishing between male and female pheromonal cues in urine, in males only. In females, they identified a population of glutamatergic VMHvl Cckar+, Esr1+ neurons projecting to the AVPV nucleus that was absent in males and essential for female sexual behavior, while VMHvl Cckar-, Esr1+ cell types were needed for maternal aggression, male sex behavior, and social aggression (Ahern et al. 2013; Bayless et al., 2019; Knoedler et al., 2022). The AVPV is home to Gonadotropin releasing hormone (GnRH) neurons, the most adventurous of all neuroendocrine cells.

Born in the olfactory bulb, they migrate all the way into the teensy tiny little AVPV, where they then project to the median eminence to release GnRH in a pulsatile fashion. This pulsatile release generates a signal that is received and interpreted by the pituitary to be in favor of LH secretion when pulse frequency is high and in favor of FSH secretion when pulse frequency is low. Follicle stimulating hormone and lutenizing hormone release are crucial to fertility for their role in the production and release of gonadal steroids. Sex steroids ultimately regulate the final GnRH signal via both positive and negative feedback from upstream ERα-expressing kisspeptin neurons. (Wang et al., 2020). Approximately three quarters of the AVPV kisspeptin neurons are GABAergic and co-express tyrosine hydroxylase (TH) and Estrogen receptor alpha. Kisspeptin neurons were shown to express c-Fos at higher levels during the pre-ovulatory LH surge and activity of these neurons is majorly dependent on sex steroid hormone levels acting in positive and negative feedback to tightly regulate GnRH release frequency to optimize fertility and reproductive success (Wang et al, 2020). This is all to say that from a neuroendocrine perspective, the developmental blueprints underlying the hypothalamic-pituitary-adrenal (HPA), -gonadal (HPG), -thyroid axis (HPT) or hypothalamic-neurohypophysis system, are all important for regulating hormone levels that are required for maintenance of reproductive cycling and stereotyping behaviors like innate sexual odor preferences. We may have gotten lucky with the timing of our function-blocking antibody injections being after most of the cell death-dependent sexual differentiation occurring in neighboring nuclei of the POA that are more crucial for hormonal regulation. It is possible, however, that the neonatal antibody blockade could have had unintended effects on the development of these other regions. It would be easiest to assess this by

measuring circulating hormone levels of all groups once sexually mature, something we did not do at the time, since all female groups were cycling normally.

In non-copulating male rats, or rats that don't show a strong preference for a female in proestrus/estrous over a female not in heat, c-Fos reactivity in the vomeronasal pathway was reduced in the MeAD, MePD, granule cell layer of the AOB, BNST, and mPOA. The greatest decrease in responsivity when compared to copulating males was observed in the mPOA of non-copulating males, where exposure to females in estrous produced half the response (same as non-estrous female) than copulating males did as revealed by half the c-Fos expression in this region (Portillo, W., & Paredes, R. G., 2004).

Over thirty percent of mouse neocortical GABAergic interneurons are removed by apoptosis, dependent on BAX during the first postnatal week of life. In the neocortex and cerebellar cortices, conditional deletion of y-Protocadherins (Pcdhgs) during the week of greatest levels of apoptosis increased apoptosis in many brain regions and resulted in a substantial decrease in GABAergic interneuron survival. However, loss of this protein has no effect on pyramidal or glial cells that also normally express it. The decreased GABAergic tone resulted in reduced AKT signaling in Pcdhg mutant interneurons, a factor that is restored when pro-apoptotic BAX is additionally genetically ablated.

Oligodendrocytes are very important for generating neuronal firing in response to sensory stimuli. Birth and maturation of oligodendrocytes begins just prior to birth of the organism, with peak oligodendrocyte proliferation and maturation occurring between birth and weaning, after neurons and astrocytes have completed their peak proliferative

interval and are at, or near, maturity in both rodents and humans (Rice & Barone, 2000).

The consequences of differential conduction velocities are abnormal neuronal integration (mostly at junctions), use of "inefficient" (compensatory) circuits, and below-threshold for integrated firing due to insufficient metabolic support for neurons. Together, these abnormalities in firing integration and capacity resulted in delayed processing of auditory stimuli, as was the behavioral assay used in this study (Carriere et al., 2020).

Now that immunology has reached the final frontier and the field of neuroimmunology is finally a burgeoning one that knows no bounds, rather than an abandoned effort due to the incorrect assumption that immune cells are not able to gain entry to the brain, scientists around the world are finally appreciating the very active role that our immune system has on brain health, where all that sickness behavior begins! Neuroscientists had considered the brain an "immune-privileged" organ in the body until recently, and it was once thought that only one immune cell type inhabited the brain, microglia. Our studies are the first showing the major role of not just microglia, but also the mast cell, in sexual differentiation of the developing brain. These two cell types are both highly mobile, sensitive, fast, and powerful responders to stimuli. Both contain a diverse repertoire of messenger systems (ligands and receptors, many reciprocal or complimentary) and for mast cells, pre-formed mediators and neurotransmitters that are packaged and stored for immediate release. Since timing of critical periods during development is so important, it's no wonder that they would be under the guidance of two of the most sensitive and quick-responding immune cell types. Neuroimmunology will surely flourish in part due to the renewed public interest in immunology and infectious

disease agents. we are sure to find novel ways the immune system regulates, maintains, and evolves with the developing and mature brain to keep it functioning optimally and pathogen-free throughout life. The discovery that mast cells are initiating developmental sexual differentiation in the preoptic area is just the tip of the iceberg. The incredible specificity with regard to the mast cell's location within the brain and maintenance of appreciable numbers of cells for such a short sensitive period of development, during which the brain is sensitive to hormonal input, AND findings that these mast cells express estrogen receptors on their surface, and store GnRH, serotonin, histamine and other neurotransmitters inside their granules indicate that there are many important functions for these cells that have yet to be uncovered.

Research on the ancient mast cell, or the primordial neuro-endo-immuno axis cell (as I prefer to think of it) that has been around before the nervous system evolved, as evidenced by their crucial role in primitive creatures like sea squirts (Wong et al., 2014) and seems to come and go in waves. This could be because mast cells are poorly understood, difficult to phenotype (changes based on niche occupied), and present in smaller numbers than other immune cell types. These mysterious cells, although difficult to study, are truly the most unique and dynamic cell type that has been discovered from a biochemical, hormonal, neuroendocrine and immune prospective. Newer studies spanning the entire POA, and anterior hypothalamus currently being conducted by a postdoctoral researcher in our laboratory, Dr. Erin Reinl, have suggested that mast cells may be closely associated with the SDN, in terms of location; earlier studies did not survey the most caudal parts of the POA, where the SDN resides. Fresh studies with newer students are underway in our laboratory to uncover the physiological role that

hippocampal mast cells are playing during the first two weeks, when their population is temporarily expanded and restricted to certain locations within the brain (our published and unpublished observations). These studies are currently being headed by Alexa Blanchard and are already going in an exciting direction.

The role of mast cells in sexual differentiation of the SDN is not fully known, at present. Although we do know that activating them with the classic degranulator, c48/80, prior to its differentiation, reduces phagocytosis and increases the SDN volume in females, more studies are needed to understand the mechanisms and signaling molecules that are used/regulated by mast cells in this region. Future studies into the role of mast cell to microglia signaling during the first weeks of postnatal life continue and as we discover better pharmacological agents and tools for manipulating these cells without off-target effects (as the mast cell degranulator, compound 48/80, also has excitatory effects on neurons (Heppner, 1992; Eglezos, 1992)). Additionally, as we now have access to techniques to better visualize their location and population expansion during embryonic and postnatal periods, such as using CLARITY to clear rat embryos, we can directly visualize mast cells to understand exactly when and how they enter the SDN or hippocampus during development and, also, how they maintain and expand their population during development and then reduce this population substantially for the remainder of life.

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