THE ROLE OF TRPC2 IN SEX-SPECIFIC BRAIN CIRCUITS AND BEHAVIOR

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ABSTRACT

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The transient receptor potential cation channel 2 (TRPC2) is canonically known for carrying pheromonal information from the vomeronasal organ (VNO) to the brain in rodents. Mice with a disabled TRPC2 gene display drastic changes in sex-specific behaviors, including sexual and aggressive behavior. Specifically, male-male and maternal aggression is absent while *both* males and females show male-typical mounting behavior directed towards *both* sexes in a non-preferential manner. In short, sexual preference seems to be severely disrupted. Several groups have shown that the VNO of TRPC2 knockout (KO) mice show a markedly reduced activation of the VNO in response to pheromones, suggesting that pheromonal signaling via TRPP2 channels in the VNO shape these sex-specific behaviors. However, TRPC2 is also expressed in other tissues, including the reproductive organs, raising the possibility that disruption of TRPC2 function outside the VNO also contributes to changes in adult sex-specific behavior. My dissertation research aims to understand the underpinnings of this behavioral change, examining how the loss of TRPC2 function influences pre- and postnatal development, reproductive success and morphological sex differences in the brain. First, I found that mice lacking TRPC2 display defects in their development, with effects on pubertal timing and pup survival, along with effects on reproductive success. While maternal experience rescued pup survival in TRPC2 KO mice, it did not improve reproductive outcomes. Next, I examined two brain regions implicated in the control of mounting and aggression, the posterodorsal aspect of the medial amygdala and ventromedial hypothalamus. Utilizing a Nissl stain and glial fibrillary acidic protein immunohistochemistry, I determined that TRPC2 KO mice show altered patterns of sex differences at the cellular level in both these regions, offering insight into the neural mechanisms underlying impaired sexual and aggressive behavior. Finally, I examined whether sexual experience can reverse deficits in behavior and rescue the brain's response to pheromones. I found that prolonged sexual experience did not reinstate normal sexual preference nor recover the brain response to pheromones. These experiments suggest TRPC2 function, driven by pheromones and possibly other

incoming signals, participates in organizing sex-specific behavior and brain circuitry. TRPC2 function outside the VNO may also impact adult sex-specific behaviors.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

AGD	anogenital distance
AOB	accessory olfactory bulb
AOC	accessory olfactory cortex
ARC	arcuate nucleus
BAOT	bed nucleus of the accessory olfactory tract
BMA	basomedial nucleus
BNST	bed nucleus of the stria terminalis
E2	estradiol
ER	estrogen receptor
GDX	gonadectomized (male)
GFAP	glial fibrillary acidic protein
-ir	-immunoreactive
KO	knockout
MEA	medial amygdala
MePD	posterodorsal aspect of the medial amygdala
MePV	posteroventral aspect of the medial amygdala
MHC	major histocompatibility complex
MOB	main olfactory bulb
MOE	main olfactory epithelium
MPOA	medial preoptic nucleus
mya	million years ago
NAcc	nucleus accumbens core
NGS	normal goat serum
NWM	New World monkeys
OT	optic tract
OVEX	gonadectomized (female)

OWM	Old World monkeys
PBS	phosphate buffered saline
PBS-GT	phosphate buffered saline with 0.3% triton and 0.1% gelatin
PCR	polymerase chain reaction
PD	postnatal day
PMCO	posteromedial cortical amygdaloid nucleus
PVN	paraventricular nucleus
SEM	standard error of the mean
SSSB	same-sex sexual behavior
T	testosterone
TRPC2	transient receptor potential cation channel 2
VMH	ventromedial hypothalamus
VMHdm	ventromedial hypothalamus dorsomedial subdivision
VMHvl	ventromedial hypothalamus ventrolateral subdivision
VNE	vomeronasal organ neuroepithelium
VNO	vomeronasal organ
VR	vomeronasal receptor
VSN	vomeronasal organ sensory neuron
WT	wild type

CHAPTER 1: INTRODUCTION

Pheromones and rodents

Social communication and behavior is facilitated by chemicals in many species (Chamero, Leinders-Zufall, & Zufall, 2012; Keverne, 2002a, 2002b; Stowers & Spehr, 2015; Woodley, 2014). Chemicals released by one animal to trigger a behavioral or physiological response in another are called pheromones (Stowers & Kuo, 2015). Pheromones themselves represent a diverse array of complex molecules. There are heavy protein pheromones, such as the major urinary proteins produced by males (Chamero et al., 2007), and volatilized pheromones, such as (Z)-5-tetradecen-1-ol (Yoshikawa, Nakagawa, Mori, Watanabe, & Touhara, 2013). Many of these protein and steroid pheromones are sex-specific and/or related to circulating hormone levels (Chamero et al., 2007; Korzan, Freamat, Johnson, Cherry, & Baum, 2013; Roberts et al., 2010; Wysocki & Lepri, 1991) providing general social information for each conspecific. For example, major urinary proteins typical of adult males depend on high circulating testosterone (Chamero et al., 2007). In contrast, major histocompatibility complex peptide 1 (MHC) pheromones are non-volatile (Rajendren & Dominic, 1984) and specific to each mouse. The MHC is produced based on individualized genetic components related to immunological function and signals aspects of relatedness to avoid inbreeding in wild populations (Peele, Salazar, Mimmack, Keverne, & Brennan, 2003). The detection and sending of these chemicals provides vast amounts of information regarding their social environment, e.g. territories, conspecific presence, reproductive status of males/females (Kaur et al., 2014; Lisa Stowers & Liberles, 2016). Pheromones play a critical role in the life history of rodents.

Vomeronasal organ and main olfactory organ detection of pheromones

Pheromones are largely detected by the vomeronasal organ (VNO) (Hasen & Gammie, 2009; Keverne, 2002a; Meredith & Fewell, 2001). The VNO is a blind-ended pouch located ventrally in either nasal passage (Stowers & Spehr, 2015). The medial side of the pouch contains vascular tissue which gates access to the VNO lumen and provides pumping action to draw chemicals in (Keverne, 1999). The other side contains bipolar VNO sensory neurons (VSN), a single dendrite from each VSN ends in a microvilli structure extending into the VNO lumen (Døving & Trotier, 1998). The VNO may be separated into two regions, apical and basal, based on g-protein and vomeronasal receptor (VR) expression. Apical

VSNs express the V1R group of receptors and their transduction pathway involves the use of Gαi2 g-protein (Norlin, Gussing, & Berghard, 2003; Oboti et al., 2014). Basal VSNs express V2R and the Gαo transduction molecule (Chamero et al., 2011; Oboti et al., 2014). Both V2Rs and V1Rs g-coupled receptors are formed by separate multigene families (Francia, Pifferi, Menini, & Tirindelli, 2014; Xia, Broad, Emson, & Keverne, 2010; Young, Massa, Hsu, & Trask, 2010) allowing the detection of many ligands. Detection of a pheromone by a VR leads to cytosolic release of diacylglycerol which activates TRPC2 and allow an influx of calcium and other cations, calcium activated chloride channels then allow efflux of chloride leading to depolarization and an action potential (Kim et al., 2012; Kiselyov, van Rossum, & Patterson, 2010). The axons of VSNs terminate exclusively in glomeruli of the accessory olfactory bulb (Omura & Mombaerts, 2014; Rodriguez, Feinstein, & Mombaerts, 1999; Yoshihara et al., 1997). This accessory olfactory circuit exists in parallel to another chemical detections system, the main olfactory system.

Some pheromones are detected in the main olfactory system by the main olfactory epithelium (MOE) (L. Stowers & T. H. Kuo, 2015). Both volatile and non-volatile pheromones are detected by the MOE, like trimethylamine and MHC respectively (Stowers & T. H. Kuo, 2015), although, data suggests the VNO is important for MHC detection too (Bellringer, Pratt, & Keverne, 1980). Trimethylamine and other volatilizable pheromones are used to attract females to male urine (Yoshikawa et al., 2013; Zhang, Block, & Katz, 2005) while MHC provides individual recognition (Leinders-Zufall et al., 2004). Unlike the VR in the VNO, trace-amine associated receptors in MOE neurons are thought to bind to pheromone molecules (Liberles & Buck, 2006) but pheromone reception in the MOE is less understood compared with the VNO. General activation of MOE receptors leads to downstream activation of g-protein G_{olf} and adenylyl cyclase second messenger systems (Norlin & Berghard, 2001; Wang et al., 2006). It is known that the transduction molecule TRPM5 helps to produce the electrical signal to the main olfactory bulb (MOB) for pheromones specifically (Lin, Margolskee, Donnert, Hell, & Restrepo, 2007). Neurons that detect pheromones in the MOE activate several glomeruli on the ventral aspect of the MOB (Kang, Baum, & Cherry, 2009) which then sends main olfactory signals further into the brain, in pathways separate from the accessory olfactory circuit (Scalia & Winans, 1975). The main and accessory olfactory systems work together to detect pheromones.

Vomeronasal brain circuit:

The VNO utilizes a specialized brain circuit to alter behavior and physiology. Relevant pheromonal information first detected in the VNO is sent to the AOB. Information from the apical and basal VNO are segregated into the anterior and posterior glomeruli of the AOB respectively (Jia & Halpern, 1996). Mitral-tufted cells connect to specific populations of these glomeruli and transfer information to several downstream brain regions (Binns & Brennan, 2005; Salazar & Brennan, 2001). The mitral-tufted cell axons end in several nuclei termed the accessory olfactory cortex (AOC); the bed nucleus of the accessory olfactory tract (BAOT), bed nucleus of the stria terminalis (BNST), medial amygdaloid nuclei (MEA) and posteromedial cortical amygdaloid nucleus (PMCO) (de Olmos, Hardy, & Heimer, 1978; Salazar & Brennan, 2001; Scalia & Winans, 1975). The segregated information from the anterior and posterior AOB converge throughout the entire AOC (Von Campenhausen & Mori, 2000). The AOC processes social information then sends signals to the hypothalamus to initiate appropriate behavioral or physiological responses (Chamero et al., 2012; Insel & Fernald, 2004). For example, the ventromedial hypothalamus (VMH) receives signals from the MEA (Kevetter & Winans, 1981; Petrovich, Canteras, & Swanson, 2001) which may initiate behavior like aggression and lordosis (Pfaff & Sakuma, 1979a) via the periaqueductal grey (Lonstein & Stern, 1998). Physiological responses to pheromones, like increased testosterone in the presence of a receptive female, are due to mediobasal hypothalamic gonadotropin releasing hormone neurons receiving signals from the MEA (Wersinger & Baum, 1997). Detection of pheromones can lead to behavior and physiological changes through AOC activity.

Pheromones influence rodent behavior:

Rodents may respond to the pheromones in a scent mark, such as urine, or to the presence of pheromones on a conspecific by expressing specific behaviors. Urine marking provides wild rodents with the ability to communicate territories and conspecific status. The interplay between a naïve female mouse and male urine provides a well-studied example of a stereotyped olfactory signaling event. While the volatile components of urine are unique to each male, it is the novelty of the scent which prompts initial investigations by females (Todrank & Heth, 2003), leading the mouse to draw close enough that non-volatile compounds may be detected. A female mouse in contact with a male urine mark will be able to detect the compound darcin, a major urinary protein produced in the liver. This pheromone activates the

female hippocampus (Hoffman, Pickavance, Thippeswamy, Beynon, & Hurst, 2015) leading to associative learning (Hoffman et al., 2015; Roberts et al., 2010). At the scent mark, non-volatile major urinary proteins (Kaur et al., 2014) and major histocompatibility complex class I peptides (Leinders-Zufall et al., 2014) provide the female with socially relevant information regarding the male that deposited the urine (Roberts et al., 2010), such as the degree of relatedness. Up to this point, all components of the male urine have been attractive due to their novelty. It is only after direct contact with darcin that females can produce the appropriate behavioral paradigm in response to the volatile pheromones that initially led her to the scent mark. Females will become attracted to the volatile pheromones of unrelated males or repelled by highly-related male urine (Moncho-Bogani, Martinez-Garcia, Novejarque, & Lanuza, 2005). In short, closer investigation of novel volatile pheromones allows non-volatile pheromones, only detectable at close proximity, to assign taxic value to the previously identified volatiles. In the wild, this helps females avoid inbreeding.

Pheromones become associated with aversive or attractive stimuli and take on new meaning based on developmental and environmental interactions (Pankevich, Cherry, & Baum, 2006; Stowers & Kuo, 2015). Like females, experience with pheromones plays an essential role in male behavioral development. For male mice, female pheromones are pro-copulatory and male-pheromones inhibit copulation and induce aggression (Connor, 1972) but the development of this behavior is more nuanced. In rats, c-fos, an indication of cell activation, and dopamine levels in the nucleus accumbens (NAcc) core are elevated by exposure to receptive female urine in experienced, but not naïve, males. So, alterations in NAcc activation may be involved with changes to experienced male behavior. Mice given prior experience with pheromones before using a pheromone to induce a c-fos response also show a VNO-dependent increase in NAcc activation (Pankevich et al., 2006). While pheromones themselves can alter brain activation, they are often paired with other socially relevant stimuli. For example, a male detecting urine from another male may simply countermark unless the other male is physically present, in which case he will display aggressive behaviors (Maruniak, Wysocki, & Taylor, 1986; Novotny, Harvey, Jemiolo, & Alberts, 1985). So, previous pheromone exposure and non-pheromonal environmental cues interact to alter mouse behavior. Still, other aspects of mouse biology, beyond behavior, are altered by pheromones themselves.

Pheromones influence rodent physiology:

Pheromonal contributions to behavior may coincide with physiological responses as well. Perhaps one of the more striking examples is the “Bruce effect”, where a novel male scent delays embryo implantation, inducing spontaneous abortion, in mated females (Lloyd-Thomas & Keverne, 1982). This physiological process occurs via individual identity detection utilizing the MHC (Thompson, McMillon, Napier, & Wekesa, 2007). This large non-volatile pheromone derives its structure from a region of DNA that shows high allelic diversity within populations and is involved with immunological responses. These compounds allow female mice to preferentially seek out males with a greater number of heterozygous alleles (Leinders-Zufall et al., 2014). Female development may also be affected by pheromonal intervention. In wild mouse populations, calorically-challenged females release 2-heptanone in their urine which delays puberty in prepubertal pups (Massey & Vandenbergh, 1980; Stowers & Kuo, 2015) while males secrete 6-hydroxy-6methyl-3-heptanone which accelerates puberty in juvenile females (Flanagan, Webb, & Stowers, 2011; Mucignat-Caretta, Caretta, & Cavaggioni, 1995). Physiology may also be altered in males, the VNO is necessary to induce rapid release of gonadotropin releasing hormone in response to female pheromones (Coquelin, Clancy, Macrides, Noble, & Gorski, 1984), which leads to increased circulating testosterone levels (Wysocki, Katz, & Bernhard, 1983). Such changes to physiology may prime rodents for reproductive or antagonistic interactions.

Sex differences in rodent CNS and behavior

Sex differences in the rodent accessory olfactory system:

Sex differences exist within the neural circuitry underlying the accessory olfactory system. These differences are implicated in the sex-typical behaviors of mice. A significant amount of work has gone into describing the sex differences which are present in the VNO itself (Alekseyenko, Baum, & Cherry, 2006; Baum, 2009). Starting at the molecular level, gonadal hormones modulate the levels of particular vomeronasal receptor (VR) types, including the large V2R subfamilies V1R and V4R., Males express more V1R and V4R receptors than females and treating males with estradiol reduces the number of V1R. Conversely, treating castrated males with testosterone increases V4R receptors (Alekseyenko et al., 2006). Interestingly, the male bias in V1R appears in the caudal portion of the VNO (Alekseyenko et al.,

2006). Moreover, the VNO shows sex differences in the expression of estrogen receptor α and β and estrogen producing enzymes (Cherian, Lam, McDaniels, Struziak, & Delay, 2014), suggesting steroid hormones drive sex differences by acting directly on the VNO. Indeed, estradiol rapidly modulates VSN responses to female urine in both males and females (Cherian et al., 2014). Sex differences in circulating estradiol levels could lead to sex differences in VNO function. The different pheromone sensitivities seen across the estrous cycle are likely due to altered hormone levels, a process that does not occur in males (Dey et al., 2015). Circulating steroid hormone levels and the VNO interact, allowing varying physiological and behavioral responses to VNO activation between males and females (Halem, Baum, & Cherry, 2001). Activation of VSNs varies between sexes because sex differences in hormone levels alter urine-induced currents in the VNO (Alekseyenko et al., 2006; Kang, Janes, Baum, & Cherry, 2006). The sex differences in VR expression and VSN activation suggest signaling from the VNO is sexually differentiated. Indeed, c-fos activation in the accessory olfactory bulb (AOB), which receives information directly from the VNO, is sexually dimorphic in response to various stimuli (Halem et al., 2001; Halem, Cherry, & Baum, 1999). Reproductive status may also modulate the response to the same stimulus, for example, a novel males scent can be attractive to a reproductive female (Roberts et al., 2010; Todrank & Heth, 2003) but initiate aggressive behavior in lactating dams (Chamero et al., 2011). Because sex differences exist in the mouse VNO anatomy and physiology, downstream brain structures receive sexually differentiated signals. Continued processing within sexually dimorphic brain circuitry may lead to further sex-specific modifications. The differences between the male and female AOC might be one mechanism producing sexually dimorphic behaviors.

Posterodorsal aspect of the medial amygdala (MePD):

The MePD is a region involved with social recognition (Choleris et al., 2007) and exerts influence over sexual behavior based on direct input from the VNO (Harris & Sachs, 1975; Wood & Newman, 1995). Lesions here reduce male copulatory behaviors but have very little effect on aggression (A. McGregor & J. Herbert, 1992). Similar to the BNST, the MePD is important for sexual partner preference (Adekunbi et al., 2018). The MePD is profoundly sexually differentiated in rats, with adult males having a much larger MePD than adult females (Cooke, Tabibnia, & Breedlove, 1999). Substantial work has gone into understanding the cellular basis of this sex difference in regional volume. Morphometric analyses

reveal that the rat MePD contains more neurons and astrocytes in males than in females (Johnson, Breedlove, & Jordan, 2008; Morris, Jordan, & Breedlove, 2008). MePD neurons and astrocytes are also sexually differentiated in size, with larger neuronal somata and more extensive astrocytic processes in males than females (Cooke, Breedlove, & Jordan, 2003; Cooke et al., 1999; Johnson, Schneider, DonCarlos, Breedlove, & Jordan, 2012; Morris, Jordan, & Breedlove, 2008). Interestingly, the MePD is also highly lateralized in rats, with both males and females having more MePD neurons in the left hemisphere than in the right (Morris, Jordan, & Breedlove, 2008). The number of astrocytes and the complexity of their arbors is also highly lateralized, with more MePD astrocytes in the right hemisphere and more complex astrocytes on the left side (Johnson et al., 2008; Johnson et al., 2012). Consequently, sex differences in astrocytes are also highly lateralized, a sex difference in astrocyte number is seen only on the right side and sex difference in arbor complexity is found on the left (Johnson et al., 2008).

The MePD in C57Bl/6J mice is both sexually dimorphic and lateralized, it is larger in regional volume in males compared with females and both male and female C57Bl/6J mice have a larger left MePD (Pfau, Hobbs, Breedlove, & Jordan, 2016). In BALB/c mice, the MePD is larger in males than females, however, lateralized in volume only in females. Male soma are larger than females in the BALB/c strain and, unlike regional volume, male-typical soma size requires high circulating testosterone levels (Morris, Jordan, King, Northcutt, & Breedlove, 2008). The MePD of male C57Bl/6J mice contains more astrocytes and neurons than females, also in males, left hemisphere soma are larger. In females, the left MePD has more astrocytes. These cellular contributions to morphology may influence laterality and sex differences seen in volume. (Pfau et al., 2016). These analyses also indicate that strain and species influence MePD sex and laterality differences (Cooke et al., 1999; Morris, Jordan, King, et al., 2008; Pfau et al., 2016). How these sex differences develop has been examined as well. In rats, the sex difference in neuron number is independent of circulating levels of hormones but regional volume and soma size are larger in intact males and gonadectomized females (Morris, Jordan, & Breedlove, 2008). In mice, the sex difference in volume and soma size is lost after castration in male mice while in females, gonadectomy masculinizes the volume and soma size (Morris, Jordan, King, et al., 2008). While sex differences and laterality exists in the morphology of the mouse MePD (Pfau et al., 2016), it is unknown

what guides this during development. Still, the influence of sex hormones and chromosomes induces changes to the overall medial amygdala (Vousden et al., 2018).

Ventromedial hypothalamic nucleus ventrolateral subdivision (VMHvl):

Neurons within the VMH regulate many behaviors but those within the ventral lateral VMH (VMHvl) specifically are involved with male and female sexual behavior (Mohedano-Moriano et al., 2007; Yang et al., 2013). The VMHvl is involved with the hormonal response to pheromonal signals (S. Wersinger & Rissman, 2000) and exerts control over receptivity (Pfaff & Sakuma, 1979a, 1979b) through progesterone receptor expressing neurons (Yang et al., 2013). Like females, the VMHvl regulates male sexual behavior, specifically their ability to mount intruder females (Lee et al., 2014). Interestingly, increasing activation of the same populations of cells that produce mounting can induce antagonistic interactions with an intruder (Falkner, Grosenick, Davidson, Deisseroth, & Lin, 2016; Lee et al., 2014). However, the VMHvl does not appear to be involved with female aggression (Yang et al., 2013). Aggressive circuitry in males starts to develop when synapses within the VMHvl begin to change from GABAergic to glutamatergic, a process facilitated by astrocytes (Chen, Trombley, & van den Pol, 1995). However, it is activation of GABA neurons that facilitates aggression in adults (Hong, Kim, & Anderson, 2014). Interestingly, GFAP expression density, a measure which may represent astrocyte number or complexity, is increased in the hypothalamus after androgen receptor loss (McQueen, Wright, Arbuthnott, & Fink, 1990), suggesting they may have a sex-typical function in the region or influence the development of sex differences (McCarthy, Todd, & Amateau, 2003). Female rats appear to show a relatively higher number of neurons and more neurons expressing specific hormone receptors when compared with males (Patisaul, Fortino, & Polston, 2007) but a full stereological analysis has not been performed. In mice, the neurons which express the estrogen receptor 1 are more numerous in females (Yang et al., 2013) but an examination of the entire neuron population has not been carried out.

Sex differences in behavior:

Like their brains, rodent behavior shows many sex differences. Some of these are based on the disparate roles taken by female and male mice i.e. male-male aggression, mounting and intromission for males, lordosis, pup rearing and maternal aggression for females. Aggressive and reproductive behaviors in adult males rely on the sex-typical levels of endogenous testosterone (Edwards, 1969; Luttge & Hall,

1973) seen 30 days postnatally (Barkley & Goldman, 1977). While a naïve WT male will mount receptive females, nonreceptive females, and very rarely mount other males, experience with a receptive female induces a change. WT males exposed to receptive females decrease mounting of nonreceptive females and males (Hayashi & Kimura, 1974; Pankevich, Baum, & Cherry, 2004; Wysocki & Lepri, 1991). Aggressive behaviors displays a similar change after experience; naïve WT males aggress towards intruder males and aggressive behavior is increased after aggressive experiences (Lagerspetz & Hautojärvi, 1967). Females show unique behaviors as well. Female mice display sexual receptivity in adulthood based on hormone cycles; endogenous estradiol (Rissman, Early, Taylor, Korach, & Lubahn, 1997) and progesterone levels (Edwards, 1970). They also display maternal aggression while lactating (Hasen & Gammie, 2009). Hormones and experience interact to induce pregnancy via sex-specific behaviors.

Many aspects of these sex-typical behaviors rely on the VNO for both organization and activation. Removal of the VNO in adulthood reduces male-male aggression and sexual behaviors but sexual experience prior to ablation of the VNO can prevent sexual behavior loss. Male aggression is directed at other males, which they will chase, attack and bite while they perform olfactory investigations on and mount intruder females (Wysocki & Lepri, 1991). So, activation of the VNO in later adulthood appears dispensable after behavioral paradigms are organized in naïve adults. Once developed, activation of this VNO circuit is powerful enough to induce mounting of a male intruder swabbed with female urine (Connor, 1972). Female mice will display aggression towards males as well but only when nursing (Joseph S Lonstein & Gammie, 2002) and VNO removal eliminates maternal aggression (Wysocki & Lepri, 1991). Aspects of female reproduction involve the VNO, such as the ability to shift the estrous cycle based on detection of male pheromones (Bronson & Whitten, 1968). Females also use pheromones in the development of mate recognition (Oboti et al., 2011). The ability to detect their mate allows lactating females to direct maternal aggression towards novel males (Baum, 2012). Many experiences which influence the expression of sex-typical behaviors involve the detection of pheromones through the VNO.

TRPC2

TRPC2 expression and function:

Activation of VNO pheromone receptors (e.g. V2Rs) causes release of Gαi2 or Gαo g-proteins into the cytoplasm. These g-proteins lead to DAG production which interacts with the l transient receptor potential cation channel 2 (TRPC2). Activation of TRPC2 allows cations in the cell, mainly Ca 2+. Calcium activates chloride channels and the resulting efflux of chloride depolarizes the cell (Kim et al., 2012; Kiselyov et al., 2010) This results in activations of several channels which lead to depolarization of the cell and the propagation of action potentials (Kim et al., 2012). Signal regulation can occur through Ca 2+-calmodulin interactions with TRPC2 (Spehr et al., 2009). In the VNO, TRPC2 is expressed by embryonic day 14.5 (Omura & Mombaerts, 2014) into adulthood (Stowers, Holy, Meister, Dulac, & Koentges, 2002) and this system is open and functioning at birth (Hovis et al., 2012). This ion channel may assist in the production of VNO signals throughout development into adulthood.

Extra-VNO tissues have been shown to express TRPC2 as well. TRPC2 mRNA appears in the mouse endometrium (De Clercq et al., 2017), testis (Hofmann, Schaefer, Schultz, & Gudermann, 2000; Yildirim, Dietrich, & Birnbaumer, 2003), erythroid cells (Chu et al., 2004). Some have suggested a splice variant of TRPC2 has also been found in the brain but this product cannot form a channel and has not been found in other studies (Chen et al., 2017; Hofmann et al., 2000; Yildirim et al., 2003). Expression in sperm is related to the acrosome reaction in mice (Jungnickel, Marrero, Birnbaumer, Lémos, & Florman, 2001; Stamboulian et al., 2005), and TRPC2 appears important for thyroid function in rats (Törnquist, Sukumaran, Kemppainen, Löf, & Viitanen, 2014).

TRPC2 KO mouse behavior:

TRPC2 KO mice display a variety of behavioral differences from WT mice. TRPC2 KO females are capable of mounting both male and female intruders (Kimchi, Xu, & Dulac, 2007) but lack maternal aggression (Hasen & Gammie, 2009). Such males take on a subordinate role when paired with WT males (Leypold et al., 2002). TRPC2 KO males also lack aggression (Stowers, Holy, Meister, Dulac, & Koentges, 2002) due to the loss of aggressive seeking behavior (Falkner et al., 2016). Like TRPC2 KO females, KO males are also well-known for expressing same-sex sexual behavior (Leypold et al., 2002). Despite these differences, some aspects of their sexual behavior remain intact, such as ultrasonic

vocalizations and male-female copulatory behavior (Kimchi et al., 2007; Stowers et al., 2002). Overall, TRPC2 KO reduces aggression while increasing and augmenting their sexual behavior. In 2007, Kimchi et al., removed the VNO from female mice and found they displayed the same male-typical mounting behaviors as TRPC2 KO females. Based on this result they offered a VNO-centric model and suggest the VNO is responsible for inhibiting mounting circuitry already present in females. Still, others feel this VNO-centric view of TRPC2 KO behavior has limited our understanding of the model and extra-VNO expression requires more examination (Yu, 2015). TRPC2 expression in the VNO *and* outside may provide mechanisms leading to sex-typical adult sexual and aggressive behaviors. A VNO-centric model implies removing the peripheral portion of the AOC, the VNO, is sufficient to induce the same behaviors expressed by TRPC2 KO mice, i.e. the VNO is gating activation of sex-typical circuitry present in both sexes. However, another group could not replicate male-typical mounting in females after VNO removal (Martel & Baum, 2009). Therefore, the mechanism by which TRPC2 influences adult behaviors cannot be limited to VNO function in adulthood. TRPC2 expression and influence outside the VNO may drive the appearance of sex-typical sexual and aggressive behaviors in adult mice.

Overview of dissertation chapters

The olfactory system is essential for the expression of murine social behavior. Both the main and accessory systems work together to produce behavioral and physiological responses to social cues. TRPC2 is an important signaling molecule for the accessory olfactory system and is expressed from pre-natal development into adulthood. Mice lacking TRPC2 show drastic changes in behavior. I hypothesized that TRPC2 is essential for sex-typical brain circuitry and behavior.

In Chapter Two I examine measures that are related to sex-typical development and TRPC2 expression within and outside the VNO to determine how the KO affects growth and reproduction. I find several differences in KO development and reproductive deficits which may challenge the VNO-centric model of TRPC2 studies. In chapter three I examine the hypothesis that TRPC2 KO affects more than just the VNO by examining brain structures related to the sex-typical behaviors altered in KOs. Consistent with my hypothesis, the MePD and VMHvl of male and female mice were altered after TRPC2 KO. In chapter four I investigate whether sexual behavior can overcome altered VNO function through extended

exposure to environmental cues known to alter male mouse behavior. After additional experience with relevant cues, the behavior of TRPC2 KOs does not conform to typical mice. Finally, in Chapter five I discuss the implications of TRPC2 loss and KO behavior on primate evolution and overall findings.

CHAPTER 2: TRPC2 KNOCKOUT MICE SHOW DEVELOPMENTAL DELAYS AND REDUCED FERTILITY

Abstract

The transient receptor potential cation channel 2 (TRPC2) is a key transducer of pheromonal information in the vomeronasal organ (VNO). TRPC2 expression is high in the VNO but also found in extra-VNO locations, notably the testis, sperm and endometrium. Prior studies focused on the altered social behaviors of TRPC2 knockout (KO) mice with little attention to whether TRPC2 also affects reproduction and development. We addressed this gap, measuring several reproductive outcomes in homozygous KO mice. We report that loss of TRPC2 leads to severe reproductive deficits, including increased postnatal mortality, although maternal experience reversed this effect. We also find increased latencies to parturition and delayed puberty for both sexes, but delayed puberty occurred only for second and not first KO litters from the same dams. Crossing KO mice with WT mice reversed the defects in reproductive success and parturition length. Circulating testosterone level and anogenital distance was comparable in KO and WT mice, arguing against a role for testosterone in producing these effects. These results bear important considerations for future studies utilizing the TRPC2 KO mouse model and stress the need for understanding if and how altered reproduction and prepubertal development contribute to changes in adult behavior.

Introduction

The transient receptor potential cation channel, subfamily C, member 2 (TRPC2) (Francia et al., 2014; Stowers & Spehr, 2015) is a critical transducer of pheromone signaling in the vomeronasal organ (VNO), a sensory structure in the nasal passage specialized in detecting conspecific pheromones (Kiselyov et al., 2010; Stowers & Spehr, 2015). Under specific social conditions, the VNO can trigger aggressive (Bean & Wysocki, 1989; Chamero et al., 2011; Clancy, Coquelin, Macrides, Gorski, & Noble, 1984) and sexual behaviors (Kimchi et al., 2007; Leypold et al., 2002; Wysocki & Lepri, 1991) in mice. These same behaviors are altered in mice lacking a functional TRPC2 gene (KO) (Kimchi et al., 2007), making it likely that TRPC2 in the VNO mediates these effects. However, TRPC2 mRNA is also found in other tissues, such as sperm (Jungnickel et al., 2001), where it is involved with fusion of the egg and

sperm (Jungnickel et al., 2001), the testis itself (Yildirim et al., 2003) , and the uterine endometrium (De Clercq et al., 2017). Such data raise the question of whether TRPC2 has broader effects than currently recognized, potentially affecting reproductive competence and maturation of TRPC2 KO mice, which may ultimately contribute to shaping adult behavior.

Besides the fact that TRPC2 is expressed in tissues critical for reproduction, aspects of VNO function suggest TRPC2 affects reproduction and development of progeny. For example, urine detection by the VNO, which is severely disrupted after TRPC2 loss (Hasen & Gammie, 2011), exerts powerful control over female developmental rates. Urine from calorically-challenged females delays puberty (Massey & Vandenbergh, 1980) while urine from a mature male accelerates puberty in juvenile females (Kaneko, Debski, Wilson, & Whitten, 1980; Mucignat-Caretta et al., 1995). Losing signals that facilitate development, such as those controlling pubertal onset timing, may lead to differences between KO and WT mice development. Organization of reproductive behaviors in males depends on the VNO detecting female pheromones. A naïve WT male will mount both receptive and nonreceptive females (Hayashi & Kimura, 1974), but with sexual experience, mounting of nonreceptive females decreases, as long as the VNO is intact (Wysocki & Lepri, 1991). Reproductive capacity is also likely influenced by pheromones (Connor, 1972), given that male mouse pheromones accelerate the estrous hormone cycle in females (Bronson & Whitten, 1968), whereas detecting female urine increases circulating testosterone levels in males (Coquelin et al., 1984). With such a complex interplay between pheromones, reproduction and development in rodents, it is likely that aspects of reproduction and development would be altered in TRPC2 KO mice.

The existing literature suggests that TRPC2 KO mice reproduce and develop normally (Stowers et al., 2002). However, while producing KO and WT mice in our colony, differences in fertility seemed obvious, prompting the current study to systematically measure several outcomes related to reproductive success and development of KO offspring. We find that breeding pairs of homozygous KO male and female mice displayed severe deficits in fertility, including increased latency to parturition. These deficits were reversed by crossbreeding KO mice with WTs, regardless of KO sex. Gene KO influenced prepubertal body weight and pubertal timing for both males and females, with some effects depending on whether progeny were from the first or second litter of the same breeding pairs. These data implicate

TRPC2 in murine reproduction and development that could also ultimately contribute to the effects of the KO on adult behavior.

Material and methods

Breeding methods:

Homozygous TRPC2 KO (B6;129S1-Trpc2tm1Dlc/J JAX stock number: 021208) mice and homozygous TRPC2 WT (B6129SF2/J021208; JAX stock number: 101045) mice were obtained from Jackson labs and bred to maintain homozygosity. PCR was used to confirm genotypes and homozygosity from ear clip samples taken upon weaning. Mice were paired at 56 to 87 postnatal days (PD) of age (n=8 WT pairs, n=13 KO pairs). Males were singly caged for one week prior to introducing the female into his cage. After two weeks, paired mice were singly housed. Females were re-paired with the same male one week after separation if not visibly gestating. Females visibly gestating were checked daily for parturition. On the day of pup birth, the number of days from first day of pairing to parturition (referred to as "time to reproduce") and number of newborn pups were noted. At 21 days after birth, litters were weaned, noting the number of offspring at this age, their sex and body weight. A week after weaning, dams were re-paired with the same breeder male.

Crossbreeding experiment:

Proven breeder WT male and female mice were paired with sexually naïve homozygous TRPC2 KO female or male mice (PD 56 to 87, n=7/sex). Mice were singly housed after two weeks of paired housing. All visibly gestating females were checked daily and the day of parturition noted. Pups were removed on the day of birth, counted, sacrificed and a tail sample collected for PCR to identify pups that carried the SRY gene, which allowed us to determine sex ratios within the litters of newborn heterozygous pups. Anatomical data was collected on all adult mice used in this crossbreeding experiment. Upon sacrifice, a blood sample was collected for serum testosterone (T) analysis and the testes, seminal vesicles, ovaries and uterine horns of the successful and unsuccessful breeders were weighed.

Peripubertal measures in homozygous KO and WT mice:

At weaning, the anogenital distance, a biomarker of T influence during development (Ryan & Vandenbergh, 2002) and body weight were measured for each offspring. They were then group-housed

with same-sex littermates. Weanlings were checked daily to determine a proxy for pubertal onset, vaginal opening for females and preputial sheath separation for males (Hoffmann, 2018). Vaginal opening was determined by examining the cornified tissue that seals the vagina. With gentle downward movement of the clitoris, a closed vagina is characterized by little movement of the clitoris and the presence of cornified tissue keeping the vagina closed whereas greater movement of the clitoris and the presence of a gap in vaginal tissue (glabrous tissue) indicated vaginal opening. Preputial sheath separation was determined by gently palpating both sides of the penis. An intact sheath would remain covering the penis while a separated sheath slides down from the tip of the penis.

Adult testosterone (T) levels:

Adult KO and WT mice (PD 56-87) produced during the colony breeding experiment were sacrificed. Their weight was noted and at least 300 µL of whole blood was placed into a 1.5 mL tube (previously filled with 250 µL of heparin then dried) then placed into crushed ice until centrifugation for measurements of plasma T. For the crossbreeding experiment, blood was collected from breeders and placed into a 1.5 mL tube and allowed to coagulate on crushed blue ice until centrifugation for measurements of serum T. After centrifuging, plasma or serum was collected and 150 µL sent to the Diagnostic Center for Population and Animal Health at Michigan State University (https://www.animalhealth.msu.edu/) to perform T immunoassay (Minimum detectable level: 0.7 nmol/l; Intra-assay CV: 11%).

Statistical analysis:

Mann-Whitney nonparametric analysis was used to assess the effects of genotype on breeding success. Mixed-design ANOVA was used to examine effects of breeding experience (first versus second pairing as a within-subjects factor) and genotype (between-subjects factor) on latency to parturition (number of days from day of pairing to day of parturition), litter size at birth, pup loss, sex ratio at weaning, and body weight at weaning. The day of sheath separation and vaginal opening were analyzed separately using Kaplan Meier analyses (providing a X^2 value), multiple tests required splitting the alpha across three analyses. AGD, adult body weights and plasma T levels were analyzed using a two-way ANOVA with genotype and sex as between subject factors. A one-way ANOVA was used to examine the effect of genotype on latency to parturition, litter size at birth, and sex ratio of newborns within litters (in

the cross breeding experiment) using posthoc Dunnett's analysis to compare reproductive outcomes from cross-bred pairs (WT male with KO female, and KO male with WT female) to that of WT pairs. Reproductive success of the three pairing types for the crossbreeding experiment was analyzed using a nonparametric Kruskal-Wallace test.

Results

TRPC2 KO reduces mouse fertility:

Loss of TRPC2 significantly reduced breeding success (U=6.58, p<0.001, d=4.84; fig 1A). Examining those pairs that produced progeny for at least one pairing indicated KO X KO pairings show fewer pregnancies than WT pairings (U=596.5, p<0.001; fig 1B). Pup loss, defined as the number of pups at birth minus the number at weaning, varied based on litter order ($F_{(1,20)}$=10.81, p=0.004, d=3.29) and genotype ($F_{(1,20)}$=10.81, p=0.004, d=3.29, fig 1C). Interestingly, mortality for KO pups was significantly higher (~3 fold) than for WT pups but only for the first and not the second litter produced from the same dams, resulting in a significant interaction between genotype and litter order number ($F_{(1,20)}$=11.85, p=0.003). Posthoc analysis with Bonferroni correction confirmed that significantly more pups are lost from first-time KO dams than for second (p<0.001, d=4.22). In contrast, loss of WT pups was negligible for both the first and second litters from the same dams (p=0.90). Latency to parturition was longer for KO dams than WT dams ($F_{(1, 17)}$=7.59, p=0.016, d=0.77; fig 1D) and was comparable for first and second litters ($F_{(1,13)}$=1.99, p=0.182), with no significant interaction ($F_{(1,13)}$=0.94, p=0.351). Some reproductive measures were unperturbed by the TRPC2 KO, including the size of litters at birth (table 1) and the sex ratio of weanling pups ($F_{(1, 17)}$=0.47, p=0.504; table 1). Pups raised to adulthood showed a sex difference in weight with males weighing significantly more than females ($F_{(1,52)}$=61.72, p<0.001, d=7.86), as expected, but no effect of genotype ($F_{(1, 52)}$=0.153, p=0.697) nor a significant genotype by sex interaction ($F_{(1, 52)}$=0.079, p=0.78). At weaning, KO pups weighed significantly less than WT pups ($F_{(1,112)}$=74.28, p<0.001, d=17.24; fig 1E), with no sex difference ($F_{(1,112)}$=1.94, p=0.167) evident at this age. A significant effect of litter order on body weight was found ($F_{(1, 112)}$=11.95, p=0.002) with a significant interaction between litter and genotype ($F_{(1,112)}$=4.01, p=0.048). Pup weight at weaning was greater in second litters (p<0.001, d=2.89; fig 1E) but only for WT, and not KO mice (p=1.00).

KO pups show developmental delays:

While there was no overall effect of genotype on pubertal timing when data was collapsed across litters (females: X^2=1.3, p=0.25, adjusted α=0.017 and males: X^2=4.92, p=0.027, adjusted α=0.017; fig 2A, B), after examining age of preputial sheath separation and vaginal opening separately for first and second litters, we found that the pattern of genotype differences were opposite across litters (fig 2C, D). In first litters, KO females and males reach sheath separation and vaginal opening earlier than WTs (females: X^2=14.04, p<0.001, adjusted α=0.017 and males: X^2=6.403, p=0.011, adjusted α=0.017; fig 2E, F). However, for second litters, KO mice showed delays in these proxies for pubertal onset (females: X^2=26.9, p<0.001, adjusted α=0.017 and males: X^2=18.15, p<0.001, adjusted α=0.017; fig 2G, H).

Pairing KO with WT mice rescues reproductive deficits:

Pairing WT with KO mice of either sex (WT male X KO female or WT female X KO male) rescued the reproductive success of KO mice (X^2=0.204, p=0.903; fig 3A). Latency to parturition ($F_{(2, 20)}$=1.075, p=0.362; fig 3B), number of pups at birth ($F_{(2, 19)}$=0.059, p=0.943) and sex ratios ($F_{(2, 19)}$=0.588, p=0.566; table 2) were all similar across pairing types. Anatomical measures from adults used for crossbreeding indicates successful female dams weighed more than unsuccessful females ($F_{(3, 20)}$=6.326, p=0.023; table 3) regardless of genotype ($F_{(3, 20)}$=0.238, p=0.632), and there was no interaction between success and genotype ($F_{(3, 20)}$=1.055 p=0.320). KO males had significantly smaller testis than WT ($F_{(3, 22)}$=7.095, p=0.016; table 3) regardless of reproductive success ($F_{(3, 22)}$=1.864, p=0.189) and with no interaction ($F_{(3, 22)}$=0.815 p=0.378). Other anatomical measures (ovarian, uterine horn and seminal vesicle weights) from crossbreeding pairs showed no differences (table 3).

Adult testosterone (T) levels are the same for WT and KO mice:

Plasma T levels in adult KO and WT mice showed the expected sex difference with males having significantly higher T levels than females ($F_{(1,49)}$=30.95, p<0.001, d=5.56; fig 4A). Genotype however did not affect T levels ($F_{(1,49)}$=0.481, p=0.492) nor was there an interaction ($F_{(1,49)}$=0.598, p=0.444). Serum T levels for mice used in the crossbreeding experiment also showed the same pattern, with a sex difference and no effects of genotype (data not shown). The AGD of both WT and KO weanlings showed the expected sex difference ($F_{(1,72)}$=327.26, p<0.001, d=1.62; fig 4B), with WT and KO males having

significantly longer AGDs than their respective females. There was no main effect of genotype on AGD ($F_{(1,72)}$=2.176, p=0.145) nor an interaction of genotype and sex ($F_{(1,72)}$=0.617 p=0.435).

Discussion

Focus on the transient receptor potential cation channel 2 (TRPC2) has largely centered on its critical function as a transducer of pheromonal signals in the VNO. Without TRPC2, mice show profound changes in sex-specific behaviors such as sex and aggressive behaviors, behaviors that are known to be heavily influenced by pheromonal cues. While effects on reproductive competence of homozygous KO mice are reportedly null (Lisa Stowers et al., 2002), we find otherwise, with significant defects in fertility. Fewer (~45% less) KO pairings result in progeny compared to WT pairings, a deficit that remains stable regardless of sexual experience. Day to reproduction (number of days from first day of pairing to birth) is also significantly longer for KOs than WT dams, independent of the number of litters that a given dam has had. An increase in the day to reproduction may indicate the dams had took longer to reproduce, it is also possible gestation was longer. KO pups show a higher mortality rate than WT pups during the preweaning phase, but notably, only for the first and not the second litter from the same pairing. Regardless, latency to parturition remains protracted. We also find a complex interaction between the effect of the KO with litter order on our proxy for pubertal timing, with pubertal onset occurring significantly earlier for KO mice than WT mice for both males and females of first litters. However, this pattern flips for second litters, with puberty beginning significantly later in KO males and females compared to WT mice. While we hoped that crossing homozygous KO mice with WT breeder mice would uncover the source of impaired fertility, we found that both sorts of pairing (KO male with WT females, or KO female with WT male) reversed deficits in reproductive success and on parturition latency. Finally, we find that circulating T levels are unaffected by genotype, contrary to earlier reports (Kimchi et al., 2007; Stowers et al., 2002). Clearly, the loss of TRPC2 has significant effects that encompass both the likelihood of conceiving, the survival of offspring and pubertal timing. Such factors may also contribute to the altered behavior of adult KO mice.

Despite robust changes in some aspects of TRPC2 KO male sex behavior (Kimchi et al., 2007; Leypold et al., 2002), they reportedly mount and intromit females at normal WT-levels (L. Stowers et al.,

2002) suggesting that reproductive deficits may be in the testes themselves. The TRPC2 protein is involved in the sperm acrosome reaction, which allows entrance into the egg (Jungnickel et al., 2001). Additionally, knocking out acrosin, another protein involved in the acrosome reaction, delays fertilization (Adham, Nayernia, & Engel, 1997) and thus, defects in the acrosome reaction caused by lack of TRPC2 in sperm may also be a contributing factor in the lower success rate or increased parturition latency of homozygous pairs. However, pairing TRPC2-/+ mice leads to the production of some KO offspring, suggesting TRPC2- sperm can compete with TRPC2+ sperm. TRPC2 expression is also lost in the endometrium of KO mice (De Clercq et al., 2017), which may influence reproductive success as well. Maternal communication with embryos and vice versa involve calcium signaling via TRP channels (De Clercq & Vriens, 2018; Dörr & Fecher-Trost, 2011). In TRPC2 KO animals, VNO neurons show a 40% reduction in calcium-activated channel openings (Kim et al., 2012; Yu, 2015) suggesting a large disruption of intracellular calcium signals wherever TRPC2 is normally expressed. Within the endometrium, TRPC2 mRNA is most prevalent within stromal cells (De Clercq et al., 2017) that are known to prepare the uterine lining for trophoblast attachment and mediate placental invasion (Gellersen, Brosens, & Brosens, 2007). Stromal cells also express TRPC6 (De Clercq et al., 2017) which interacts with TRPC2 (Chu et al., 2004) and may allow for adaptive responses during invasion. Calcium-activated channels that rely on TRPC2 in the VNO (Kim et al., 2012) are functionally expressed in the endometrium and placenta, promoting placental vascularization (Rada, Murray, & England, 2014) and endometrial development (Lu et al., 2014). Intracellular calcium changes also mediate responses to oxygen tension during pregnancy. Placental and endometrial adaptations to maternal blood oxygen tension through angiogenesis and metabolic changes rely on low oxygen sensing (De Marco & Caniggia, 2002). Concurrently, sensing low oxygen environments through olfactory circuits involves TRPC2 (Bleymehl et al., 2016). So, TRPC2 may have a conserved function in these transient reproductive organs where responses by the mother or embryo to changing oxygen levels are vital for maintaining the pregnancy. Because TRPC2 KO mice were paired for two weeks, defects in fertilization and/or maintenance of the pregnancy could each be contributing factors underlying the longer latency to parturition. While TRPC2-dependent mechanisms outside the VNO may influence reproduction, TRPC2 function in these regions remains unexamined.

However, it is already well-established that TRPC2 loss changes mouse sex behaviors (Stowers et al., 2002). Male KOs show the stereotypical courtship and copulatory behaviors of WT animals (Leypold et al., 2002) but pheromones typically guide these behaviors towards periods of maximum fertility. For example, experienced males will preferentially mount cohoused females during the females' fertile period, using changes in urinary pheromones as a cue (Wysocki & Lepri, 1991). Additionally, females are more receptive to sexual behavior during their fertile periods (Rissman et al., 1997). Synchronizing mounting behavior with receptive fertile periods is important because males exhibit refractory periods between mounting/intromission/ejaculating bouts that can last up to 24 hours (McGill, 1962). If KO males do not pattern their ejaculation bouts based on female urinary status, they may mount and intromit outside their partners fertile period. Additionally, the fertile periods of KO females may not coincide with behavioral receptivity. Uncoupling insemination from the fertile window could delay fertilization until the next cycle. This would explain pairing failures and the increase in the time to reproduction measure. Indeed, the difference in time to reproduction between KO x KO and WT x WT pairings is approximately the duration of one ovulatory cycle. The presence of either WT sex in KO x WT pairings seems to rescue the time to reproduction measure which may indicate the fertile period was once again coupled with ejaculation and receptivity. Directly measuring their behaviors during these types of pairings may indicate in which KO sex a behavioral change occurs. This suggests maintaining TRPC2 KO mice under laboratory conditions may benefit from heterozygous pairings. It is also possible that different breeding paradigms and housing conditions are more amenable to KO reproduction as these reproductive deficits have not been seen previously.

Interactions between mother and pup often depend on chemical communication through the main olfactory system (Distel & Hudson, 1985; Gandelman, Zarrow, Denenberg, & Myers, 1971; Moriceau & Sullivan, 2004; Tirindelli, Dibattista, Pifferi, & Menini, 2009) and possibly the VNO as well (Del Cerro, 1998; Del Punta et al., 2002; Lepri, Wysocki, & Vandenbergh, 1985). Notably, both systems express TRPC2 (Kiselyov et al., 2010; Omura & Mombaerts, 2014). Here, we find that maternal experience can reverse the deleterious effects of TRPC2 KO on pup survival, restoring survival to WT levels (fig 1C). This result clearly demonstrates that deficits caused by the disabled TRPC2 gene are not necessarily static but can be overcome by experience. The different survival rates of KO offspring across litters may well

lead to different behavioral outcomes in adulthood and warrants consideration in future studies using homozygous pairings. Because nursing is disrupted in KO animals (Hasen & Gammie, 2009; Kimchi et al., 2007) this may explain the lower body weight seen at weaning (fig 1E). However, because their weights remain low in the second litter, when survival increases, deficits in nursing are unlikely to explain the reduced survival of offspring from first litters.

TRPC2-expressing neurons in the vomeronasal organ exert control over the timing of puberty in female mice (Oboti et al., 2014) but there was no information to date about the role of TRPC2 in regulating the timing of male puberty. Thus, we recorded the day of preputial sheath separation in males (Korenbrot, Huhtaniemi, & Weiner, 1977) and vaginal opening in females (Ahima, Dushay, Flier, Prabakaran, & Flier, 1997) as indicators of pubertal onset and found that the effect of the KO on pubertal timing was opposite depending on whether the pups were from the first or second litter from the same dams. Puberty was accelerated for both male and female KOs of first litters but delayed in the second (fig 2C, D). While the effect of knocking out TRPC2 may disrupt GnRH migration to the hypothalamus (Schwanzel-Fukuda, 1999), and delay puberty, this possible mechanism would not explain the acceleration of puberty in the first. Given that maternal experience improves pup survival in the second litter but that development is slowed raises the possibility that there is a benefit conferred by smaller (first) litters that enhances the rate of development, allowing them to survive on their own sooner in the face of defective maternal care. However, the pubertal timing curves for second litters of KO parents appear to be bimodally distributed, especially in males, i.e. both sexes show an initial group of faster developing mice followed by a period when few mice show sheath separation or vaginal opening and then another group of pups appear to develop later on (fig 2G, H). So, pups from within the same KO litter may show different phenotypes, i.e. pups intrinsically showing accelerated or delayed development. On the other hand, deficits in olfactory cues may well be the determining factor driving the overall delays in puberty for second litters, which are also larger, possibly putting pups at a competitive disadvantage for maternal resources. While complicated, these data underscore the fact that the effects of the gene KO are different across different litters from the same dam, which may well lead to variance in the apparent effects of the KO on adult behaviors. An important next step would be swapping pups between naïve and experienced

KO females at birth. This would indicate if the effects of litter on pup measures are due to altered maternal care after previous maternal experience.

The first study reporting male-typical sexual behavior in KO females also found they had higher free T levels (Kimchi et al., 2007), albeit the increase was small. Because treating females with T also induces male-typical sex behavior (Martel & Baum, 2009), it remained controversial whether T has a role in the expression of male sex behaviors in KO females. Consequently, we examined this possibility in the current study, measuring circulating T levels directly, and anogenital distance (AGD), a sensitive biomarker of androgen levels in development. We were also interested in whether T was elevated before weaning and therefore could contribute to defects in fertility for KO females. Our measures of AGD revealed that male and female weanlings show the expected sex difference, with males having a much larger AGD than females (fig 4B). On the other hand, the AGD of KOs was no different than WTs (fig 4B), suggesting that T levels were normal during embryonic and prepubertal development. We also find the expected sex difference in adult circulating T, without effects of the KO (fig 4A), suggesting that elevated T levels are unlikely to explain the observed effects on reproduction and development.

TRPC2 plays an important role in both reproduction and development. The presence of at least one functional TRPC2 gene, maternal or paternal, rescues fertility. The current data add critical new information regarding the wide reaching and early effects of TRPC2 KO on reproductive capacity and development, which likely shape adult behavior. Moreover, while experience seems capable of rescuing some deficits, it appears unable to rescue others. This changing landscape over the reproductive history of the dams means that offspring from different litters, or possibly within, have different development trajectories that may well contribute to altered KO behavior in adulthood.

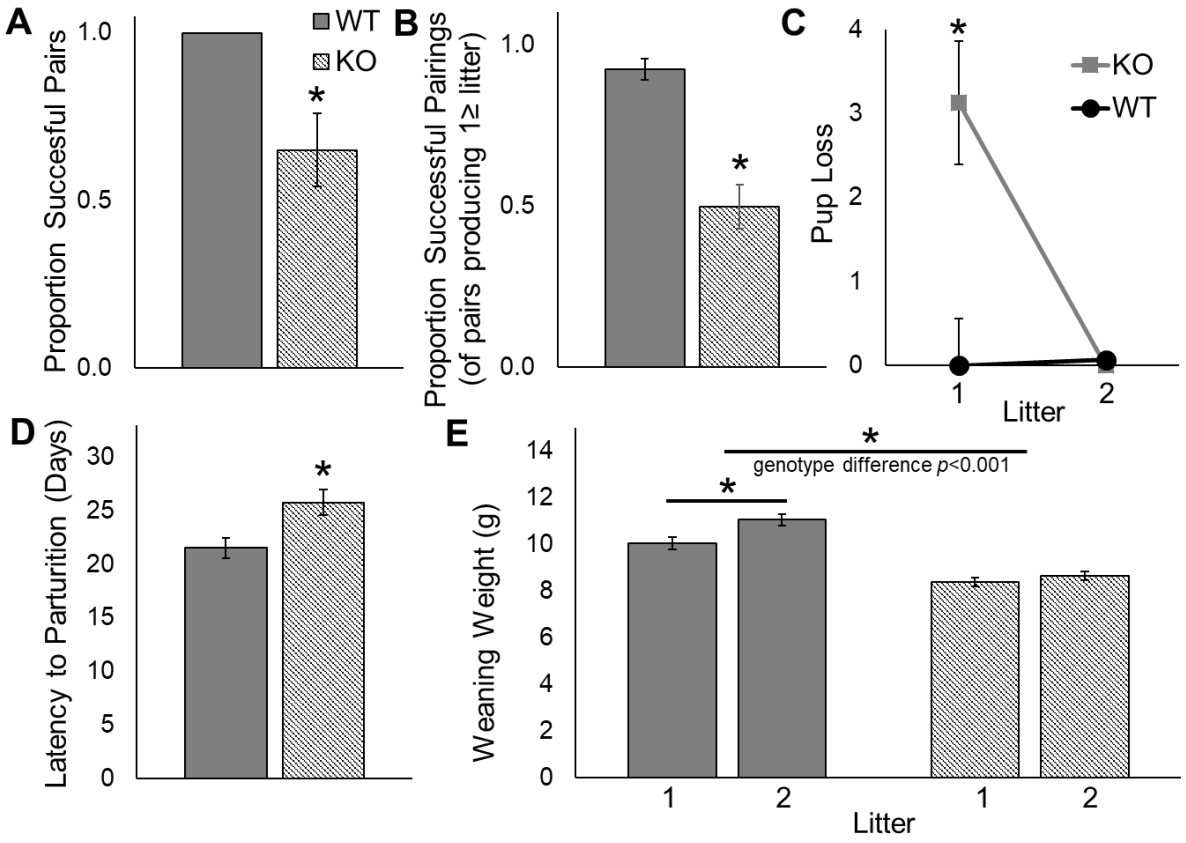

Figure 1: *TRPC2 loss leads to deficits in reproduction, pup survival and pup growth.* (A) The proportion of successful pairs is lower for TRPC2 KO mice than age-matched WT's, with only 13 of 19 KO pairs producing progeny across the two pairings while all WT pairings produced progeny in the first, second or both pairings. (B) While male/female KO pairings could produce offspring, they nonetheless showed a pronounced deficit in the proportion of pairings resulting in pregnancies compared to successful WT pairs. (C) More pups are lost between birth and weaning for first litters of KO dams compared to WT dams. A significant decrease in pup loss across KO litters indicates previous experience with a litter may rescue maternal care deficits. (D) The amount of time between the start of pairing and parturition resulting from that pairing was also significantly increased, an effect seen for both pairing cycles, suggesting that prior sexual and gestational experiences cannot overcome this defect. (E) Despite the increase in pup survival with maternal experience, body weight of KO weanlings was comparable across the two pairings and was overall significantly less than WT weanlings. Note also that body weight of WT weanlings from the second pairing was also significantly more than from the first. (*p<0.05, error bars ±SEM).

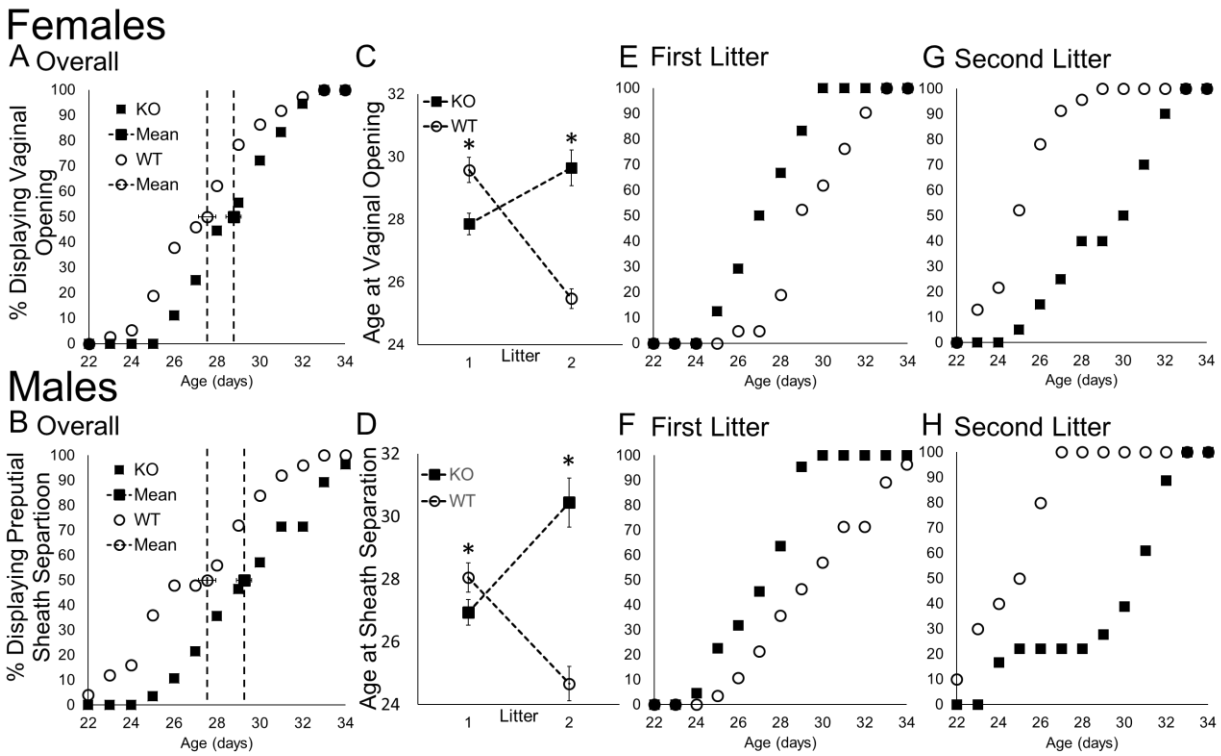

Figure 2: *TRPC2 influences timing of vaginal opening in female and preputial sheath separation in male mice.* (A, B) Overall pubertal onset was similar between KO and WT mice. (C, D) Pubertal onset is delayed for TRPC2 KO mice in second but not first litters. (E, F) When broken down by litter order, first-litter KO mice reached puberty significantly earlier than WTs in females and around the same time as WTs in males, but (G, H) second-litter KO mice born from the same dams showed pronounced delays for both female and male KOs. (*p<0.05, error bars ±SEM)

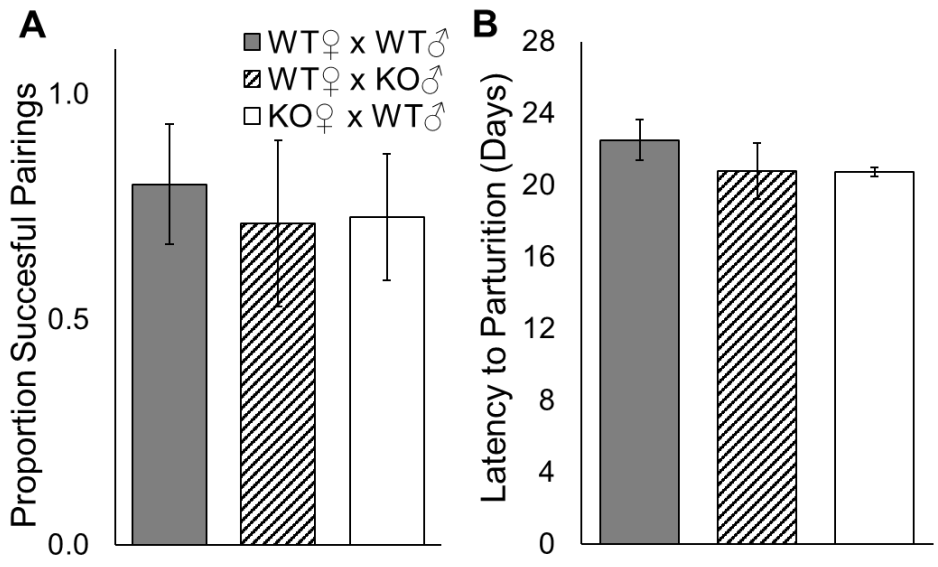

Figure 3: *Crossbreeding pair data.* (A) Pairing KO males with WT females or vice versa brought the proportion of successful pairings and (B) the number of days to parturition to that of WTs suggesting that both male and female KOs have subtle defects in fertility that are evident only when both parents are KOs (error bars ±SEM).

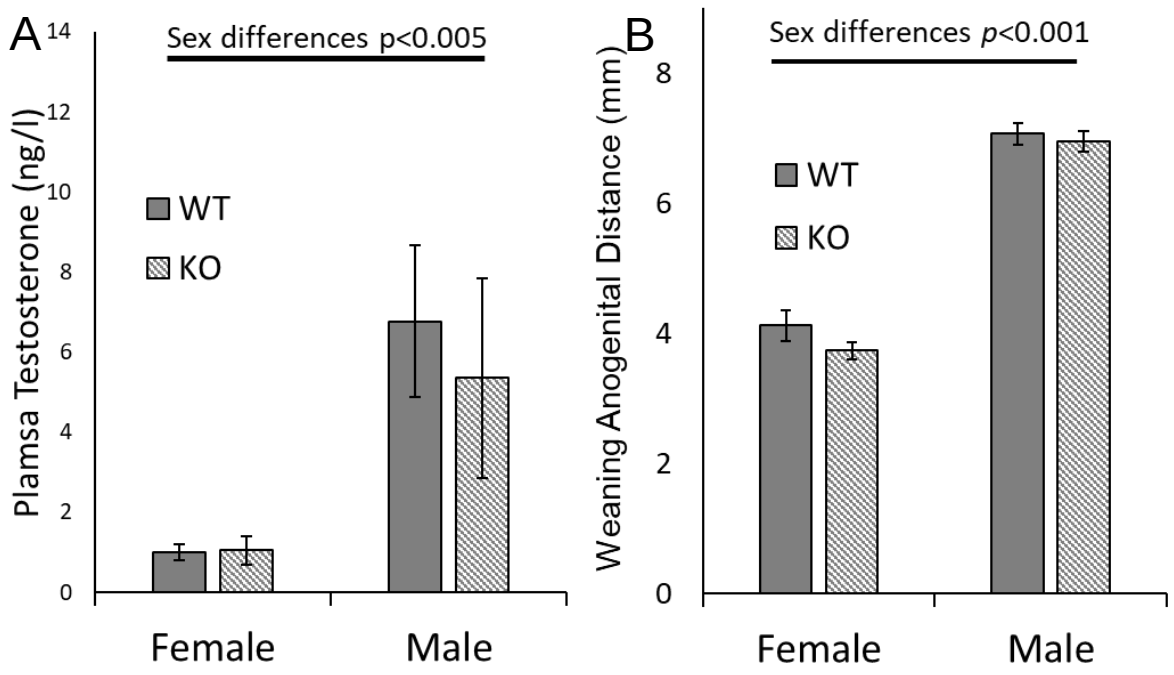

Figure 4: *TRPC2 loss does not alter adult plasma testosterone levels or anogenital distance at weaning.* (A) Both adult KO and WT mice show the same robust sex difference in circulating testosterone with no apparent effect of genotype on testosterone levels. (B) The anogenital distance (AGD), a sensitive biomarker of androgens during development, was also comparable between KO and WT mice. For both KOs and WTs, the AGD of males was nearly twice that of females. Likewise, serum testosterone levels showed the expected sex difference and no genotype difference (data not shown). (error bars ±SEM)

Table 1: *Colony breeding litter data*

	WT♀ x WT♂		**KO♀ x KO♂**	
litter	**First**	**Second**	**First**	**Second**
# pups at weaning	7±1.8	6.9±1.8	7.2±1	7.1±1.9
% females at weaning	54.5±6.6%	47.3±5.4%	63.3±6.4%	49±5.4%

Table 2: *Crossbreeding Litter data*

	WT♀ x WT♂	**WT♀ x KO♂**	**KO♀ x WT♂**
# pups at birth	6.57±0.92	6.2±0.5	6.5±0.63
% females at birth	48.7±10%	41±11%	56.9±8.6%

Table 3: *Effects of TRPC2 KO on body, gonad and accessory sex organ weight of adult KO and WT male and female breeders that were successful or not in producing offspring.* (Bolded terms are significantly greater $p<0.05$)

	WT		**KO**	
Females	Successful	Unsuccessful	Successful	Unsuccessful
Body Weight	**28.4±1.2**	26.1±1.8	**30.74±1.2**	25.27±1.8
Ovary Weight	0.25±0.03	0.3±0.04	0.33±0.03	0.32±0.04
Uterine Horn Weight	1.9±0.3	1.4±0.5	2.1±0.4	2.4±0.5
Males				
Body Weight	28.12±0.82	30.05±2.95	28.59±0.96	26.5±1.3
Testis Weight	**2.5±0.07**	**2.3±0.1**	2.18±0.08	2.13±0.15
Seminal Vesicle Weight	0.31±0.03	0.3±0.02	0.26±0.01	0.27±0.01

CHAPTER 3: LOSS OF TRPC2 FUNCTION ALTERS BRAIN SEXUAL DIMORPHISMS

Abstract

The transient receptor potential cation channel 2 (TRPC2) assists in transducing pheromonal information from the vomeronasal organ (VNO) to the brain, by way of the accessory olfactory bulb which signals the vomeronasal amygdala. Mice lacking the TRPC2 (KO) gene have reduced VNO function and smaller accessory olfactory bulbs compared with WT mice. Additionally, KO mice express altered sexual behavior and aggression. Specifically, both male and female TRPC2 KO mice mount intruder males and females in an apparently non-discriminant fashion and lack male-male or maternal aggression. While brain sex differences are driven largely by gonadal hormones, environmental signals also have a role shaping sexually dimorphic brain circuitry. I asked whether pheromones ordinarily conveyed by TRPC2-mediated signals affecting the sexually dimorphic development of the brain. I focused on the posterodorsal aspect of the medial amygdala (MePD) and ventromedial hypothalamus ventrolateral subdivision (VMHvl), central nuclei within the vomeronasal amygdala that are also sexually dimorphic in organization and function. I find the MePD of TRPC2 KO mice display lateralized absence of sexually dimorphic neuron populations; KO females show neuron loss on the right vs. WT females, while KO males display neuron loss on the left. Despite the loss of these neuron populations, the sex difference in neuron number seen in WT animals persists in KO mice, although, in different hemispheres. However, astrocyte numbers appear unaltered by the KO. The VMHvl of KO females is like WT females but the KO appears to drastically alter the VMHvl in males. I report a sex difference in the VMHvl astrocyte population as females have more astrocytes than males. Male KOs have more astrocytes than their WT counterparts, eliminating the sex difference in astrocyte number. These data suggest TRPC2-mediated signaling influence development of sexually dimorphic traits in the MePD and VMHvl, which may mediate some of the behavioral effects of the TRPC2 KO.

Introduction

Considerable evidence supports the idea that brain sexual dimorphisms underlie sexually differentiated behaviors. A large body of research also supports the idea of that sex differences in gonadal hormones determine such sex differences in the brain. While the sex chromosomes may also

contribute, data for their direct role in the formation of sexually differentiated circuits remains scant. However, recent data suggest that factors from other peripheral systems may also have a role (Ashlyn Swift-Gallant, Niel, & Monks, 2012). In mice, the accessory olfactory system receives information from the vomeronasal organ (VNO), a peripheral sensory tissue that detects pheromones. Within VNO neurons, the transient receptor potential cation channel 2 (TRPC2) transduces pheromonal signals to action potentials sent to the accessory olfactory bulb (AOB) (Kiselyov et al., 2010). Ultimately, these signals are processed in the amygdala and hypothalamus (Insel & Fernald, 2004), inducing sex-specific responses in sexual and aggressive behavior (Clancy et al., 1984). Notably, these same behaviors are altered in mice without a functional TRPC2 gene (KO) (Leypold et al., 2002; Stowers et al., 2002). The amygdala and hypothalamus are highly sexually differentiated structures (Baum, 2009; Dugger, Morris, Jordan, & Breedlove, 2007; Pfau et al., 2016) which play key roles in the expression of sex-specific sexual and aggressive behavior (Lee et al., 2014; C. F. Yang et al., 2013). For example, male mate recognition requires the integration of newborn neurons into the *female* amygdala based on the pheromonal cues they receive during sexual experience (Oboti et al., 2011) resulting in a sexually dimorphic behavior, maternal aggression. These observations raise the question of whether pheromonal signals that require TRPC2 also normally participate in the formation and/or maintenance of morphological sex differences in these same brain areas. We examine the possible influence of TRPC2 loss on brain sex dimorphisms in the current study.

Many sex-typical behaviors are altered after manipulating VNO function. For example, incoming pheromonal signals to the VNO of male rodents normally directs their sexual behavior towards females, but both an intact VNO and sexual experience are required to produce this preference (Pankevich et al., 2006; Wysocki & Lepri, 1991). TRPC2 KO male mice lack this preference in sexual partners, mounting *both* males and females (Stowers et al., 2002). Perhaps more striking is the robust and frequent expression of male mounting behavior in TRPC2 KO females, a behavior that typically requires testosterone treatment (Martel & Baum, 2009). Like KO males, KO females will mount *both* male and female mice (Kimchi et al., 2007). In females, TRPC2 loss also eliminates maternal aggression (Hasen & Gammie, 2009) and disrupts nursing behavior (Hasen & Gammie, 2011; Kimchi et al., 2007). While males also lose male-directed aggression (Stowers et al., 2002), TRPC2 KO augments their parental behaviors

(Orikasa et al., 2017). In short, these sex-specific behaviors seem to be selectively altered in TRPC2 KO mice. The neural basis of these striking changes in social behavior is largely unknown, with only a few studies indicating anatomical changes in relevant regions of the brain. For example, neuron number in the VNO starts off similar to *trpc2* +/+ wild type (WT) mice but these connections disappear during development of the brain in TRPC2 KO mice (Stowers et al., 2002), undoubtedly reducing the transmission of pheromonal information to VNO-recipient areas. Indeed, KO mice have a smaller AOB and the effects of the TRPC2 KO vary by sex (Hasen & Gammie, 2009). Thus, pheromonal signaling via TRPC2 may also play a critical role in organizing sex differences in the brain underlying sex-typical social behavior.

Social behaviors like mounting and aggression are critically tied to several nuclei along the VNO-recipient pathway, namely the posterodorsal aspect of the medial amygdala (MePD) (DA Adekunbi et al., 2018; D. R. Pfau et al., 2016) and ventromedial hypothalamus (VMH) (Yang et al., 2013). For example, the MePD mediates courtship/copulatory behavior typical of WT male mice (DA Adekunbi et al., 2018; Aggarwal et al., 2018) and shows striking sex differences in its volume, neuron number and soma size, and astrocyte number (Pfau et al., 2016). The VMH ventrolateral subdivision (VMHvl) is also critical for the expression of male mounting and aggression (Lee et al., 2014). This region governs both male and female sexual behavior through sexually dimorphic neuronal populations (Yang et al., 2013). Here we ask whether mice lacking TRPC2, which display robust changes in sex-specific behaviors, also exhibit changes in MePD and VMH morphology. To answer this question, we conducted an in-depth morphological analysis of each region in both Nissl- and GFAP (glial-stained tissue, allowing us to analyze neurons, glia and astrocytes in these two regions of KO and WT male and female mice. We find that KO mice show overall deficits in the number of cells in the MePD and VMHvl although the extent of these deficits depends on sex in both regions, as well as hemisphere in the MePD. These morphological changes triggered by a loss of TRPC2 function may underlie or contribute to the unique behaviors displayed by such mice.

Materials and methods

Animals:

TRPC2 -/- (B6;129S1-Trpc2tm1Dlc/J JAX stock number: 021208) mice and TRPC2 +/+ (B6129SF2/J021208; JAX stock number: 101045) were purchased and used to establish homozygousTRPC2 KO and WT colonies of mice, both maintained on the matched background strain (B6129SF2). Pups from these homozygous pairings were weaned at postnatal day (PD) 21, ear punched, housed with same-sex littermates and remained unmanipulated until tissue collection. Adult (PD 84-105) age-matched WT and KO mice were overdosed with sodium pentobarbital (210 mg/kg) and perfused transcardially with 0.9% saline followed by phosphate buffered (0.1M, pH 7.4) 4% paraformaldehyde. Brains were harvested and postfixed 2 hours in the same paraformaldehyde solution (room temperature) followed by cryoprotection in 20% sucrose in 0.1M phosphate buffer for at least 48 hours (at 4° C). Brains were scored along the right dorsal surface of the cortex to mark side and then cross sectioned at 30 microns on a freezing sliding microtome. Three adjacent series were collected and stored at -20°C in de Olmos cryoprotectant (de Olmos et al., 1978) until stained. All animal procedures were approved by the Institutional Animal Care and Use Committee at Michigan State University.

Nissl staining:

One series was brought to room temperature, rinsed and mounted onto gel-subbed slides. Once dry and defatted, sections were stained with thionin (D. R. Pfau et al., 2016), dehydrated, cleared, and coverslipped with Permount (Fisher Scientific). This protocol results in distinct staining characteristics for neurons and glial, allowing for counts of each. Neurons were identified based on presence of a cytoplasmic shell surrounding a distinct nucleus and nucleolus. Glial cells, which included astrocytes, were identified based on the absence of an apparent cytoplasmic compartment but with a distinct nucleus often containing lobed nucleoli.

Glial fibrillary acidic protein immunohistological staining:

The second series was brought to room temperature and rinsed with phosphate buffered saline (0.1M, pH 7.4) containing 0.3% Triton and 0.1% gelatin (PBS-GT). This same solution was used throughout as the vehicle for immunoreagents and rinses. Following a published protocol for GFAP immunostaining of astrocytes in mice (D. R. Pfau et al., 2016) sections were incubated in 0.5% sodium

borohydride in PBS-GT (15 min), blocked for nonspecific avidin-biotin binding (Avidin and Biotin blocking kit, SP-2001, Vector, Burlingame, CA), followed by an overnight incubation at 4°C in monoclonal mouse anti-GFAP (EMD Millipore Cat# MAB360, RRID:AB_2109815, 1:50,000 dilution). Sections were then incubated in biotinylated horse anti-mouse secondary antibody (Vector Laboratories Cat# BA-2001, RRID:AB_2336180,, 1:2,500 dilution) followed by incubation in half-strength peroxidase avidin-biotin complex solution (Elite Avidin Biotin complex kit, PK-6100, Vector Laboratories, Burlingame, CA) and reacted for 5 minutes using 0.025% diaminobenzidine (DAB, Sigma, St. Louis, MO) with 0.0125% H2O2 in Tris buffer (0.05M, pH 7.2), to visualize horse radish peroxidase. After rinsing to quench the reaction, sections were mounted onto gel-subbed slides and allowed to dry before counterstaining with Harris's Hematoxylin solution (Sigma, #010M4354) followed by alcohol dehydration, clearing, and coverslipping with Permount.

Stereological analysis:

Stereological analysis was performed using a Zeiss Axioplan II microscope equipped with an Optronics MicroFire digital video camera and StereoInvestigator software (v. 7.0, MBF Bioscience, Williston, VT). The total number of neurons, glia, and astrocytes were determined in the MePD of each hemisphere and in two subregions of the VMH; dorsomedial (dm) and ventrolateral (vl), sampled throughout the rostrocaudal extent of each region. At low magnification, regional boundaries were traced in each section. Regions were identified based on a mouse atlas (Paxinos & Franklin, 2004) and previously defined parameters within our laboratory (Dugger et al., 2007; Morris, Jordan, King, et al., 2008). To outline the MePD, landmarks visible in both Nissl and hematoxylin stained sections included the optic tract, shape of the stria terminalis and posteroventral medial amygdala. Dense cell populations surrounded by a capsule in the medial hypothalamus form the VMHdm and VMHvl with a cell-poor central region between them (Dulce Madeira, Ferreira-Silva, & Paula-Barbosa, 2001). Landmarks for the VMHdm and VMHvl included the arcuate nucleus and paraventricular nucleus.

Neurons and overall glia counted in Nissl-stained sections using a 100X Zeiss plan-neofluar oil-immersion objective (na=1.3). Slides were coded making the observer blind to the sex and genotype of the animal. The optical fractionator probe was used to produce unbiased counts of the number of neurons and glial cells in Nissl-stained sections. To measure the size of neuronal soma, neurons from five

randomly selected sections were analyzed. The StereoInvestigator optical fractionator setup was used to pick five random sites within each section's contour (total of 10 cells per hemisphere) and the soma of a neuron closest to the optical fractionator probe was traced using the nucleator probe an 100X objective. Astrocytes were counted using the optical fractionator probe in GFAP-labeled sections with a 40X Zeiss plan-neofluar objective (na = 0.75). Astrocytes were identified based on having GFAP-labeled fibers surrounding a distinct hematoxylin-stained nucleus in the same plane of focus.

These methods provided estimates of the total number of neurons, overall glial cells, and astrocytes, along with regional volumes and cell densities. If one section was missing, the average cell number of adjacent sections was included in the analysis, but if more than one section was damaged or missing, the brain was excluded from the analysis. However, the program cannot produce estimations of regional area, accounting for the lower sample sizes reported for volume measures. Neurolucida software (v. 7.0, MBF Bioscience) was used to trace and estimate the complexity of astrocyte arbors. In five randomly selected MePD and VMHvl regions from each animal, the complete astrocytic arbor of two astrocytes randomly selected by an observer blind to sex and genotype were traced using the 100X objective, with ten astrocytes sampled from each hemisphere. Based on these traces, the average number of primary processes, average number of branch points, average number of branch endings, total length and average branch length were calculated for each animal and hemisphere.

Statistical analysis:

Separate ANOVAs were used to examine each measure (regional volume, neuron number, overall glial number, neuron soma size, astrocyte number and process complexity) within each brain region or sub-region (VMHvl and VMHdm). For the MePD and VMH regions, a 3-way mixed design ANOVA was utilized, with laterality as the within-subjects variable, and sex and genotype as between-subject variables. This was followed by conservative posthoc Bonferroni comparisons to examine significant main effects and interactions. Results reported as means $\pm$ SEM (standard error of the mean) alpha=0.05. We examined more closely the potential relationships between cellular components and volume in the two sexually differentiated regions, the MePD and VMHvl. Separate correlations were run for each experimental group, hemisphere and brain region. Principle component analysis allowed dimension reduction for measurements of astrocyte complexity to reduce the number of correlations

examined and increase power. With the complexity factors, correlations were carried out with alpha level 0.01, family wise error rates were calculated separately based on the number of principle components extracted in a region. For the left MePD, twelve correlations were run with a family wise error rate of 0.11, in the right MePD, nine correlations were run with a family wise error rate of 0.09. For the left and right VMHvl, a total of twelve correlations were run with a family wise error rate of 0.11.

Results

Posterodorsal aspect of the medial amygdala (MePD):

Volume: MePD volume in Nissl stained slices depended on both genotype ($F_{(1,25)}$=8.84, p=0.006, d=1.06) and sex ($F_{(1,25)}$=19.7, p<0.001, d=1.58), with the MePD larger overall in WT mice compared to KO mice and also larger overall in males compared to females (Fig 5A). There was a main effect of laterality, with the left MePD larger than the right ($F_{(1,25)}$=3.65, p=0.019, d=0.44) and a three-way interaction between hemisphere, sex and genotype ($F_{(1,25)}$=11.6, p=0.01). Posthoc analyses indicated that the WT MePD volume is sexually differentiated on both the *left* (p<0.001, d=2.03) and *right* (p=0.035, d=1.24) whereas TRPC2 KO mice had a sex difference only on the *right* (p=0.002, d=1.83) but not left (p=0.11). Moreover, the TRPC2 KO reduced MePD volume in both a sex- and side-specific manner, with KO females having a smaller *right* MePD compared to WT females (p=0.049, d=1.15), and KO males having a smaller *left* MePD than WT males (p=0.002, d=0.95). TRPC2 KO eliminated the laterality in male MePD volume (p=0.45) that is normally present in WTs (p=0.008, d=1.2) and produced a hemispheric difference in KO females (p=0.028, d=2.1) that is absent in WT females (p=0.64). Interestingly, comparable analysis in GFAP stained tissue (fig 5B) did not reveal significant effects of sex ($F_{(1,17)}$=2.47, p=0.134), genotype ($F_{(1,17)}$=1.73, p=0.206) nor hemisphere ($F_{(1,17)}$=0.333, p=0.572), nor any interactions between these variables. The lower N for this analysis may have contributed to the lack of statistical differences (see methods), since the trends look quite similar to what is shown based on Nissl staining.

Neuron number and somal size: Cell counts in Nissl stained slices revealed significant effects of sex ($F_{(1,29)}$=8.24, p=0.008, d=0.99), with males having more neurons overall than females, as well as laterality ($F_{(1,29)}$=7.44, p=0.011, d=0.29), with *left* MePD containing more neurons overall than the right. While there was no main effect of genotype ($F_{(1,29)}$=3.16, p=0.086), a three-way interaction between all

variables appeared ($F_{(1,29)}$=12.47, p=0.001) and posthoc analysis revealed the following. WT mice had a sex difference in MePD neuron number on the *left* (p=0.011, d=1.34) while KO mice showed a sex difference in neuron number on the *right* (p=0.014, d=1.38). Moreover, TRPC2 KO reduced neuron number in the *right* hemisphere of females (p=0.046, d=1.03) and the *left* hemisphere of males (p=0.041, d=0.93; fig 6a). As a result of these lateralized effects on neuron number, KO females had significantly more MePD neurons on the left (p=0.001, d=0.79), like WT males (p=0.02, d=0.52), while KO males were now like WT females, exhibiting no laterality in MePD neuron number (WT female p=0.59, KO males p=0.8; Fig 6a). We also found an overall male-biased sex difference in the size of MePD neuronal soma ($F_{(1,25)}$=12.62, p=0.002, d=1.67; fig 6B) but soma size was independent of TRPC2 gene KO ($F_{(1,25)}$=0.1, p=0.754) and hemisphere ($F_{(1,25)}$=2.55, p=0.12). No interactions reached significance, indicating that different mechanisms control the number and size of neurons.

Number of glia: Males had more glia in Nissl-stained tissue than females ($F_{(1,29)}$=7.86, p=0.009, d=1.21) and WTs had more glia than KOs ($F_{(1,29)}$=11.59, p=0.002, d=1; fig 6C). However, there was no effect of hemisphere ($F_{(1,29)}$=0.88 p=0.357) nor any significant interactions between main effects.

Number and branch complexity of astrocytes: Like total glia, astrocytes showed an overall sex difference in number, with males having more MePD astrocytes than females ($F_{(1,26)}$=16.44, p>0.0001, d=1.17; fig 6D), similar to previous findings (Pfau et al., 2016). However, unlike overall glial number, TRPC2 had no effect on the number of MePD astrocytes ($F_{(1,26)}$=0.299, p=0.59), suggesting that TRPC2 regulates glia in the MePD that don't express GFAP. While no overall effects of laterality were found ($F_{(1,26)}$=3.85, p=0.06), the effect of sex interacted with hemisphere ($F_{(1,26)}$=6.17, p=0.02), with both KO and WT females having more astrocytes on the left (p=0.004, d=0.49). The complexity of MePD astrocytes was unaffected by sex, laterality and genotype (table 4) with the one exception that the loss of TRPC2 significantly reduced the number of branch endings in KOs ($F_{(1,16)}$=4.48, p=0.05, d=0.9). However, with the number of independent F tests on related parameters from the same cells, this may be a type I error.

Overall, the MePD displays robust sex differences in most cellular and volumetric analyses. The effect of TRPC2 on these sex differences are complex, with sex differences conserved, absent, minimized, or introduced depending on the cellular parameter being measured in mice lacking the TRPC2 gene.

Ventromedial hypothalamus ventrolateral subdivision (VMHvl):

Volume: The volume of the VMHvl in Nissl stained tissue was unaffected by sex ($F_{(1,19)}$=2.7, p=0.12), genotype ($F_{(1,19)}$=2.69, p=0.12) and hemisphere ($F_{(1,19)}$=0.99, p=0.33; fig 7A). However, overall volumes from GFAP stained tissue were larger in males ($F_{(1,24)}$=8.25 p=0.009) and WTs ($F_{(1,24)}$=5.65, p=0.027; fig 7B) without an effect of hemisphere ($F_{(1,24)}$=0.49, p=0.49). The interactions between sex and genotype showed a marginally significant relationship in *both* staining methods (Nissl tissue $F_{(1,19)}$=4.3, p=0.052, GFAP tissue $F_{(1,24)}$=4.08, p=0.056), prompting us to explore this interaction further using posthoc Bonferroni analyses. The volume of the VMHvl is normally the same in males and females, in both Nissl (p=0.36) and GFAP stained tissue (p=0.56; Fig 7A, B). Disabling the TRPC2 gene significantly reduced VMHvl volume from WT levels but only in males (Nissl p=0.01, d=1.5; GFAP p=0.003, d=0.89) not females (Nissl p=0.76; GFAP p=0.58). Thus, the TRPC2 KO introduced a volume sex difference (Nissl p=0.017, d=1; GFAP p=0.002, d=0.99) that is not normally present in WT mice (Nissl p=0.36; GFAP p=0.56).

Neuron number and somal size: The number of neurons in the VMHvl varied by both sex ($F_{(1,25)}$=6.88, p=0.015; fig 8A) and genotype ($F_{(1,25)}$=7.39, p=0.012) but not hemisphere ($F_{(1,25)}$=0.053, p=0.82). A significant interaction between sex and genotype was found ($F_{(1,25)}$=7.57, p=0.011). Posthoc analyses indicated that while WT mice showed no sex difference (p=0.927) KO mice did, with KO males having fewer neurons than KO females (p=0.001, d=1.86), aligning with the effect of the KO on VMHvl volume. KO males also had significantly fewer neurons than WT males (p<0.001, d=1.9). Overall, the size of neuronal somata were similar between sexes ($F_{(1,25)}$=1.2, p=0.28; fig 8B) and hemisphere ($F_{(1,25)}$=1.5, p=0.3) but WT mice had larger neuron soma than KOs ($F_{(1,25)}$=4.28, p=0.049, d=2), indicating a main effect of genotype but not sex. These two factors did not interact ($F_{(1,25)}$=0.96, p=0.34). In male mice, the processes controlling VMHvl neuron number and soma size are both influenced by TRPC2. In females, TRPC2 affects VMHvl soma size while separate mechanisms influence neuron number.

Glial number The overall number of glia in Nissl stained material was not affected by genotype ($F_{(1,25)}$=0.22, p=0.64; fig 8C), hemisphere ($F_{(1,25)}$=2.7, p=0.12) nor sex ($F_{(1,25)}$=0.51, p=0.48). Unlike VMHvl volume and neuron number, there was no interaction between sex and genotype ($F_{(1,25)}$=0.025, p=0.88).

Number and branch complexity of astrocytes: Counting the number of astrocytes in GFAP-stained material also revealed no overall effect of sex ($F_{(1,30)}$=1.31, p=0.26; fig 8D), hemisphere ($F_{(1,30)}$=0.71, p=0.41) or genotype ($F_{(1,30)}$=3.36, p=0.08) but there was a significant interaction between sex and genotype ($F_{(1,30)}$=10.34, p=003). In WT mice, females had more VMHvl astrocytes than males (p=0.004, d=1.6) but this sex difference disappeared in KO mice (p=0.162) because the number of astrocytes in the VMHvl showed a selective increase in KO males compared to WT males (p=0.002, d=1.8). Table 5 contains complexity analyses of VMHvl astrocytes, with significant main effects and interactions summarized here. The number of branch nodes of VMHvl astrocytes was affected by genotype but not sex, with the TRPC2 KO significantly reducing the number of branch nodes compared to WT astrocytes ($F_{(1,17)}$=8.04, p=0.012, d=1.15). The effect of the TRPC2 KO also depended on sex ($F_{(1,17)}$=4.47, p=0.05). Posthoc analysis revealed a sex difference in the number of astrocyte branch nodes in KO mice, with VMHvl astrocytes having more branch nodes in KO females than KO males (p=0.027, d=1.4). (p=0.554) but did in KO mice, with KO males having significantly fewer branch nodes than KO females (p=0.027, d=1.4). Consequently, KO males also had fewer nodes than WT males (p=0.002, d=2). An overall sex difference was also found in the number of branch endings as female mice had more endings regardless of genotype ($F_{(1,17)}$=5.68, p=0.029, d=1.1). However, the effect of sex on the number of branch endings depended significantly on genotype ($F_{(1,17)}$=4.65, p=0.046), with a sex difference in only KO and not WT mice. Posthoc analysis indicated that KO males had fewer branch endings than KO females (p=0.004, d=2.1) and compared to WT males (p=0.017, d=2). There was also a significant interaction between sex, genotype and hemisphere in branch length ($F_{(1,17)}$=6.8, p=0.019) without significant main effects of these variables separately (table 5). Posthoc analysis indicated that WT females displayed laterality in total branch lengths, with greater lengths in the right hemisphere (Total branch lengths mean±SEM, Left: *306.2±27.3*, Right: *352.1±33.36* p=0.033, d=0.33). This was the only effect of hemisphere found during the analysis of the VMHvl. In sum, the VMHvl of B6129S mice displays few sex differences in overall morphology and virtually no laterality. TRPC2s' role in VMHvl anatomy appears specific to males, resulting in several sex differences in KO mice not ordinarily seen in WT mice.

Ventromedial hypothalamus dorsomedial subdivision (VMHdm):

We found no sex, genotype nor hemisphere differences in VMHdm anatomy. Specifically, volume measurements in Nissl stained tissue had no significant sex ($F_{(1,19)}$=0.006, p=0.94; fig 9A), laterality ($F_{(1,19)}$=0.002, p=0.97) nor genotype ($F_{(1,19)}$=0.847, p=0.34) differences. Neuron number was also independent of sex ($F_{(1,22)}$=0.001, p=0.97; fig 9B), hemisphere ($F_{(1,22)}$=2.23, p=0.15) and genotype ($F_{(1,22)}$=0.008, p=0.93), as was overall glial cell number (Sex $F_{(1,22)}$=0.224, p=0.64; Hemisphere $F_{(1,22)}$=0.006, p=0.94; Genotype $F_{(1,22)}$=0.056, p=0.82; fig 9C).

Correlations and principle component analysis:

For Nissl stained tissue, a single significant correlation was found. The number of glia in the MePD of WT males correlated positively with neuron number in the left hemisphere (p=0.006, r^2=0.81). No such correlation was found in WT females. In contrast, KO males were more like females, WT and KO, as no correlations between neuron and glia number, neuron soma size or volume were found. When examining the VMHvl in Nissl tissue, soma size in WT males was positively correlated with the regional volume in the left hemisphere (p=0.009, r^2=0.85), but not the right (, while measures from the KO male VMHvl had no significant relationships, similar to females of both genotypes. The WT female VMHvl showed a positive correlation between soma size and total glia in the *right* hemisphere (p=0.003, r^2=0.85), however, this relationship was not significant in KO females (p=0.03). When performing principle component analysis with astrocyte complexity measures taken from the MePD, all measures loaded into one principle component except for branch number. This provided two principle components on the left while all measures of astrocyte complexity loaded onto one principle component for the right hemisphere. However, no group displayed significant correlations. Principle component analysis of both the left and right VMHvl produced two components, branch number loaded strongly into one and all other measures into the other. When using these principle components, no correlations reached significance in all groups. This suggests mechanisms controlling GFAP-fiber arbor in B6129S mice are unrelated to TRPC2 and sex.

Discussion

Brain anatomy is linked to behavior outcomes and sex-typical behaviors are known to arise from sexually dimorphic brain circuitry (Breedlove, Cooke, & Jordan, 1999; Yang et al., 2013). The MePD and VMHvl are both implicated in sexual and aggressive behaviors and show striking sex differences (Dugger et al., 2007; Lee et al., 2014; Pfau et al., 2016; Yang et al., 2013), the fascinating loss of aggression and augmented sexual behaviors of TRPC2 null mice (Leypold et al., 2002) prompted our study of these two brain regions. We examined the MePD and VMH of WT and KO animals from both sexes, asking whether different morphology exists between sex, genotype and hemisphere. Using two staining techniques to mark Nissl substance or immunolabel GFAP, we carried out both gross anatomical measures and analyzed cell morphology. Such techniques have previously revealed important sex and hemispheric differences in the brains of rodents (Johnson et al., 2008; Morris, Jordan, & Breedlove, 2008; Pfau et al., 2016). We now report sex differences and laterality in the MePD and VMHvl of B6129S mice. B6129S mice share many MePD characteristics with two other mouse strains. Some of these characteristics are altered in KO animals in a sex-specific manner. Our data suggest TRPC2 influences the presence of sex differences in the MePD and VMHvl. Alterations to regions associated with aggression and sexual behavior may drive the changes seen in KO behavior.

In B6129S mice, the male MePD is larger overall (fig 5A, B, C, D) with more neurons, glia and astrocytes and larger neuronal soma than females (fig 6). Several results from B6129S mice match findings from the BALB/c and C57Bl/6J mouse strains. Males from all strains have larger MePD (fig 5) with larger neuron soma than females (fig 6B, F) (Morris, Jordan, King, et al., 2008; Pfau et al., 2016). Like C57Bl/6J mice, B6129S males have more neurons (fig 6A) and astrocytes (fig 6D) than females. Males and females from both strains show laterality; males have larger left MePD than right (fig 5A) and females have more astrocytes in the left MePD than the right (fig 6H) (Pfau et al., 2016). Unlike C57Bl/6J mice, B6129S mice display a sex difference in overall glia (fig 6G) and males have more neurons in the left hemisphere compared to right (fig 6A). TRPC2 KO affected several MePD measures. KO mice have smaller MePD (fig 5A, B) and fewer MePD neurons (fig 6A) and glia (fig 6B). The number of GFAP-immunoreactive fiber endings was also lower in KOs (table 4). In contrast, neuron soma size (fig 6B, F) and astrocyte number (fig 6D, G) appear unaltered by TRPC2 loss. In KO males, deficient volumes were

seen on the *left* (fig 10A, E), eliminating the presence of larger *left* MePD seen in WT males (fig 10A, B). In KO females, the *right* MePD was smaller than that of WTs, producing laterality and a larger *left* MePD (fig 10G, H) not apparent in WT females (fig 10C, D). Male WTs and female KOs display similar laterality (fig 10A, B, G, H). These shifts in volume after TRPC2 KO are accompanied by similar changes to MePD neurons. In males, TRPC2 KO reduces neuron number on the *left* while the *right* neuron population is reduced in female KOs. As such, the smaller KO hemispheres also have fewer neurons. Overall, TRPC2 KO affects both sex differences and laterality in the MePD of both sexes (fig 10). Some morphological sex differences in the rodent MePD are critically tied to gonadal hormones (Cooke et al., 1999; Morris, Jordan, King, et al., 2008). In rats, gonadal hormones maintain larger MePD with more and larger neurons and astrocytes (Johnson, Breedlove, & Jordan, 2013; Morris, Jordan, & Breedlove, 2008; Rasia-Filho et al., 2012). Less is known about what influences the morphology of the mouse MePD but circulating testosterone is important for the maintenance of larger neuronal soma and regional volumes in males (Morris, Jordan, King, et al., 2008). We now report that after TRPC2 KO the mouse MePD displays sex-specific changes. These changes are also specific to certain cell populations. Namely, neuron and non-astrocyte glia number are altered after TRPC2 loss while astrocyte number is not. The number of new neurons in the amygdala can be influenced by pheromones (Oboti et al., 2011) while astrocytes in the rodent amygdala show differences across the estrous cycle (Martinez et al., 2006). Astrocyte number may rely on sex-specific steroid hormones while neuron number appears to rely on a TRPC2-mediated mechanism.

The MePD is known to have several functions related to sex-typical behaviors. In males, the MePD mediates sexual behavior (McGregor & Herbert, 1992) and opposite-sex urinary preferences (Baum, 2009; Maras & Petrulis, 2010). Development of an opposite-sex urine preference requires an intact VNO and sexual experience, directing experienced male sexual behavior exclusively to females based on their scent (Pankevich et al., 2006; Wysocki & Lepri, 1991). The lack of odor preference in KO males (Stowers et al., 2002) may result from fewer MePD neurons but this seems contradictory to studies finding lesions of the rodent MePD *reduce* sexual behavior (McGregor & Herbert, 1992). MePD neurons are heterogenous and those neurons missing in the KO male MePD could represent a specific subpopulation involved with the maintenance of a preference for opposite-sex urine rather than a loss of

the general neuron population. For example, disconnecting the hamster MePD from the extended medial amygdala disrupts male urinary preference (Maras & Petrulis, 2010), similar to what is seen in KO males (Stowers et al., 2002). Loss of neurons that innervate other regions of the amygdala might disrupt odor preference in KOs. This may be true for neurons that connect to other regions of the accessory olfactory circuitry too. For example, in males the bed nucleus of the stria terminalis (BNST) helps organize female urinary signals into a pro-copulatory cue after initial sexual experience (Bayless et al., 2019; de Olmos et al., 1978) and sends projections to and receives them from the MePD (Coolen & Wood, 1998). Neurons forming the connection between MePD and BNST may maintain a preference for female urine enforced by BNST cells. Cell expression pattern may also be of importance as Kisspeptin neurons in the MePD are known to mediate responses to urine (DA Adekunbi et al., 2018). Given changes in KO male-male aggression (Stowers et al., 2002), another possible subpopulation affected by TRPC2 KO are the MePD GABAergic neurons which influence male aggression (Hong et al., 2014). What hemisphere the neuron is found in may be of importance too. KO behavior and the pattern of neuron loss (fig 6E) suggest opposite-sex urinary preference in males is a product of processing in the left MePD. Interestingly, detection of familiar scents likely occurs through the left nostril (Siniscalchi et al., 2011) and the majority of VNO signals remain ipsilateral (Kucharski, Johanson, & Hall, 1986). Given KO mice have fewer neurons in the *left* hemisphere (fig 6A), neuron populations related to TRPC2 may have lateralized function. Accounting for hemisphere and staining specific neuronal subtypes, like Kisspeptin, GABAergic or BNST-projecting neurons, would provide valuable information regarding changes to the KO brain.

The MePD is important for mate recognition in females too (Binns & Brennan, 2005). Female mice vary their response to novel male scents based on internal hormonal status. For example, estrous females find novel male pheromones attractive (Moncho-Bogani et al., 2005; Roberts et al., 2010) while lactating dams will respond aggressively towards an animal carrying a novel male scent (Wysocki & Lepri, 1991). Maternal aggression is lost in KO females (Hasen & Gammie, 2009) and lactating KO dams also have deficient c-fos responses to male urine in their MePD compared with WTs (Hasen & Gammie, 2011). Interestingly, c-fos expression following exposure to a novel male intruder is increased in the *right* AOB of WT dams while no activation is seen in KO dams (Hasen & Gammie, 2009). Here we report a loss of neurons in *right* MePD of KO females compared to WT females (fig 6). In mammals, the *right*

nostril is tied to the detection of unfamiliar scents (Savic & Berglund, 2000; Zatorre & Jones-Gotman, 1990) suggesting the neurons of the *right* MePD respond to novel pheromones. It is possible the neurons lost in the female *right* MePD after TRPC2 KO (fig 6) mediate the recognition of novel male pheromones. However, recently mated KO females are capable of terminating embryo implantation via detection of a novel male MHC (Kelliher, Spehr, Li, Zufall, & Leinders-Zufall, 2006), a non-volatile pheromone that provides individual identification (Leinders-Zufall et al., 2014). These two responses to pheromones represent different domains, blocking pregnancy by delaying implantation involves a physiological response (Bellringer et al., 1980) while maternal aggression is a behavioral response (Lonstein, Simmons, & Stern, 1998). So, those MePD neurons lost in the right hemisphere may mediate the behavioral but not hormonal response to novel male urine. Indeed, studies have found differences between pheromone-induced behavior and hormone release circuits (Lo et al., 2019; Wersinger & Rissman, 2000). Given the MePD is considered a main output from the medial amygdala (Maras & Petrulis, 2010), the missing MePD neurons in KO females may be those that would have eventually activated the periaqueductal gray to induce maternal aggression (Lonstein et al., 1998; Lonstein & Stern, 1997) while neurons that lead to the hypothalamus and induce hormonal changes (Ishii et al., 2017; Pardo-Bellver, Cádiz-Moretti, Novejarque, Martínez-García, & Lanuza, 2012; Wersinger & Baum, 1997) are spared. Altered MePD volume and neuron number across both KO sexes provides a compelling argument that KO behavior results from altered organization of brain circuitry. Still, TRPC2 KO does not alter all MePD morphology, like neuron number in the right hemisphere of males and left hemisphere of females (fig 6E). However, this does not mean that the function of these neurons remains the same. For example, Kisspeptin neurons in the male MePD are involved with responding to conspecific urine (DAL Adekunbi, Li, Colledge, & O'Byrne, 2017) and are known to receive signals from the AOB (Pineda, Plaisier, Millar, & Ludwig, 2017). KO mice have smaller AOB (Hasen & Gammie, 2009) with fewer sensory fibers from the VNO (Stowers et al., 2002), which may attenuate any signals received by Kisspeptin neurons. Deficient activation of kisspeptin neurons alone may prevent urine status from altering male behavior. However, a critical target for future research in the MePD are the characteristics of neurons found in WTs but absent in KOs, such data would inform our understanding of their function in WTs and help explain changes in KO behavior.

While neurons are often considered the 'active' components of our nervous system, the important role played by glia (Allen & Barres, 2009) has highlighted a need to examine these cells as well. When examining the total glial cell population in adult B6129S mice (fig 6C, G), we found a sex difference that is absent in C57Bl/6J mice (Pfau et al., 2016). This sex difference may represent a combination of multiple glial subtypes, (i.e. microglia, astrocytes, oligodendrocytes). Astrocytes and microglia are influenced by steroid hormones and support the production of sex differences in the brain (Lenz & McCarthy, 2015; McCarthy et al., 2003; VanRyzin, 2019), the presence of sex differences in the glial population itself may facilitate this process. TRPC2 KO reduced the overall number of glia without altering the presence of an overall sex difference in glia number (fig 6G). If this glia loss after TRPC2 KO occurs early, the production of sex differences in other measures may have been disrupted, such as volume and neuron number (fig 5A, 6E). For example, variable phagocytotic behavior by microglia in the developing MePD of rats leads to sex differences during development (VanRyzin, 2019). Still, examining total glia is less informative than more specific staining like the GFAP-immunohistochemistry we carried out to mark astrocytes. In GFAP-stained tissue, we found that B6129S males have more astrocytes than females and, in females, the left hemisphere has more astrocytes than the right (fig 6H). The same sex difference and female-specific laterality appears in the C57Bl/6^{J} MePD (Pfau et al., 2016) and was unaltered by TRPC2 KO (fig 6H). That this same pattern of sex differences and laterality is present in the MePD astrocyte populations of all mice examined thus far suggests an important and conserved function. Because TRPC2 KO and WT mice have similar astrocyte numbers, MePD astrocytes may not critically mediate those sexual and aggressive behaviors altered by TRPC2 KO. Indeed, activation of MePD astrocytes has been tied to anxiety related behaviors, like fear extinction (Martin-Fernandez et al., 2017). Anxiety behaviors happen to show many sex differences (An et al., 2011; Ter Horst, Carobrez, Van Der Mark, De Kloet, & Oitzl, 2012) which may explain the sex differences in astrocytes in C57Bl/6J mice (Pfau, Jordan, & Breedlove, 2019) and here (fig 6D). As there was no loss in astrocyte number, the glia lost in KO animals are those that do not express GFAP, such as microglia and oligodendrocytes. Interestingly, microglia are known to contribute to male-typical mounting behavior (Lenz & McCarthy, 2015), which is expressed by both KO sexes.

Aside from astrocyte number, GFAP-process complexity may express sex differences (Rasia-Filho, Fabian, Rigoti, & Achaval, 2004). Rats display robust sex differences in astrocyte complexity (Johnson et al., 2008; Johnson et al., 2012), however, data from C57Bl6J mice (Pfau et al., 2016), and now B6129S mice (table 4), suggest no such sex differences exist in the complexity of mouse astrocytes. Interestingly, knocking out TRPC2 appears to reduce the number of astrocyte process endings in both sexes (table 4). It is possible this relates to changes in other cell populations of the MePD. For example, while astrocyte populations remain at WT levels, the loss of neuron number in both male and female KOs suggests the astrocyte to neuron ratio is increased. So, the loss of endings seen in KO animals may be a product of the same number of astrocytes interacting with fewer neurons or synapses. Conversely, fewer astrocytic endings may prevent neurons from producing and maintaining synaptic connections. Synapses that rely on astrocytes may play disparate roles in males and females, influencing sex-typical behavior (Ullian, Sapperstein, Christopherson, & Barres, 2001). Indeed disrupting intra-amygdala connections in males, which may rely on astrocytes for maintenance, prevents opposite-sex urinary preference (Maras & Petrulis, 2010). Such an effect may occur in KO males, loss of astrocyte process endings could disrupt the maintenance of synapses important for opposite-sex urinary preferences, resulting in no urinary preference. Astrocyte complexity in the female MePD is responsive to ovarian hormones, suggesting specific morphological changes are associated with the behaviors or physiology during the stages of the estrous cycle (Martinez et al., 2006). So, MePD astrocyte function related to reproduction in females may be altered by the loss of astrocyte endings seen in KOs. Effects of TRPC2 KO on MePD volume, neuron cell number, overall glial cell number and astrocyte process endings appear in both sexes. Like TRPC2 KO behavior, the KO MePD shows both similarities and differences to WTs that vary based on animal sex. Changes to MePD volume (fig 5), neuron cell number (fig 6A, E) overall glial cell number (fig 6B, F) and astrocyte process endings (table 4) may all uniquely contribute to TRPC2 KO behavior. Measures impervious to TRPC2 KO, namely soma size (fig 6B, F) and astrocyte number (fig 6D, H), may not critically mediate those behaviors altered in KOs.

Nissl analysis of the rat indicates males have a larger VMHvl (Matsumoto & Arai, 1983), accompanied by larger neuron soma (Dugger et al., 2007) and more overall neuropil volume (Dulce Madeira et al., 2001), neuron dendrite arbors (A. Rasia-Filho et al., 2004), and input fibers from the fornix

(Larriva-Sahd, Rondan-Zarate, & Ramirez-Degollado, 1995). It has been suggested that the sex difference in rat VMHvl size relies on increased neuropil volume in males (Dulce Madeira et al., 2001; Larriva-Sahd et al., 1995), indeed, the number of neurons is similar between the sexes (Dulce Madeira et al., 2001). In rats, both high circulating testosterone and androgen receptors are necessary for the sex differences in VMHvl volume (Dugger et al., 2007; Matsumoto & Arai, 1983). Compared with the rat, relatively little is known regarding the overall anatomy and steroid responsiveness of the mouse VMHvl. Here, we find no sex difference in VMHvl volume when analyzing Nissl (fig 7A) or GFAP stained tissue (fig 7B). However, similar to rats (Dugger et al., 2007), males have significantly larger soma than females (fig 8B). Interestingly, researchers examining subpopulations of steroid receptor expressing neurons in the mouse VMHvl found no sex differences in soma size appear (Yang et al., 2013). Yet, analysis of calbindin positive neurons in the VMHvl unveiled larger soma size in male C57Bl/6J mice compared to females (Büdefeld, Grgurevic, Tobet, & Majdic, 2008), just as we found in Nissl stained B6129S tissue (fig 8B). So, steroid hormones may not influence sex differences in soma size, alternatively, increased volume of calbindin neurons is a non-cell autonomous effect of steroid hormones. Compared to males, female mice have more VMHvl neurons that coexpress the estrogen receptor 1 (ER) and progesterone receptor (PR) (Yang et al., 2013). However, when we examined overall neuron number, the mouse VMHvl appears similar to the rat (Dulce Madeira et al., 2001) as no sex differences appear (fig 8A). Compared with neurons, very little is known about sex differences in VMHvl astrocytes but GFAP mRNA increases in the overall hypothalamus as mice and rats age (Anderson et al., 2002; Kohama, Goss, Finch, & McNeill, 1995; Nichols, Day, Laping, Johnson, & Finch, 1993). Here, we find females have more astrocytes than males (fig 8D) and their GFAP-ir fibers have more endings (table 5). This sex difference may be independent of androgens as androgen insensitivity in mice does not change GFAP staining in the VMHvl (McQueen et al., 1990). Compared with the MePD, the VMHvl displays relatively no laterality and fewer sex difference.

The loss of TRPC2 largely influenced the male VMHvl (fig 11). Compared with WTs, KO males have fewer neurons and a smaller VMHvl (fig 7, 8). So, like the KO MePD, regional volume loss coincides with fewer neurons. Within KO animals, the loss of volume and neurons in males introduced a sex difference, KO females have larger MePD with more neurons (fig 7, 8). Interestingly, TRPC2 is not the

only gene to influence this region in a sex-specific manner. Steroidogenic factor-1 (SF-1) produces sex differences in the VMHvl, however, the loss of this gene disrupts the structure of the entire VMHvl without altering neuron number (Ikeda, Luo, Abbud, Nilson, & Parker, 1995). While neuron number is decreased in KO males (fig 8A) we found they had more astrocytes than WTs, eliminating a sex difference present in WT astrocyte number (fig 8D). Despite having more astrocytes, the GFAP-ir fibers of KO males have fewer nodes and endings than any other group (table 5). The only measure altered in female KOs was the loss of laterality in GFAP fiber length, in WTs the right hemisphere has longer fibers than the left (see results section). Therefore, TRPC2 appears essential for male VMHvl anatomy while it has limited influence over the VMHvl of females.

Neurons in the VMHvl of mice are critical for male social behavior (Lee et al., 2014; Lin et al., 2011). In males, the same population of neurons expressing estrogen receptor 1 (ER) exerts control over sexual *and* aggressive behaviors. When ER neurons are weakly activated, males mount intruders (Lee et al., 2014) while increasing ER neuron activation or activating the entire VMHvl generates attack behavior (Lee et al., 2014; Lin et al., 2011). TRPC2 function may influence ER neurons as KO males are capable of mounting but not aggression (Leypold et al., 2002). Given knocking out ER entirely within the VMH greatly *reduces* male sexual behaviors (Sano, Tsuda, Musatov, Sakamoto, & Ogawa, 2013), the ER neuron population in KOs must be sufficient to induce mounting but incapable of reaching the activation required for seeking aggression. Put another way, signals that reach the VMHvl may activate aggression seeking in WT animals but KO males lack the requisite number of neurons to reach an attack threshold, resulting in mounting behavior. It is also possible that the VMHvl neurons lost in TRPC2 male mice are a yet unrecognized subpopulation involved with aggression seeking behavior, leaving ER neurons intact. That ER neurons are unperturbed by TRPC2 loss is corroborated by the results from KO females. Like males, disruption of ER neurons in the VMHvl leads to a large *reduction* in female sexual behavior (Yang et al., 2013) but stimulation of the ER neurons in females induces close examination of intruders and sometimes mounting (Lee et al., 2014), common behaviors in KO females (Kimchi et al., 2007). Still, we see relatively no changes to the female VMHvl after TRPC2 KO (fig 7, 8, table 5). In all, changes to the VMHvl after TRPC2 KO may contribute to altered KO behaviors in males, including the loss of aggression.

As the social control of aggression is broader than a single node of behavioral circuitry (Yang, Yang, Chizari, Maheswaranathan, Burke Jr, et al., 2017), the neural mechanisms of VNO-mediated social behavior may rely on non-MePD and non-VMHvl neurons, i.e.. altered input to these regions may be enough to alter the behaviors associated with them. This was one of the original hypotheses used to explain KO behaviors (Kimchi et al., 2007). In KOs, it is probable that fewer excitatory signals reach the VMHvl from downstream regions as TRPC2 loss decreases AOB volume (Hasen & Gammie, 2009), VNO sensory neuron number (L. Stowers et al., 2002) and induces several changes to the MePD (fig 5, 6). Maternal aggression is mediated by ER neurons in the VMHvl (Hashikawa et al., 2017) and lost in KO mice (Hasen & Gammie, 2009) but we find no loss of VMHvl neurons in females. A loss of signals reaching the neurons controlling maternal aggression may produce this behavioral deficit in females. Despite having fewer neurons, loss of signals may also alter VMHvl function in male KOs. Experimentally activating ER+ neurons expressing the progesterone receptor in the VMHvl of *TRPC2 KO males* induces aggression, suggesting male KOs have adequate neurons to produce attack behavior (Yang, Yang, Chizari, Maheswaranathan, Burke Jr, et al., 2017) but they cannot be sufficiently activated by other regions. Still, understanding the function and type of neuron lost in the VMHvl of KO males may inform our understanding of male sociosexual behaviors.

While females show relatively no genotype differences in the VMHvl, it remains possible that cell type changes without altering cell number. KO males, too, may show different populations despite changes in cell number. Indeed, astrocyte number but not overall glial number is increased in the KO male VMHvl, suggesting more astrocytes form at the cost of microglia or oligodendrocytes. Such processes are likely to occur during development. For example, synapses within the developing VMHvl change from GABAergic to glutamatergic, a process facilitated by astrocytes (Chen et al., 1995). This may proceed differently after TRPC2 loss; in KO males because they have more astrocytes (fig 8D) with less complexity and in KO females because of altered laterality in astrocyte complexity (table 5). Varying the numbers of GABA and glutamate synapses formed in the VMHvl would alter its function in adult KOs. Indeed, GABA and glutamate receptors in the MePD modulate aggression and asocial behavior respectively (Hong et al., 2014). Astrocyte number and complexity in adulthood may also influence VMHvl activity. For one, astrocytes are known to contribute to cellular signaling (Araque, Parpura, Sanzgiri, &

Haydon, 1999) and astrocytes assist in the scalable activation of another hypothalamic region (Gordon et al., 2009). Additionally, cultured hypothalamic astrocytes release progesterone in response to estradiol (Micevych et al., 2007), so they may play a role in modulating VMHvl progesterone receptor neuron activity too (Yang et al., 2013). The cellular changes after TRPC2 loss that decrease the neuron to glia ratio in the VMHvl of males are accompanied by less astrocyte complexity. So, like the MePD, more astrocytes interacting with fewer neurons may lead to this loss in GFAP-ir fiber complexity or vice versa. Despite the robust morphological changes discovered here, non-anatomical differences between WT and KO animals may still influence unique KO behavior.

After TRPC2 loss, we found specific changes in two brain regions associated with those behaviors altered in TRPC2 KOs, however, it is possible these changes are the result of a global disruption in brain development. We feel that several of our findings refute this notion. First, many anatomical measures in KO animals match their WT counterparts, including most measures in the female VMHvl. Second, measuring volumes (fig 9A) and counting cells (Fig 9B, C) in the VMHdm in experimental animals indicated no effect of the KO. This region lies outside the accessory olfactory system (Keverne, 1999) which also suggests the effects of TRPC2 loss are limited to regions affected by the VNO. However, TRPC2 is expressed in the main olfactory epithelium (Omura & Mombaerts, 2014) and reproductive organs (De Clercq et al., 2017; Jungnickel et al., 2001) so a VNO-centric view regarding KO behavior (Kimchi et al., 2007) may bias interpretations of findings. Indeed, some researchers have found increased testosterone levels in KO mice (Kimchi et al., 2007). Future research may examine how the altered KO MePD and VMHvl responds to gonadal hormones. Still, focusing on brain regions involved with aggression and sexual behaviors means that, while these results may not directly relate to the VNO, they very likely relate to KO loss of aggression and the expression of male-typical sexual behavior directed towards both males and females in both sexes. Understanding the underlying neural circuits that produce the fascinating KO behaviors is important regardless of where TRPC2 loss induces the change.

In sum, MePD regional volume and cellular measures of B6129S mice show sex and laterality differences while virtually no laterality and few sex differences appear in their VMHvl. Robust sex- and hemisphere-specific changes appear in several measures after TRPC2 KO. Overall, the MePD contains more effects of the KO than the VMHvl, suggesting the influence of TRPC2 loss is reduced as information

is passed down vomeronasal brain circuitry and converges with other pathways. Most MePD measures were affected by TRPC2 KO but the differences varied by sex. Interestingly, TRPC2-dependent influence over the VMHvl is specific to males. Together, our data indicate the anatomy of two forebrain regions critical to social behavior are altered in TRPC2 KO animals. That these appear differently in male and female KO mice suggests TRPC2 has different functions in male and female mice. Future research may address how TRPC2 loss allows these sex-specific changes to appear and how the unique TRPC2 KO brain leads to their distinctive behaviors.

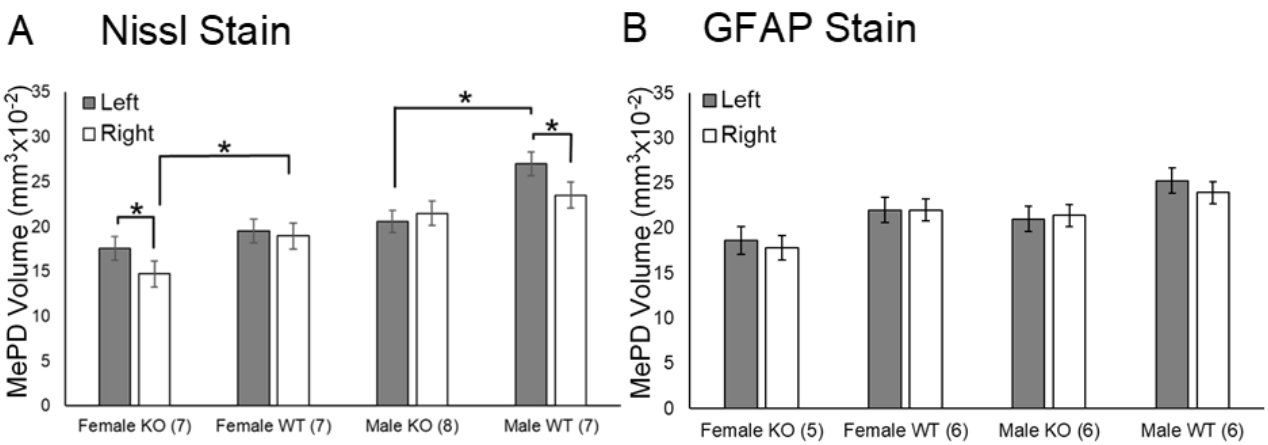

Figure 5: *Loss of TRPC2 alters MePD regional volume in a sex- and side-specific manner based on measures from Nissl stained brain sections in adult B6129S mice.* A) The MePD is lateralized in WT males, with the left MePD larger than the right and a sex difference appear in both hemispheres, males having larger MePD than females (left $p<0.001$, $d=2.03$; right $p=0.035$, $d=1.24$). TRPC2 KO decreases the size of the MePD in both sexes but in different hemispheres. Compared with WTs of the same sex, KO males have smaller *left* MePD and KO females have smaller *right* MePD. As such, the sex difference in the left hemisphere disappears in TRPC2 KO mice, with only the right MePD being larger in males than females ($p=0.002$, $d=1.83$). Which sex carries laterality is flipped after TRPC2 loss as KO females have a larger *left* MePD and no laterality exists in KO males. B) Measures of MePD volume in GFAP/hematoxylin stained sections showed no such significant differences between groups. (*p<0.05, error bars ±SEM)

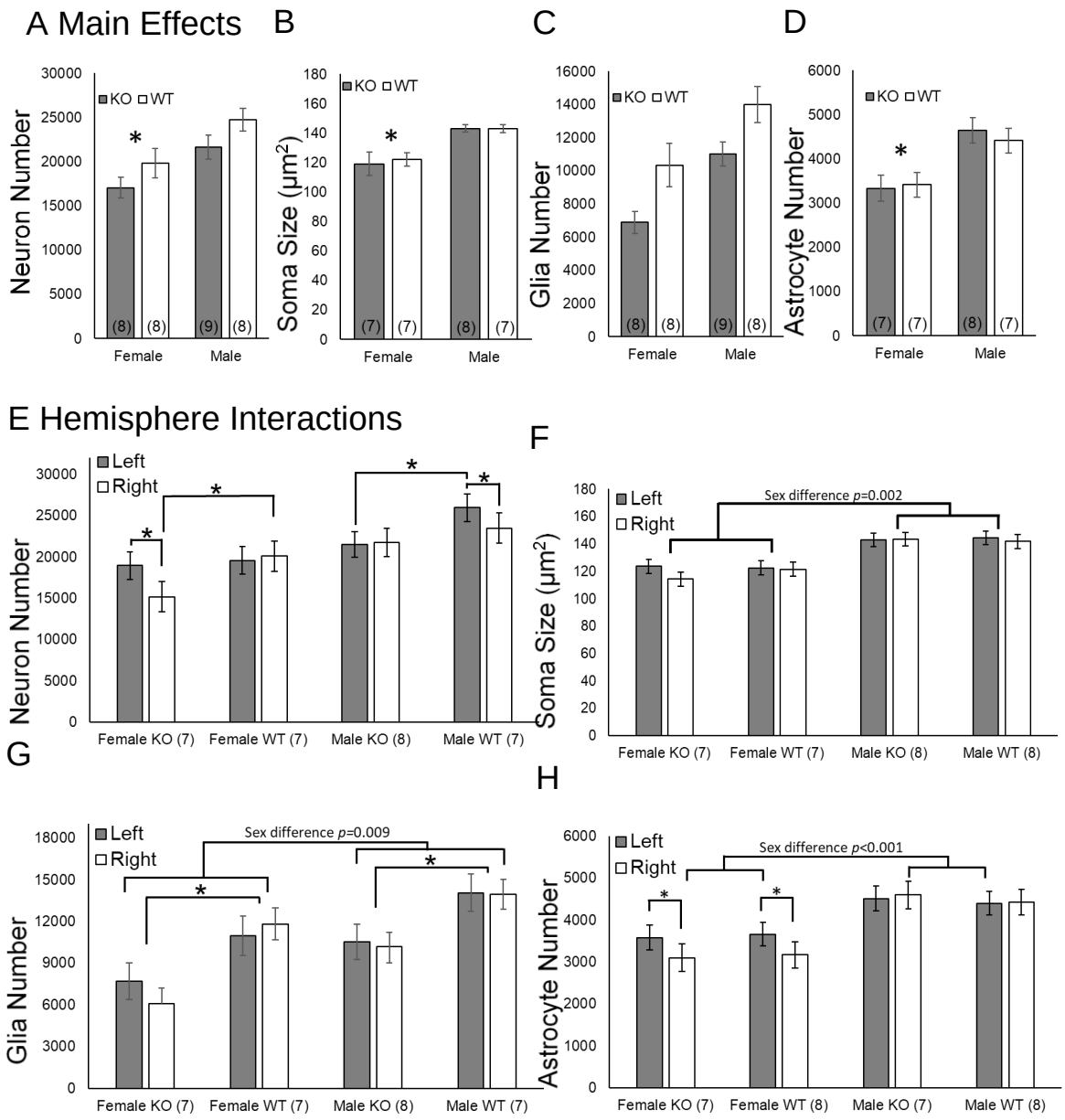

Figure 6: *Both laterality and sex differences appear in the MePD of B6129S mice while TRPC2 presence affects neuron and overall glia number only.* (A,E) The MePD of male mice has more neurons but this sex difference is mediated by both hemisphere and genotype. WT females show no laterality in neuron number, *left*-biased laterality is present in WT males. Compared with WTs, KO males have fewer neurons in the *left* hemisphere while KO females have fewer neurons on the right. Within sexes, male KOs have fewer neurons in the *left* MePD while females display neuron deficits on the *right*. As a result, laterality in KOs swaps sexes, KO females have more neurons in the *left* hemisphere while no laterality appears in

KO males. (B, F) Soma of MePD neurons are larger in males regardless of genotype or hemisphere. (C, G) Glia number is greater in WTs and both genotypes display a male-biased sex difference. (D, H) Male B6129S mice have more astrocytes than females. Females have more astrocytes in the left hemisphere, regardless of genotype. (*$p<0.05$, error bars ±SEM)

Table 4: *Statistical measures of MePD astrocyte complexity. Bolded numbers are significantly greater than plain text measures. Mean±SEM.*

	FEMALES		**MALES**		**Sex**		**Genotype**		**Laterality**	
	TRPC2 +/+	**TRPC2 -/-**	**TRPC2 +/+**	**TRPC2 -/-**	$F_{(1,16)}$	***p***	$F_{(1,16)}$	***p***	$F_{(1,16)}$	***p***
Branch number	5.47 ± 0.14	5.01 ± 0.21	5.47 ± 0.17	5.37 ± 0.13	1.22	0.29	1.17	0.3	1.38	0.26
Node Number	16.31 ± 1.31	14.42 ± 0.8	14.85 ± 1.2	12.89 ± 0.59	2.11	0.17	3.48	0.08	1.83	0.19
End Number	**22.18 ± 1.27**	19.5 ± 0.95	**20.2 ± 1.05**	18.58 ± 0.71	2.04	0.172	**4.48**	**0.05**	2.4	0.14
Total Length	291.9 ± 12.85	257.6 ± 20.17	260.7 ± 12.85	252.1 ± 13.2	1.79	0.2	2.43	0.14	1.53	0.24
Mean Branch Length	53.03 ± 3.86	52.95 ± 2.96	48.93 ± 1.37	46.43 ± 1.31	4.14	0.06	0.245	0.63	0.058	0.81

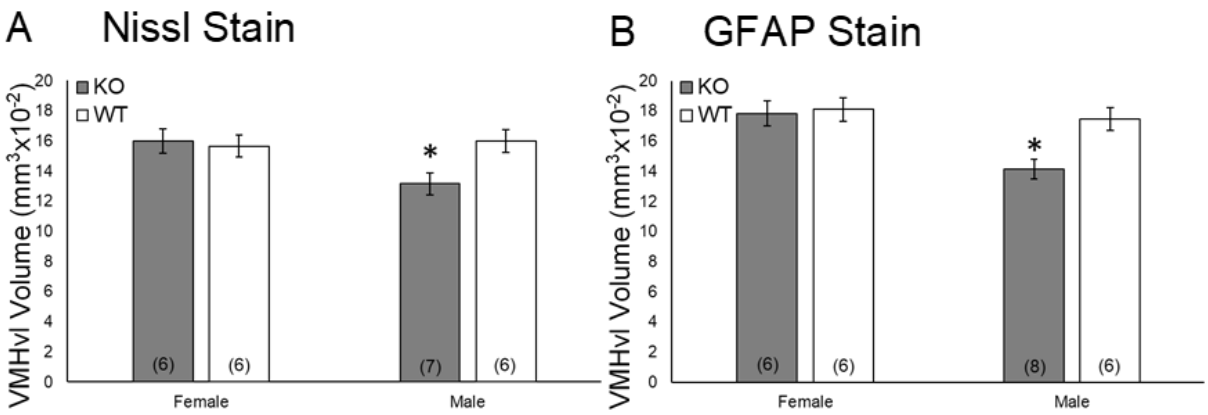

Figure 7: *Sex does not influence VMHvl regional volume in B6129S mice.* However, volume loss in the KO male VMHvl is accompanied by a sex difference in KO animals as revealed by (A) Nissl stained and (B) GFAP-stained tissue. (*$p<0.05$, error bars ±SEM)

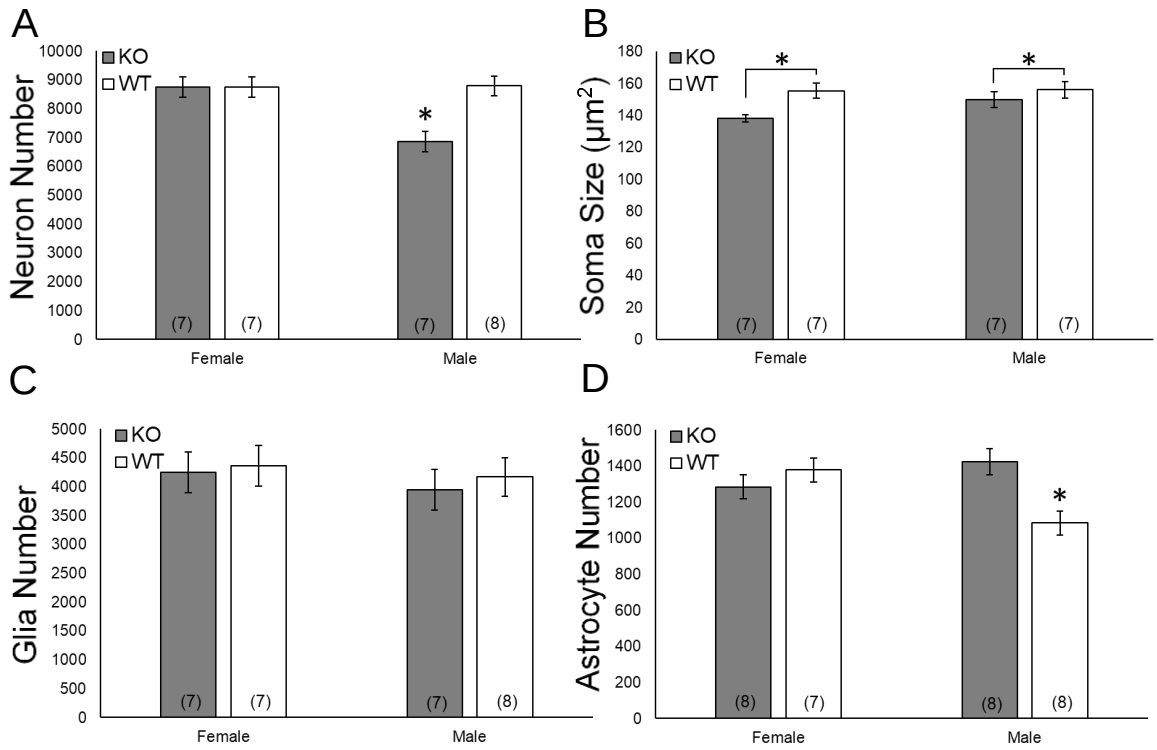

Figure 8: *The VMHvl of B6129S mice shows no sex differences apart from a female-biased number of astrocytes.* The presence of TRPC2 influenced soma size in both sexes and neuron and astrocyte number in males (A) Neuron number is similar between WT sexes. KO males have fewer neurons than WTs and this leads to a female-biased sex difference in KOs. (B) Soma size is influenced by genotype alone; WT neuron soma are larger. (C) Glial number was unaffected by sex and genotype but D) B6129S females have more astrocytes than males. The number of astrocytes in KO males is typical of WT females (*$p<0.05$, error bars ±SEM).

Table 5: *Statistical measures of VMHvl astrocyte complexity.* Bolded numbers are significantly greater than nonbolded measures based on main effects and interactions of independent ANOVAs on each branch measure. Significant interactions are described in results; Mean±SEM.

	FEMALES		**MALES**		**Sex**		**Gene**		**Laterality**	
	TRPC2 +/+	**TRPC2 -/-**	**TRPC2 +/+**	**TRPC2 -/-**	$F_{(1,17)}$	***p***	$F_{(1,17)}$	***p***	$F_{(1,17)}$	***p***
Branch number	5.1 ± 0.22	5.15 ± 0.18	5.22 ± 0.23	5 ± 0.21	0.002	0.97	0.14	0.71	0.01	0.98
Node Number	**20.2 ± 0.54**	**19.43 ± 0.96**	**21.15 ± 0.9**	15.8 ± 1.45	1.54	0.23	**8**	**0.012**	0.58	0.46
End Number	**26.32 ± 1.52**	**27.31 ± 1.92**	**25.99 ± 1.44**	20.51 ± .13	**5.68**	**0.029**	2.25	0.15	0.013	0.91
Total Length	329.15 ± 43.5	305.21 ± 25.41	333.86 ± 13.53	275.26 ± 23.36	0.2	0.66	2.13	0.16	1.39	0.25
Mean Branch Length	64.51 ± 8.09	59.66 ± 4.01	65.19 ± 5.23	55.29 ± 4.14	0.113	0.74	1.8	0.2	0.78	0.39

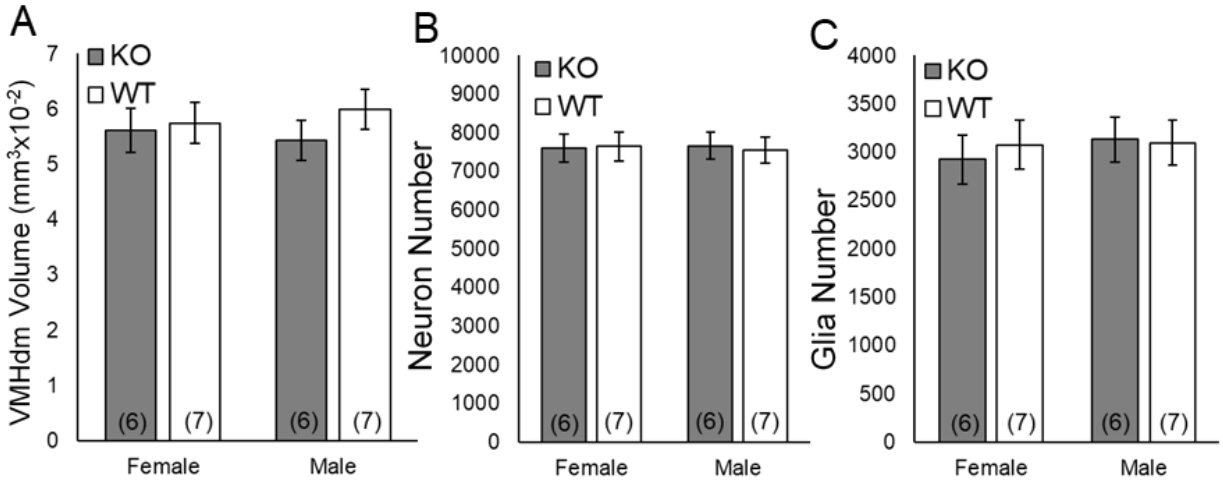

Figure 9: *Neither sex nor genotype influence VMHdm morphology in Nissl tissue.* A) Volume, B) neuron number and C) glial number are similar regardless of sex or TRPC2 presence (error bars ±SEM)

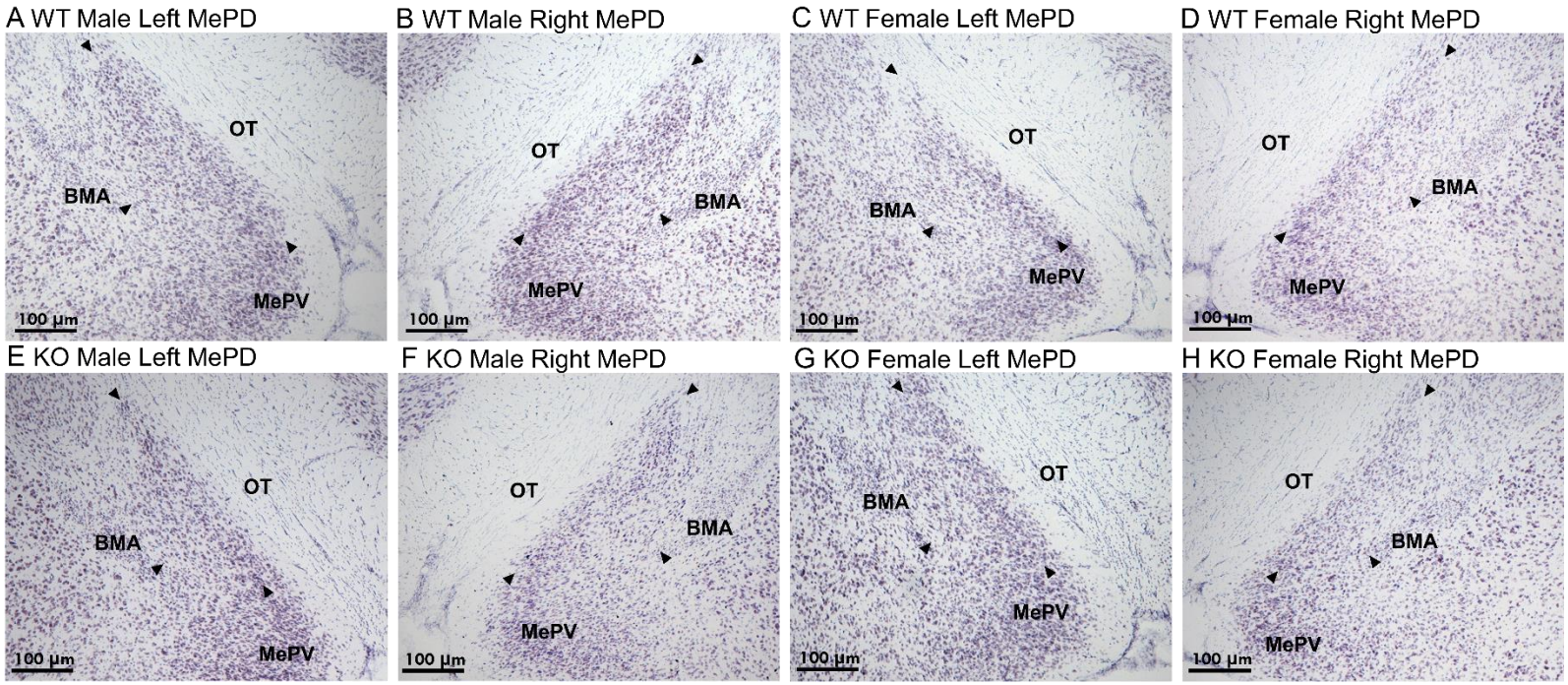

Figure 10: *The posterodorsal aspect of the medial amygdala (MePD; black arrowheads) in adult B6129S (WT) mice and TRPC2-/- (KO) mice was stained for Nissl substance with thionin, revealing sex differences, laterality and effects of TRPC2 loss.* WT males (A, B) have larger MePD than females (C, D) within both hemispheres. The left MePD of male WTs is larger than the right (A, B) while no laterality exists in WT females (C, D). TRPC2 loss affects these measures. TRPC2 KO in males reduces the volume of the left MePD (A, E) and, unlike male WTs, the KO males show no laterality (C, D). In contrast, TRPC2 KO reduced volume of the right MePD in females (D, H), resulting in female KOs having larger left MePD than right (G, H). Abbreviations: basomedial nucleus (BMA), posteroventral medial amygdala (MePV), optic tract (OT).

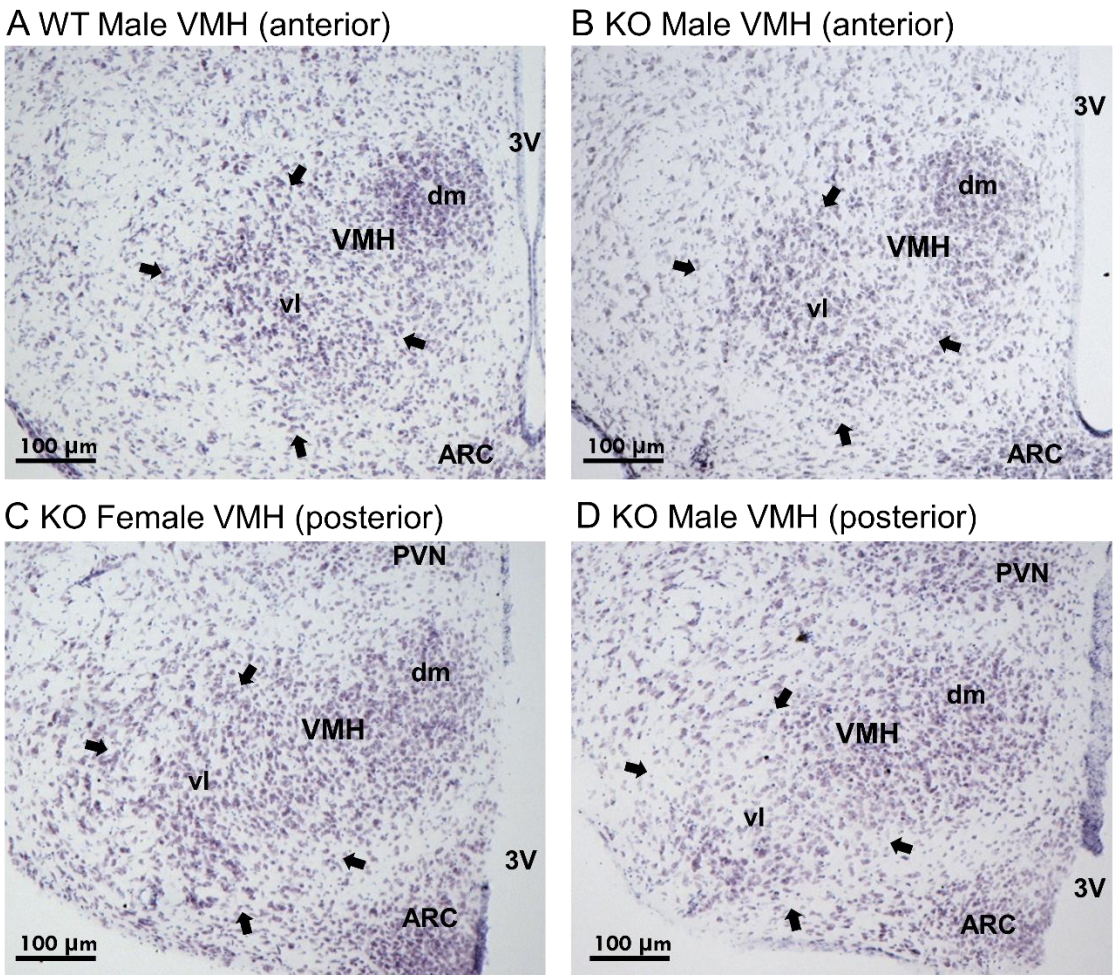

Figure 11: *The ventromedial hypothalamus (MePD; Black arrows) in adult B6129S mice (WT) and TRPC2 -/- (KO) males stained with thionin, revealing an effect of genotype through-out the region.* WT males (A) have larger VMHvl than KO males (B) and KO females (C) have larger VMHvl than KO males (D). Abbreviations: paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), ventrolateral subdivision (vl), dorsomedial subdivision (dm), arcuate nucleus (ARC).

CHAPTER 4: PROLONGED SEXUAL EXPERIENCE ALTERS TRPC2 KO MALE BEHAVIORAL AND FOREBRAIN RESPONSES TO URINE

Abstract

Pheromones secreted in female urine facilitate male copulatory behavior. For WT mice, female pheromones are pro-copulatory and male pheromones inhibit copulation and induce aggression, but the development of these behaviors is more nuanced. A naïve WT male will mount non-estrous females, and even other males, but experience with a receptive female induces a change in their behavior. WT males exposed to receptive females decrease mounting of diestrus (non-receptive) females. Thus, sexual experience seems to refine mounting behavior such that an estrous urinary signal is required to induce high levels of mounting. This preference for mounting estrous females relies on the vomeronasal organ (VNO) for organizing the behavioral cue and activating it. TRPC2 is an ion channel that assists the vomeronasal organ (VNO) in sending signals to the brain and knocking it out reduces VNO function. Naïve male TRPC2 knockout (KO) mice show anogenital investigations, mounting, and pelvic thrusts towards *both* males and females, at levels comparable to WT male behavior towards receptive females. While KO males seem to no longer require female pheromones for mounting, they retain the ability to detect chemical signals specific to female urine. Counter to evidence in WT males, reports indicate that KO males show *increased* male-male mounting after cohabitation with a female for only 10 days. If VNO signals are already disrupted by TRPC2 loss but sex detection is possible, prolonged experience may be required to organize TRPC2 KO male mounting behavior.

Introduction

Murine pheromones mediate courtship, copulation and aggression. For experienced male mice, pro-copulatory signals are produced by receptive females while male pheromones inhibit copulation and induce aggression (Connor, 1972). This preference for mounting receptive females requires an intact vomeronasal organ (VNO) to organize the behavioral response (Hayashi & Kimura, 1974; Wysocki & Lepri, 1991). Naïve males that lack the transient receptor potential cation channel 2 (TRPC2), an ion channel used in VNO signaling (Kim et al., 2012; Lucas, Ukhanov, Leinders-Zufall, & Zufall, 2003), show anogenital investigations, mounting, and pelvic thrusts towards *both* males and females, at levels

comparable to WT-male responses to receptive females (Kimchi et al., 2007; Leypold et al., 2002). Counter to evidence in WT mice, reports indicate that KO males show *increased* male-male mounting after cohabitation with a female for ten days (Leypold et al., 2002) and no preference for female urine (Bayless et al., 2019). However, the KO VNO retains the ability to detect chemical signals specific to female urine (Beny & Kimchi, 2016), albeit at lower levels than WT (Kim et al., 2012; Spehr et al., 2009). While it is reasonable to assume the behavioral paradigms of TRPC2 KO mice are unique (Yu, 2015), knocking down VNO function may simply increase the latency for the organization of WT male mounting behavior, a possibility examined here.

Sexually naïve WT males mount non-receptive females and even males (Hayashi & Kimura, 1974) while sexual experience refines mounting and aggressive behavior such that an estrous urinary signal is required to induce high levels of mounting (Hayashi & Kimura, 1974; Pankevich et al., 2004; Wysocki & Lepri, 1991). The sex-attractant signal is so strong, experienced WT males will mount a castrated male swabbed with estrous female urine (Connor, 1972; Hayashi & Kimura, 1974). This behavioral organization is apparent within the VNO recipient pathway of naïve and experienced rodents (Baum, 2009; Bayless et al., 2019; Pankevich et al., 2006). In rats, fos-immunoreactive cells and dopamine levels in the nucleus accumbens core (NAcc) are elevated after exposure to receptive female urine in experienced, but not naïve, males, suggesting a reward motivation develops after experience (Hosokawa & Chiba, 2005). Likewise, female urine activates the NAcc in the brains of experienced mice (Pankevich et al., 2006). So, sexual and pheromonal experience makes receptive female urine a rewarding stimulus for male mice. While ten days of cohabitation with a cycling female did not produce a female mounting preference, it is known that TRPC2 KO weakens VNO signals (Kim et al., 2012). So, extending pheromone exposure and increasing sexual experience may provide enough stimuli to produce a bias for female urine. However, if the rewarding experience of female pheromones requires TRPC2, extending experiences that facilitate mounting behavior organization in mice will be ineffectual in KO males.

We examine these two possibilities by providing KO males extended cohabitation with receptive females that produce pro-copulatory pheromones or non-receptive females lacking such pheromones (Hayashi & Kimura, 1974). Following treatment, mounting behavior was assessed using four different

intruders: a castrated male or receptive female painted with urine from either intact males or receptive females. Experimental males were then exposed to receptive female urine or saline and their brains analyzed for c-fos activity. Fos-immunoreactive cells were examined in brain regions associated with partner preference, sexual behavior, aggression and pheromone reception (DA Adekunbi et al., 2018; Bayless et al., 2019; Hasen & Gammie, 2009; Lee et al., 2014). If TRPC2 is dispensable for urinary preference and simply increases the latency for behavioral organization then KO males exposed to receptive females should find female scents pro-copulatory and rewarding. Such mice would mount intruders painted with female urine at a higher level than those with male urine (Connor, 1972). Concurrently, sexually naïve males housed with non-receptive females should show no urinary preference for mounting. Like rats (Hosokawa & Chiba, 2005), changes in behavioral responses to urine may be marked by altered brain activation in response to female urine. Here, we report that prolonged sexual experience in TRPC2 KO animals leads to changes in both behavior and brain activation. However, the pattern of behavioral development is reversed, sexual experience decreased reactions to urine.

Materials and methods

Animals:

TRPC2 -/- (B6;129S1-Trpc2tm1Dlc/J JAX stock number: 021208) mice and TRPC2 +/+ (B6129SF2/J021208; JAX stock number: 101045) were purchased and used to establish a colony by breeding KO to KO and WT to WT. Pups from homozygous pairings were weaned at PD 21, ear punched to confirm genotype with PCR, and housed with same-sex littermates. To induce receptive and non-receptive behavioral states, all WT female mice (PD 56-85) were gonadectomized (OVEX) then randomly provided a silastic capsule (1.98 mm id/3.17 mm od) filled with sesame oil (Blank) or sesame oil containing 17β-estradiol (E2) (50 µg in 0.025 ml). This provided control over sexual behavior and pheromone production of estrous urinary signals (Malkesman et al., 2010) but removed the need to switch out pregnant females. Intruder WT females (PD 56-85) were given OVEX+E2 and intruder WT males (PD 56-85) were castrated. After surgery, animals were given ten days to recover. Adult KO males (PD 56-85) were singly housed for a week then randomly assigned to two receptive or two non-receptive

females. Cohabitating females were introduced to the singly housed experimental male then given 35 days of cohabitation.

Urine collection:

Urine was collected in a metabolic cage from OVEX+E2 females or intact males. All urine was pooled, placed in 75 µl aliquots and stored at -80°C until used.

Behavioral assays:

Experimental males were exposed to a castrated male or receptive female painted with opposite- or same-sex urine. In all, experimental males interacted with four different intruders; 1) ♀OVEX+E2+♀urine, 2) ♂GDX+♂urine, 3) ♀OVEX+E2+♂urine, 4) ♂GDX+♀urine. On day 36, female cohabitants were removed from experimental male cages. Behavioral assays began on day 37 then occurred every other day while the order of exposure was randomized for each male. Two to five hours prior to behavior, female intruders were injected with progesterone (500 µg in 0.1 ml of peanut oil) to ensure receptivity. Behavioral assays began one hour after onset of the dark cycle and took place under dim red lighting. Experimental males were transported in their home cage to a behavioral room and then given ten minutes of acclimation. The intruder animal was brought into the room, their anogenital region painted with 75 µl of undiluted urine and then introduced to the experimental males' cage for a ten-minute recorded interaction. After, experimental males were returned to the colony room in the same cage and intruders were placed in a new cage to avoid contaminating cage-mates with urine. Behavior of experimental males was coded using Noldus The Observer XT 10.5. After interacting with each intruder type, experimental males were re-housed with the same females from the 35-day cohabitation period, given 10 days of interaction, then singly housed for 24 hours.

Olfactory exposure and brain collection:

Following 24 hours of isolation, males were randomly assigned to an exposure group, saline or urine pooled from OVEX+E2 females. Using a sterile cotton-tipped applicator, 75 µl of saline or OVEX+E2 urine was swabbed onto the noses of males ninety minutes prior to perfusion. Males were overdosed with sodium pentobarbital (210 mg/kg) and transcardially perfused with 0.9% saline followed by phosphate buffered (0.1M, pH 7.4) 4% paraformaldehyde. Brains were postfixed 2 hours in the same 4% paraformaldehyde solution (room temp) and cryoprotected in 20% sucrose in 0.1M phosphate buffer for

at least 48 hours (at 4° C). The right dorsal surface of the cortex was scored, to mark hemisphere, then sectioned at 30 microns on a freezing sliding microtome. Three adjacent series were collected and stored at -20°C in de Olmos cryoprotectant until stained (de Olmos et al., 1978). Animal procedures were approved by the Institutional Animal Care and Use Committee at Michigan State University.

C-fos immunohistological staining:

A brain series was brought to room temperature and rinsed with phosphate buffered saline (PBS, 0.1M, pH7.4). A phosphate buffered saline solution containing 0.3% Triton and 0.1% gelatin (PBS-GT) was used as a vehicle for immunoreagents and PBS or PBS-GT was used for rinsing. Sections were incubated in 0.3% H_2O_2 in PBS-GT (10 min) then 20% normal goat serum (NGS) in PBS-GT. Tissue was rinsed in PBS then placed in primary antisera (c-Fos rabbit polyclonal: 1:10,000, Santa Cruz Biotechnology, sc52) in 1% NGS overnight (4° C). Sections were rinsed once more with PBS then incubated 1 hour in biotinylated goat-anti rabbit secondary antibody (1:500, Vector Laboratories, Burlingame, CA) in 1% NGS and rinsed in PBS. Then incubated 1 hour in avidin-biotin peroxidase complex (Elite Avidin Biotin complex kit, PK-6100, Vector Laboratories, Burlingame, CA). After PBS rinses, c-Fos immunoreactive cells were visualized with a diaminobenzidine (0.0025% DAB, Sigma, St. Louis, MO), H_2O_2 (0.15%) and nickel chloride solution (0.2%) in Tris buffer (0.05M, pH 7.2) for 4 minutes. Slides were rinsed in PBS to quench the reaction then mounted onto gelatin subbed slides, dehydrated in ethanol, cleared and coverslipped with Permount.

C-fos-immunoreactive cell counts:

Using a Zeiss Axioplan II microscope equipped with an Optronics MicroFire digital video camera and StereoInvestigator software (v. 7.0, MBF Bioscience, Williston, VT), regions of interest were outlined at low magnification. These included the accessory olfactory bulb, nucleus accumbens core, medial preoptic area, bed nucleus of the stria terminalis, posterodorsal aspect of the medial amygdala and ventromedial hypothalamus ventrolateral portion. Landmarks included the third and lateral ventricles, optic tract, and anterior commissure using a standard mouse atlas (Paxinos & Franklin, 2004). Briefly, the StereoInvestigator optical fractionator setup was used to randomly select a location within outlined regions, four pictures were captured per region in each hemisphere using a 20X Zeiss plan-neofluar objective. Pictures were uploaded into Image J v1.52a and, particles were analyzed after placing a mask

with the adjust threshold setting (Swift-Gallant, Coome, Srinivasan, & Monks, 2016). The threshold was reduced to 20 and excluded pixels smaller than ten. Particles meeting these criteria were automatically counted. Analysis was carried out by an experimenter blind to subject treatment and exposure.

Statistical analysis:

For behavioral assays, mixed design ANOVA was utilized, examining each behavioral event across the four types of intruder (within-subjects) and treatment group (between-subjects). Significant main effects and interactions were examined with posthoc Bonferroni correction. A mixed design ANOVA will also be used to examine c-fos immunoreactive cells in the MPOA (medial preoptic nucleus), BNST, MePD and VMHvl, with hemisphere as a within-subjects variable and olfactory exposure and treatment as between-subject factors. The hemisphere of the AOB was not readily determine in all sections so differences here were examined using a 2-way ANOVA with between-subject factors olfactory exposure and treatment.

Results

Behavior:

Housing condition had no effect on the number of AGIs displayed by males ($F_{(1,16)}$=0.12, p=0.73), or interacted with the type of intruders ($F_{(3,16)}$=2.17, p=0.137). However, the intruder type mediated AGIs for all males ($F_{(3,16)}$=3.06, p=0.045; fig 12). Both experimental groups investigated the anogenital regions of ♂GDX+♂urine more than ♀OVEX+E2+♀urine (p=0.009, d=0.91) or ♀OVEX+E2+♂urine (p=0.016, d=0.93). Latency to AGI was not affected by treatment ($F_{(1,19)}$=0, p=0.99; fig 13A) nor it's interaction with intruder type ($F_{(3,19)}$=1.77, p=0.19). However, intruder type influenced the AGI latency of all males ($F_{(3,19)}$=17, p=0.001; fig 13). Compared with ♀OVEX+E2+♂urine, experimental males showed increased latencies to AGI for intruders carrying a male urinary signal regardless of sex (vs. ♂GDX+♂urine p=0.026, d=0.95; vs. ♀OVEX+E2+♂urine p=0.008, d=0.41). Prolonged cohabitation with receptive females increased total mount number ($F_{(1,29)}$=9.61, p=0.004, d=0.58; fig 14A) and the different intruders elicited different mounting responses experienced and naïve males ($F_{(3,29)}$=3.06, p=0.045). Responses also influenced experience ($F_{(3,29)}$=3.65, p=0.025). Posthoc analysis (fig 14B) indicated that prolonged sexual experience increased mounting of ♀OVEX+E2+♂urine intruders (p<0.001; d=1.5) while inexperienced

males mounted ♀OVEX+E2+♂urine intruders less than ♂GDX+♂urine (p=0.004, *d*=1.04) and ♀OVEX+E2+♂urine (p=0.022, *d*=0.97). Mounting latency was similar regardless of intruder type ($F_{(3,18)}$=2.3, *p*=0.117) and treatment ($F_{(1,18)}$<0.01, *p*=0.99), while their interaction was not significant ($F_{(3,18)}$=0.83, *p*=0.5; fig 15). Experienced males displayed more intromissions than inexperienced males ($F_{(1,20)}$=5.24, *p*=0.033; *d*=0.99; fig 16A) and, while no overall effect was seen for female urinary status ($F_{(1,20)}$=2, *p*=0.18), it interacted with treatment ($F_{(1,20)}$=5, *p*=0.037). Posthoc analysis (fig 16B) found that a male urinary status reduced intromissions by naïve males (*p*=0.001 *d*=1.72), but not experienced males (*p*=0.67), compared to ♀OVEX+E2+♀urine. Intruder urinary status did not influence latencies to intromission ($F_{(1,5)}$=1.7, *p*=0.25) nor did it's interaction with treatment ($F_{(1,5)}$=2.2, *p*=0.2), however, experienced males were quicker to intromit than inexperienced ($F_{(1,5)}$=6.7, *p*=0.049 *d*=0.97; fig 17).

C-fos-immunoreactive cell analysis:

Overall mean number of c-fos-immunoreactive cells for each brain region are found in table 6. Exposure to urine (fig 18A, B) did not lead to increased activation in the AOB ($F_{(1,20)}$=0.17, *p*=0.83) regardless of housing condition ($F_{(1,20)}$=0.09, *p*=0.77). C-fos-ir cell number in the BNST was unaffected by housing condition ($F_{(1,20)}$=4.32, *p*=0.051) and hemisphere ($F_{(1,20)}$=1.63, *p*=0.21). However, exposure to urine significantly increased the number of BNST c-fos-ir cells ($F_{(1,20)}$=11.16, *p*=0.003, *d*=1.29). The effect of exposure was influenced by cohabitating female status ($F_{(1,20)}$=6.47, *p*=0.019) and hemisphere ($F_{(1,20)}$=8.5, *p*=0.009). Urine exposure increased c-fos-ir cells in the BNST of naïve males in both hemispheres (left *p*=0.002, *d*=2.05; right *p*<0.001, *d*=2.48), however, the *right* hemisphere had significantly more c-fos-ir cells than the *left* in inexperienced males exposed to urine (*p*=0.04, *d*=0.64; fig 18A). Conversely, males housed with receptive females showed no increase in BNST activity after urine exposure (*p*=0.58; fig 18B) or laterality (*p*=0.45). Looking at the BNST of males exposed to urine, naïve males showed greater activation than experienced (*p*=0.004, *d*=1.8; fig 19B). The number of c-fos-ir cells in the MPOA was unaffected by housing condition ($F_{(1,20)}$=0.23, *p*=0.63) and hemisphere ($F_{(1,20)}$=0.38, *p*=0.54). However, the MPOA had increased activation after urine exposure ($F_{(1,20)}$=15.96, *p*=0.001, *d*=1.52; fig 18A, B), regardless of sexual experience ($F_{(1,20)}$=1.17, *p*=0.29). Urine exposure did not activate the MePD ($F_{(1,19)}$=0.54, *p*=0.47) nor was there an overall effect of sexual experience ($F_{(1,19)}$=1.17, *p*=0.29). However, an effect of hemisphere appeared ($F_{(1,19)}$=5.52, *p*=0.03), which was mediated by experience

($F_{(1,19)}$=4.45, p=0.048). Posthoc analysis found that prolonged sexual experience alone produced greater activation in the *right* MePD compared with the *left* (p=0.008; d=0.5; fig 19B), interestingly, this laterality did not appear in males housed with non-receptive females (p=0.87; fig 18A). The number of c-fos-ir cells in the NAcc was increased after exposure to urine ($F_{(1,20)}$=9.3, p=0.006; d=1.23; fig 18A, B) but unaffected by treatment ($F_{(1,20)}$=2.49, p=0.131) and hemisphere ($F_{(1,20)}$=0.075, p=0.79). Finally, the VMHvl showed no activational differences across all three variables, urine exposure ($F_{(1,18)}$=0.001, p=0.96), treatment ($F_{(1,18)}$=0.13, p=0.72) and hemisphere ($F_{(1,18)}$=0.13, p=0.72), nor any significant interactions. The means and SEM for each region are summarized by hemisphere in table 6.

Discussion

VNO signaling relies on a number of channels and second-messenger systems (Kim et al., 2012; Norlin et al., 2003; Stowers & Spehr, 2015), some of which seem dispensable for male-typical behavior. For example, removal of $G_{\alpha}i2$ and $G_{\alpha}o$, g-proteins found in the VNO, disrupts aggression but does not change sexual behavior (Chamero et al., 2011; Norlin et al., 2003). However, knocking out the TRPC2 gene leads to drastic changes in aggression and sexual behaviors (Kimchi et al., 2007; Stowers et al., 2002). In lactating females, TRPC2 KO disrupts aggressive responses towards novel males (Hasen & Gammie, 2009; Wysocki & Lepri, 1991). This is accompanied by changes to the activation of their accessory olfactory brain circuitry when exposed to males or male urine (Hasen & Gammie, 2009, 2011). Interestingly, TRPC2 KO males can detect sex via olfaction after an aversive training paradigm (Beny & Kimchi, 2016) and their aggression can be activated by stimulation of the VMHvl (Yang, Yang, Chizari, Maheswaranathan, Burke, et al., 2017). So, TRPC2 KO males can change their behavior in response to olfactory cues and can express aggression given the proper stimulus. These findings prompted our study of the urinary preferences of sexually naïve and experienced TRPC2 KO males. We sought to determine if KO mice require prolonged pheromone and sexual behavior exposure to produce the female urinary preference seen in mice after minimal experience. We find inexperienced and experience male courtship behaviors seem to rely on both intruder sex and urinary status. In contrast, the presence of a female urinary signal appears important for the copulatory behavior of *inexperienced* males alone. We also find that urine activates some regions of the accessory olfactory brain circuit in both experimental groups, but

experience decreased activation within a region tied to urinary preference. Prolonged experience appears to decrease the influence of urine on the behavior and brain of TRPC2 KO males

Sniffing of the anogenital region is a courtship behavior that precedes copulation (Wysocki & Lepri, 1991). In Swiss Webster and C57Bl/6J mice, males reduce olfactory investigations of male urine compared with female urine (Pankevich et al., 2004). However, Swiss Webster males lacking a VNO investigated male and female urine the same number of times (Pankevich et al., 2004; Pankevich et al., 2006). We found that naïve KO mice do not alter the number of anogenital investigations (AGI) of female intruders based on urinary status and the AGI response to male intruders did not depend on urinary status either (fig 12). In a typical social context, male urine causes Swiss Webster and C57Bl/6J mice to delay their chemical investigations of an intruder (Pankevich et al., 2004; Pankevich et al., 2006). We also found this to be the case if the male odor was paired with a male intruder (fig 12). In comparison, females swabbed with *male or female* urine were investigated quicker than the males with male urine while latencies to investigating males with female urine were like all other measures (fig 13). So, TRPC2 KO males from both groups appear interested in the sex and urinary status of the intruder when performing AGIs. Disrupting VNO activity by blocking the nasopalatine ducts increases olfactory investigations (Levy, Sofer, Brumfeld, Zilkha, & Kimchi, 2019), suggesting increased olfactory investigations result from continued attempts to detect an odor. This is unsurprising given how much mice rely on odors to understand their social context (Keverne, 2004). So, changes to the number of olfactory investigations in TRPC2 KO male mice may simple be an attempt to detect more chemical cues to provide better social context.

A completely naïve male may attempt to mount any intruder, including males, until they learn through experience (Hayashi & Kimura, 1974; Wysocki & Lepri, 1991). Sexual behavior paired with olfactory detection of female urine organizes the mounting behavior patterns of adult male mice by producing a preference for female urine (Wysocki & Lepri, 1991). Analysis with C57Bl/6J males found that removal of the VNO leaves mounting behavior intact but eliminates their preference for estrous female urine (Pankevich et al., 2006). Early studies concluded that TRPC2 KO males have no preference for female urine because they can no longer detect the chemicals that would provide such information (Stowers et al., 2002). However, TRPC2 KOs are capable of detecting the sex of an intruder (Beny &

Kimchi, 2016) and respond to some pheromonal cues (Kelliher et al., 2006). We found that inexperienced males seem to discriminate between females swabbed with female urine and females swabbed with male urine because they decrease their mounting behavior when presented with the latter (fig 14B). This appears similar to the male preference for mounting receptive females (Wysocki & Lepri, 1991). However, inexperienced males do not alter their mounting of male intruders based on urinary status (fig 14B). This finding seems paradoxical because male urine inhibits mounting of intruder females, similar to mice with an intact TRPC2 gene (Mugford & Nowell, 1970), but does not inhibit mounting of male intruders. One possible explanation is that non-urine cues alter how inexperienced KOs behave. For example, in social situations mice may produce ultrasonic vocalizations that vary by sex and context (Nunez, Nyby, & Whitney, 1978; Portfors, 2007). Like olfactory signals, auditory signaling is an important aspect of mouse social behavior (Portfors, 2007). Castrated males do not produce ultrasonic vocalizations (Nunez et al., 1978) while intruder females could produce vocalizations typical of an estrous female (Neunuebel, Taylor, Arthur, & Egnor, 2015). Attending to non-urinary signals, like ultrasonic vocalizations, may alter the salience of urine presence. So, when experiencing female ultrasonic vocalizations, naïve KOs may seek animals carrying a female urinary signal.

After prolonged sexual experience, TRPC2 KO males mount and intromit all intruders at similar levels and latencies (fig 14B, 15B, 16B, 17). This represents a reversal of the behavioral progression previously found in mice where naïve males mount and intromit intruders indiscriminately but experienced males preferentially mount those with a female urinary signal (Hayashi & Kimura, 1974). One possible explanation is the loss of VNO neurons that selectively respond to female urine. Neurons in the VNO are highly plastic and when pheromones are present without the opportunity for mating, those neurons which detect female pheromones are lost and males lose interest in female urine (Xu, Lee, & Holy, 2016). A similar ‘use it or lose it’ paradigm may play out for KOs housed with receptive females. Their detection of pheromones is limited by TRPC2 loss but there are many opportunities for mating. Dissociating these signals may help to eliminate any neurons that would facilitate the production of a female urine preference through experience. As such, inexperienced males might have more VNO neurons detecting female urine than experienced KOs. In that case, fewer environmental signals may inform the expression of sexual behaviors for experienced males. Indeed, experienced KOs intromit female intruders quicker

than inexperienced males (fig 17) suggesting they require less sensory information to induce consummation.

In Swiss Webster mice, female urine induces c-fos expression in the male accessory olfactory bulb (AOB), posterodorsal aspect of the medial amygdala (MePD), bed nucleus of the stria terminalis (BNST), ventromedial hypothalamus ventrolateral portion (VMHvl) and the nucleus accumbens core (NAcc). This pattern of activation disappears in males lacking a VNO (Pankevich et al., 2006). The accessory olfactory bulb (AOB) receives signals from VNO sensory neurons that respond to female urine (D. E. Pankevich et al., 2006) and individual experiences can alter the number of these neurons. We find that regardless of experience, female urine did not activate the KO male AOB (fig 18A, B). This matches previous findings from female TRPC2 KO mice, whose AOB displayed no response to male soiled bedding (Hasen & Gammie, 2011). Still, inexperienced males appear to use urinary signals to alter their response to female intruders. This is a possibility given some pheromones are detected by the main olfactory bulb (Wang et al., 2006), including urinary signals (Veyrac, Wang, Baum, & Bakker, 2011). TRPC2 KO mice can also use olfactory cues to identify females (Beny & Kimchi, 2016). So, TRPC2 KO responses to urine are likely occurring because of main olfactory detection.

Development of a female urinary preference corresponds with morphological and functional changes to forebrain circuitry receiving olfactory sensory information (Bayless et al., 2019; Binns & Brennan, 2005; Pankevich et al., 2006). Notably, the male BNST typically shows strong activation in response to female urine which increases after sexual experience (Bayless et al., 2019; Korzan et al., 2013). We found urine exposure significantly activated the BNST in inexperienced TRPC2 KO males but not experienced males (fig 18A, B, 19B). While the BNST is well known for its connection to the AOB (Scalia & Winans, 1975) it also receives signals from the main olfactory bulb (MOB) (Kang et al., 2009). So, detection of urine by the main olfactory epithelium (MOE) (Wang et al., 2006) may induce the BNST activation seen here. However, changes that occur in the BNST appear opposite from those found previously; increased activation of the BNST by female urine typically produces a female urinary preference after sexual experience (Bayless et al., 2019). This may explain why urine appears more salient to inexperienced KO males because they still show activation of the BNST. Regions downstream from the BNST do not show any changes after prolonged experience but are activated by urine exposure.

The MPOA receives signals from both the MOE and VNO through the BNST (Bayless et al., 2019; Spehr et al., 2006) and is activated by urine in KO males (fig 18A, B). Lesioning the MPOA dramatically reduces mounting behavior (Bean, Nunez, & Conner, 1981), which is not characteristic of TRPC2 KOs (Leypold et al., 2002). So, BNST signals originating in the MOE may be activating the MPOA and facilitating mounting behavior in naïve TRPC2 KOs. Another region that guides mounting in males is the nucleus accumbens core (NAcc) (Beny-Shefer et al., 2017). This region is well known for mediating reward (Moncho-Bogani et al., 2005) and both the MOE and VNO can provide a rewarding experience to naïve male mice after exposure to estrous female urine (Korzan et al., 2013). Sexual experience increases activation in the rat NAcc (Hosokawa & Chiba, 2005) and experienced mice exposed to female urine show NAcc activation as well (Pankevich et al., 2006). We find that in TRPC2 KO males, female urine activates the NAcc regardless of experience (fig 18A, B, 19B). However, the TRPC2 KO mouse NAcc lacks signaling from the ventral tegmental area which is essential for the development of a urinary preference (Beny-Shefer et al., 2017). Therefore, those cells activated in the NAcc in TRPC2 KOs may not produce a rewarding experience or influence urinary preferences. Unlike the BNST, experience did not alter the MPOA or NAcc signaling further suggesting the changes in male mounting that appear after experience are a product of the altered activity in the BNST.

Urine exposure had no effect on the number of c-fos-ir cells in the MePD (fig 18A, B). In female KOs the MePD is also inactive after exposure to opposite-sex urine (Hasen & Gammie, 2011). The VNO is required to activate the MePD in response to pheromones (Samuelsen & Meredith, 2009). So, a lack of activation makes sense given we saw no AOB activity. The MePD is vitally important for both sexual behavior and female urinary preference (Adekunbi et al., 2018; Maras & Petrulis, 2010) so, while the MePD may no longer critically mediate urinary preference, it may still be involved with sexual behaviors. Downstream from the MePD is the VMHvl (Pardo-Bellver et al., 2012), which contains separate neuron populations to produce behavioral or hormonal changes in males (Coquelin et al., 1984; Lee et al., 2014; Lo et al., 2019). Both inexperienced and experienced males lacking TRPC2 had no activation of the VMHvl after urine exposure (fig 18A, B, 19A). Likewise, removing the VNO in male mice eliminates VMHvl activation (Pankevich, Cherry, & Baum, 2006). VNO removal also prevents the hormonal response to female urine (Coquelin et al., 1984) and our results suggest the same is true after VNO function is

disabled by TRPC2 loss. This may have important implications for TRPC2 KO behavior as males typically receive a testosterone surge during male-female mating that is facilitated by VMHvl (Coquelin et al., 1984). Pheromonal manipulations of hormones may help guide or organize sexual behavior in WT mice. It will be important to examine forebrain activation after urine exposure in experienced and naïve WT males. It is possible these regions show similar activation in KO and WTs but still lead to different behaviors. In all, our findings suggest that female urine acting through the MOE influences TRPC2 KO behavior and brain activation in unique ways.

Mounting behavior typical of male mice is not rescued in TRPC2 KOs after prolonged sexual experience. Inexperienced males also show activation of the BNST, which is absent in experienced males. Loss of BNST signaling may reduce the salience of urine just as increased BNST signaling increases its salience in TRPC2 +/+ mice (Bayless et al., 2019). TRPC2 KOs likely detect urine through the MOE because regions associated with the VNO alone are not active. Therefore, it is important to consider the possible influence of urine detection by the MOE during cohabitation with receptive or non-receptive females. TRPC2 KO mice can be conditioned to find female social signals aversive (Beny & Kimchi, 2016) and males housed with non-receptive females experienced continued rejection of sexual advances from females who were not carrying a receptive female signal. So, MOE signaling may still provide information regarding the receptivity of females. As a result, behavioral differences between KO groups may simply be a product of non-receptive females becoming an aversive stimulus. This would cause inexperienced KO males to reduce mounting when the receptive female signal is masked by male urine. Compared with previously described paradigms, TRPC2 KOs appear to show the opposite progression of both behavioral and brain changes following sexual experience. The loss of stereotypical brain and behavioral organization may guide the development of unique KO behaviors.

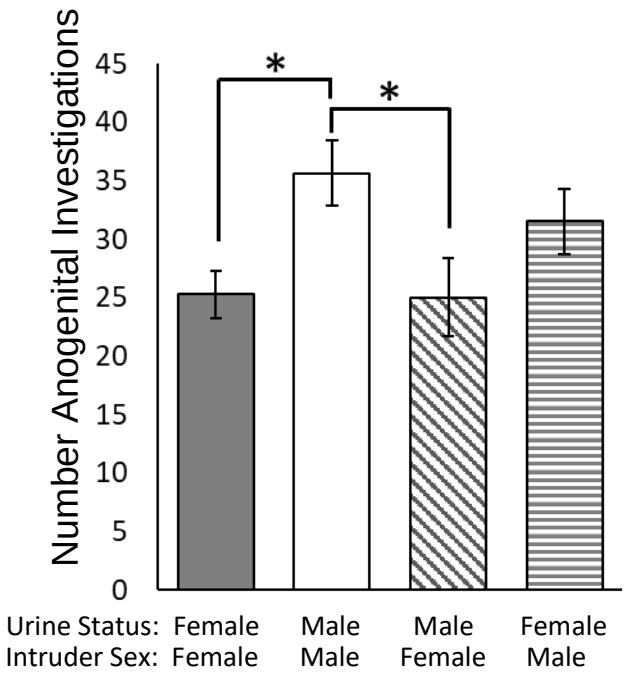

Figure 12: *Both naïve and experienced KO males examine the anogenital regions of males swabbed with male urine more than female intruders, regardless of their urine status.* (Error Bars ±SEM, *p<0.05, n=7/group).

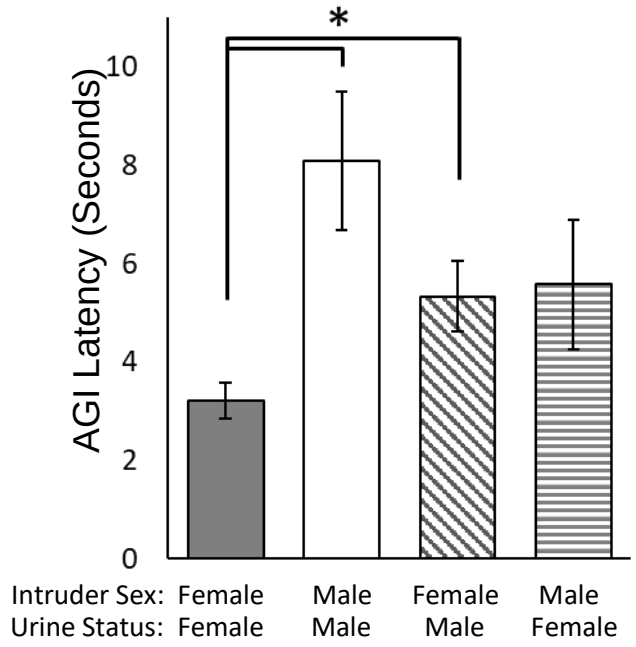

Figure 13: *The presence of a male urinary signal increases latencies to anogenital investigations in both naïve and experienced KO males.* (Error Bars ±SEM, *p<0.05, n=7/group).

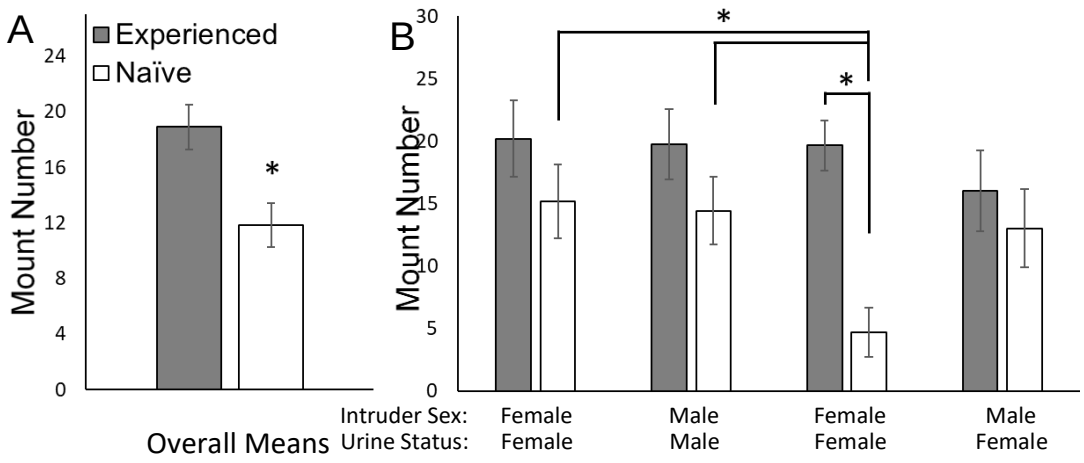

Figure 14: *Experienced males show increased mounting independent of sex and urinary status while experienced males altered mounting of females swabbed with opposite-sex urine.* A) Experienced males showed a marked increase in mounting overall B) Presence of male urine on a female inhibited mounting by naïve males while experienced males mounted all intruders equally. (Error Bars ±SEM, *p<0.05, n=7/group).

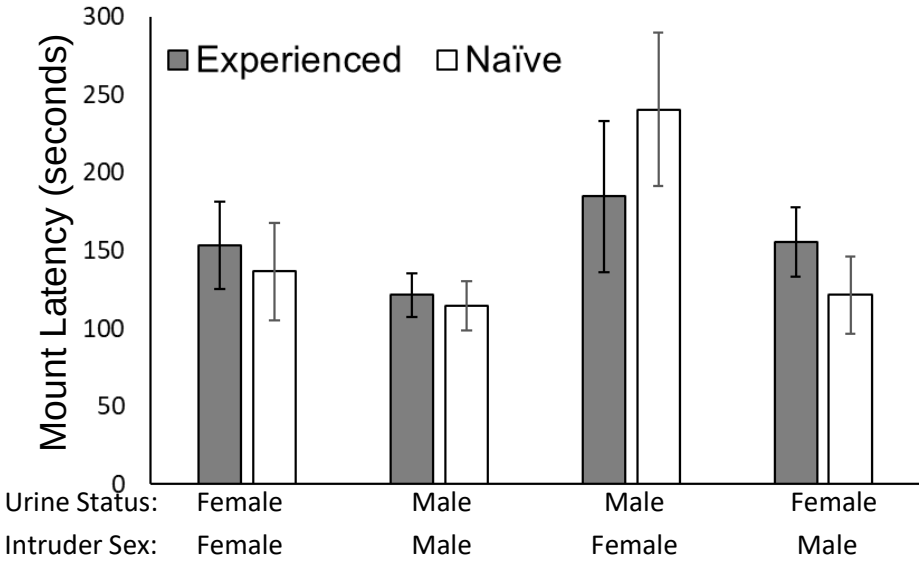

Figure 15: *Mount latency was unaltered by experience and similar across intruder types.* (Error Bars ±SEM, n=7/group).

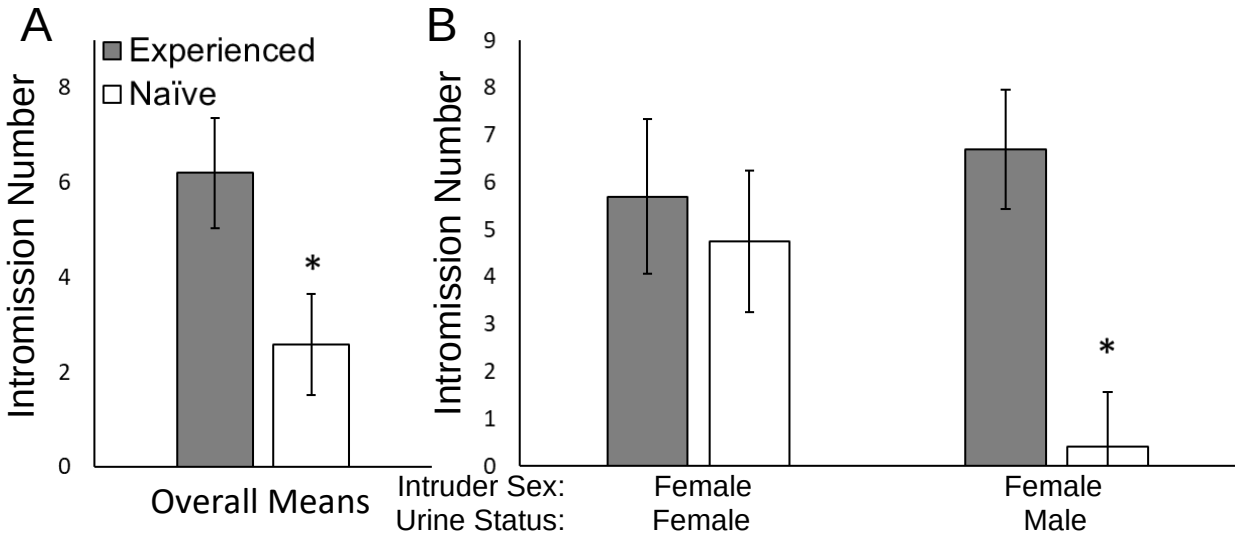

Figure 16: *Mounting by naïve males is dependent on female urinary status.* A) Experienced males intromit intruder females more than naïve males. B) Swabbing females with male urine inhibits intromissions by naïve KO males. (Error Bars ±SEM, *p<0.05, n=7/group).

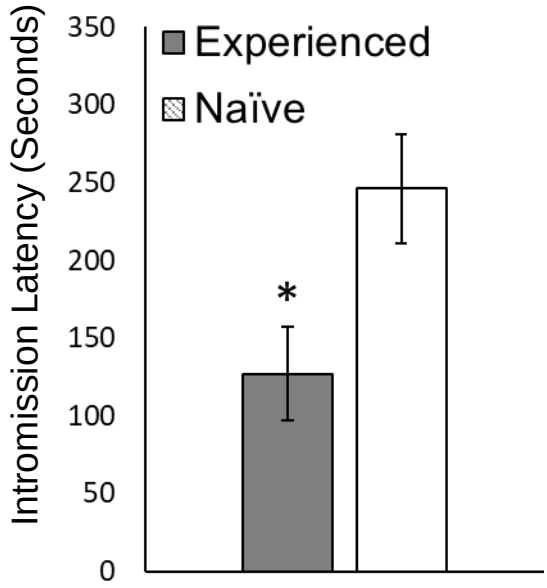

Figure 17: *Experienced males intromitted females earlier regardless of urinary status.* (Error Bars ±SEM, *p<0.05, n=7/group).

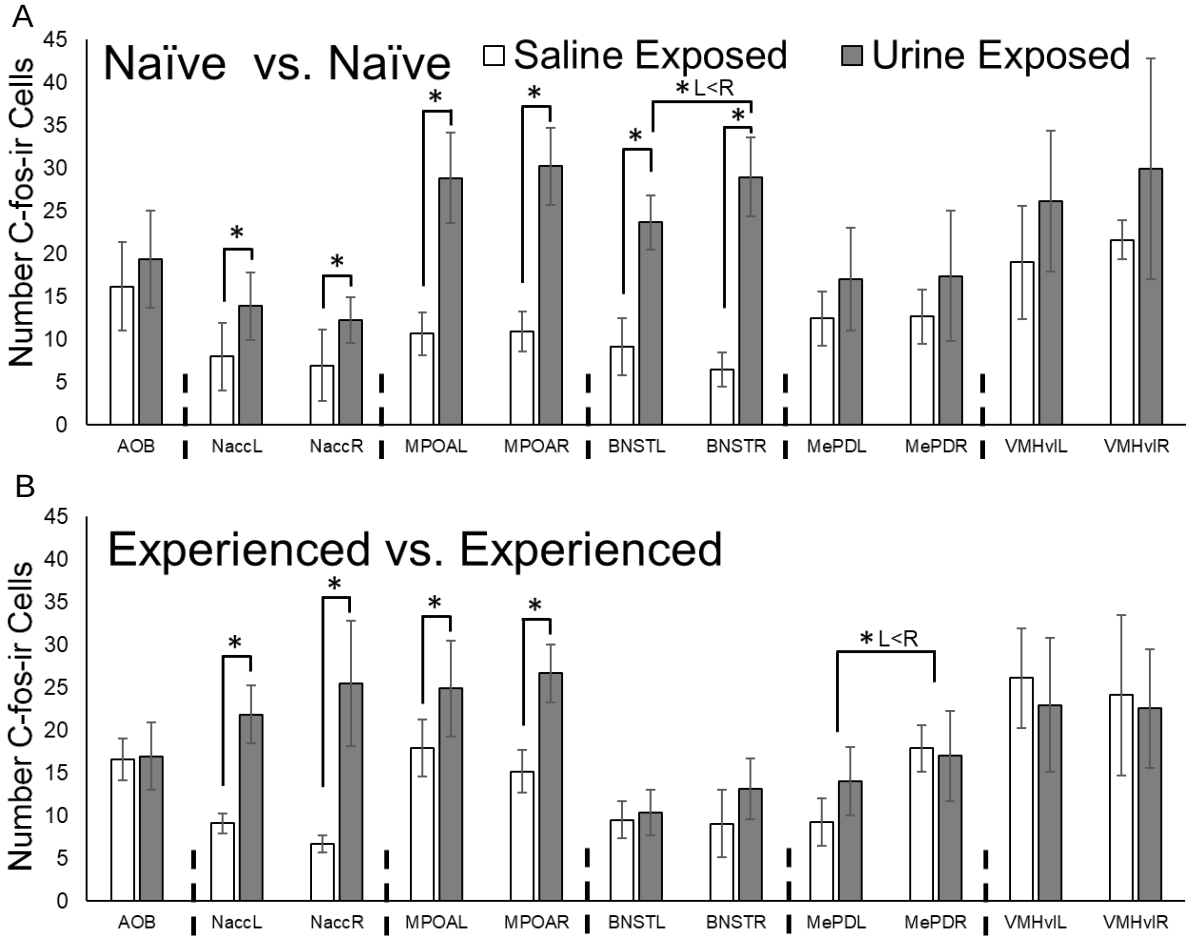

Figure 18: *Forebrain regions associated with reward and sexual behavior are activated in both experienced and naïve animals while one associated with partner preference is only active in naïve males.* A) Urine activates the NAcc, MPOA and BNST in naïve males. Right-biased activation appears in the BNST after urine exposure. B) Experienced males show urine-induced activation in the NAcc and MPOA. After prolonged sexual experience, the number of c-fos-ir cells is greater in the right MePD, regardless of exposure. (Error Bars ±SEM, *p<0.05, n=6/group).

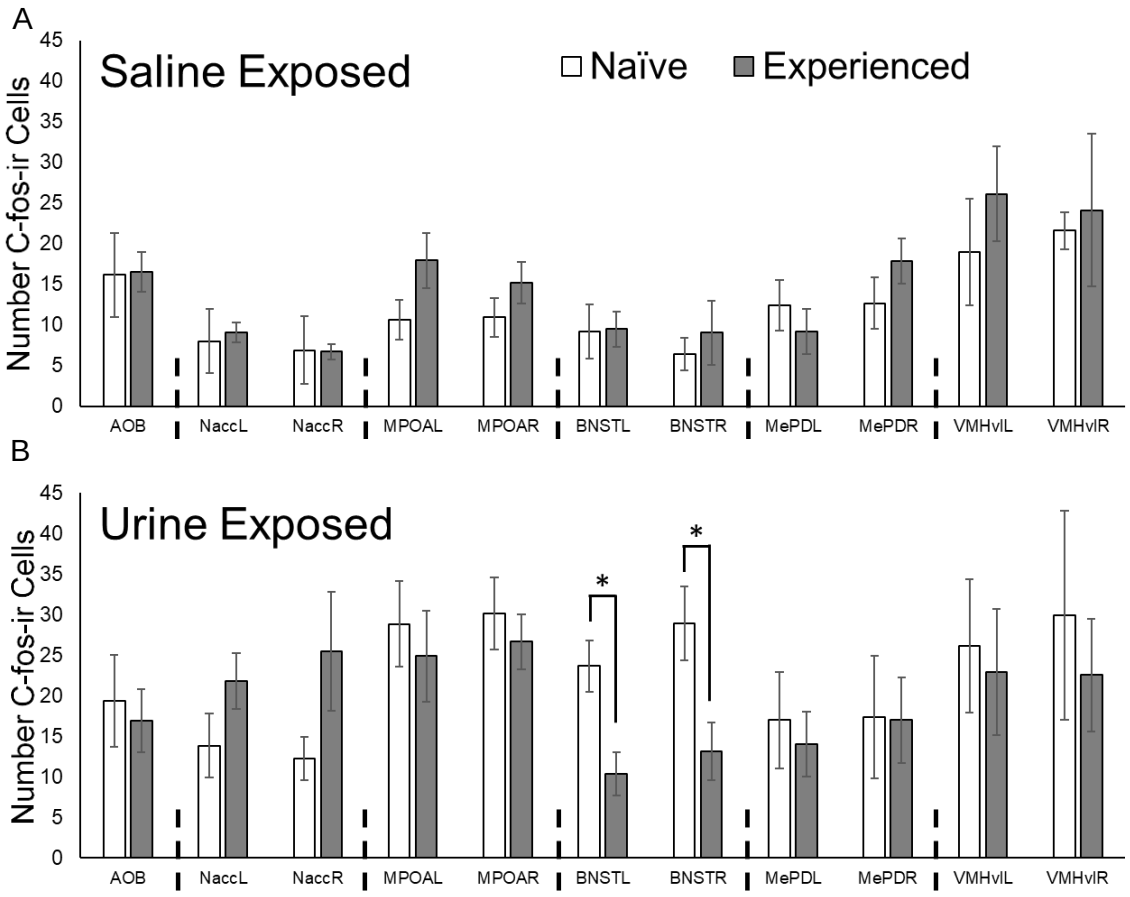

Figure 19: *Sexual experience reduces urine-induced activation of the BNST.* A) Baseline activation after saline exposure is similar between naïve and experienced animals. B) Female urine activates more cells in the BNST of naïve males compared with experienced. (Error Bars ±SEM, *p<0.05, n=6/group).

Table 6: *Average number of c-fos-ir cells in each forebrain region, means ±SEM.*

	Housed w/ Non-receptive Females		**Housed w/ Receptive Females**	
Brain Region	**Saline exposure**	**Urine exposure**	**Saline Exposure**	**Urine Exposure**
AOB	16.18 ± 5.15	19.38 ± 5.68	16.57 ± 2.44	16.98 ± 3.9
NAccL	8.0 ± 3.94	13.87 ± 3.98	9.11 ± 1.2	21.83 ± 3.4
NAccR	6.92 ± 4.17	12.25 ± 2.66	6.71 ± 0.96	25.45 ± 7.32
MPOAL	10.65 ± 2.47	28.83 ± 5.28	17.93 ± 3.36	24.92 ± 5.61
MPOAR	10.93 ± 2.36	30.19 ± 4.45	15.2 ± 2.52	29.69 ± 3.4
BNSTL	9.18 ± 3.34	23.67 ± 3.15	9.49 ± 2.17	10.4 ± 2.66
BNSTR	6.46 ± 1.98	28.94 ±4.6	9.07 ± 3.95	13.17 ± 3.56
MePDL	12.42 ± 3.13	17.0 ± 5.99	9.25 ± 2.77	14.05 ± 3.99
MePDR	12.67 ± 3.17	17.38 ± 7.59	17.88 ± 2.76	17.0 ± 5.3
VMHvlL	19 ± 6.59	26.15 ± 8.21	26.14 ± 5.83	22.95 ± 7.81
VMHvlR	21.63 ± 2.28	29.92 ± 12.87	24.13 ± 9.37	22.55 ± 6.94

CHAPTER 5: GENERAL DISSCUSSION

The de-scent of sexuality: did loss of a pheromone signaling protein permit the evolution of same-sex sexual behavior in primates?

Primate same-sex sexual behavior (SSSB) is rarely observed in strepsirrhine species, and only somewhat more common in platyrrhines, but is observed in nearly all catarrhine species, including humans, suggesting the common catarrhine ancestor as the origin of routine SSSB. In mice, disruption of the transient receptor potential cation channel 2 (TRPC2) gene, which is crucial for transducing chemosensory signals from pheromones in the vomeronasal organ, greatly increased the likelihood of SSSB. We note that catarrhine primates share a common deleterious mutation in this gene, indicating that the protein was dysfunctional in the common catarrhine ancestral primate approximately 25 million years ago (mya). We hypothesize that the loss of this protein for processing pheromonal signals in males and females made SSSB more likely in a primate ancestral species by effectively lifting a pheromonally-mediated barrier to SSSB, and that this was an important precursor to the evolution of such behavior in humans. Additional comparisons between SSSB and the functional status of the TRPC2 gene or related proteins across primate species could lend support to or falsify this hypothesis. Our current research indicates that loss of TRPC2 function in developing mice leads to the loss or attenuation of sexually dimorphisms in the adult brain, which may help us to understand the biological underpinnings of SSSB. Our hypothesis offers an ultimate evolutionary explanation for SSSB in humans.

Background

We have been working with mice in which the gene for the transient receptor potential cation channel 2 (TRPC2), an important component of vomeronasal organ (VNO) neuronal signaling (Leypold et al., 2002; Spehr et al., 2009; Stowers & Spehr, 2015), has been disabled. The VNO is a highly specialized sensory organ found bilaterally in the cartilaginous base of the nasal cavity to which it is connected by a duct. The VNO detects specific ligands called pheromones, broadly defined as chemicals released by one animal to induce a change in behavior and/or physiology in another (Stowers & Kuo, 2015). Both male and female TRPC2 knockout (KO) mice display rather vigorous same-sex sexual behavior (SSSB) (Kimchi et al., 2007; Leypold et al., 2002; Stowers et al., 2002) that is not typical of wildtype (WT) mice (Leypold et al.,

2002). Importantly, conditioned aversion to sex-specific signals indicate the TRPC2 KO mice retain the ability to discriminate male and female conspecifics (Beny & Kimchi, 2016), so their SSSB is not due to an inability to recognize their partner's sex. KO males mount other males vigorously and their mounting of receptive females continues to result in intromission and insemination, similar to WT males (Leypold et al., 2002). KO-females show higher levels of female-female mounting than WT mice (Kimchi et al., 2007). Both naïve male and female KO mice and those given experience with the opposite sex will show extensive anogenital investigations and vigorous mounting and pelvic thrusts, which are all male-typical behavior, towards both males and females. These behaviors appear indistinguishable from the typical responses of WT-males to a receptive female.

We were already aware that the TRPC2 gene is dysfunctional in humans (Vannier et al., 1999), but in surveying the state of the gene in various other primate species, we noted an interesting coincidence—the gene remains functional in several non-catarrhine primate species that are known for displaying little or no SSSB (fig 20) (Dixson, 2012; Liman & Innan, 2003; Zhang & Webb, 2003). On the other hand, it appears that the TRPC2 gene was already dysfunctional in the common ancestor to Old World monkeys (OWM) and apes (Zhang & Webb, 2003), two groups in which SSSB is well documented (Dixson, 2010, 2012; Vasey, 1995). This coincidence led us to hypothesize that the ancestral primate VNO, and more specifically, signaling via TRPC2 in the VNO, promoted the development of sex-specific brain organization to direct sexual behavior only or mostly towards opposite-sex partners. If so, then loss of this signal in the course of the evolution of catarrhines may have lifted this barrier, enabling more frequent SSSB, which is now expressed in extant OWMs and apes. We offer this tentative hypothesis in the hopes of encouraging other researchers to explore ways to test the notion.

TRPC2 and primate same-sex sexual behavior:

From lemurs to apes and humans, the selection for TRPC2 appears to relax because the gene accumulates premature stop codons, frameshifts and missense mutations (Liman & Innan, 2003). The gene is intact in the primate suborder strepsirrhine, which includes lemurs and tarsiers (Liman & Innan, 2003). Ring-tailed lemurs (*Lemur catta*), for example, have extremely complex olfactory chemical social systems (Garrett, 2015) with multiple sensory neuron layers in the VNO (Garrett et al., 2013; Schilling, 1979).

The next primate suborder, the New World monkeys (NWM) or platyrrhines, separated from strepsirrhine around 50 mya when the TRPC2 gene was presumably still functional (Liman & Innan, 2003). While the TRPC2 gene can produce a functional protein in NWM (Liman & Innan, 2003; Zhang & Webb, 2003), the VNO in which this protein must operate shows great variability within this suborder (Smith et al., 2011). This variation in vomeronasal function may be related to decreased purifying selection for the TRPC2 gene seen in some NWM species compared with prosimians (Liman & Innan, 2003). Owl monkeys (*Aotus trivirgatus*), which are considered an out-group within NWMs in terms of VNO complexity, still have a VNO with multiple neuronal layers (Hunter, Fleming, & Dixson, 1984).

Catarrhine divergence from the platyrrhines occurred 30-40 mya. Analyzing several extant catarrhine species reveals a premature stop codon mutation in exon 13 (out of 21) of all their TRPC2 gene analogues (fig 21) (Liman & Innan, 2003; Zhang & Webb, 2003). Subsequent mutations in TRPC2 have accumulated within the catarrhine lineage to further degrade its function (Liman & Innan, 2003; Zhang & Webb, 2003), along with other VNO neuron activation pathway losses (Hohenbrink, Mundy, Zimmermann, & Radespiel, 2013; Young & Trask, 2007; Zhang & Webb, 2003). As extant hominidae (apes and humans) and cercopithecoidea (OWM) have a severely reduced or absent VNO (fig 20) (Garrett, 2015; Smith et al., 2002; Smith et al., 2001), further changes to other VNO-related genes must have occurred. The loss of TRPC2 correlates with the presence of more routine SSSB in primates, while species retaining a functional TRPC2 gene show little SSSB. For example, many lemur species, with an intact TRPC2 gene, are known to display little or no SSSB (Dixson, 2012). Despite extensive observation, SSSB has not been reported for owl monkeys (*Aotus*), suggesting it is either absent or extremely rare in this NWM species, a suborder where the TRPC2 gene remains intact and the VNO is well-developed. While these suborders are not entirely devoid of SSSB (Ploog, Blitz, & Ploog, 1963; Schiml, Mendoza, Saltzman, Lyons, & Mason, 1996; Vasey, 2017), once we reach catarrhine primates (OWM, apes and humans), SSSB is well documented and seems to be much more frequent in most species of this suborder, albeit data are lacking for others (fig 21) (Dixson, 2012; Vasey, 2017). Even with the missing data, the most parsimonious explanation for SSSB patterns across phylogeny is that it became more routine and occurred in more social contexts in the common ancestor of apes and OWMs after they split from other primates (Garrett, 2015; Radinsky, 1975). One group estimates TRPC2 loss at 25-40 mya (Liman & Innan, 2003) and another around 23 mya (Zhang

& Webb, 2003). So, the inferred loss of TRPC2 and onset of SSSB converge in a common catarrhine ancestor (fig 21) approximately 25 mya, prior to splitting into OWM and apes. Perhaps the loss of a protein that previously enforced opposite-sex sexual behavior by organizing or activating brain responses to male or female pheromones made SSSB more likely in a primate ancestral species, thereby increasing such behavior in animals evolving from that line. If so, then male-male and female-female sexual behavior has likely been a characteristic of catarrhine primates for over 25 million years and has evolved into the myriad of SSSB phenotypes expressed throughout the life history of catarrhines.

In some catarrhine species, accounts of SSSB have only been published for one sex. For example, some male siamangs (*Symphalangus syndactylus*) have been observed expressing SSSB (Liebal, Pika, & Tomasello, 2004) while female SSSB in this genera has not yet been documented (Vasey, 2017). In mountain gorillas (*Gorilla b. beringei*), both female-female and male-male sexual behavior is reported (Grueter & Stoinski, 2016; Yamagiwa, 1987). Other catarrhine species have been observed expressing SSSB only during certain developmental periods. In colobus monkeys (*Colobus vellerosus*), young males may engage in SSSB (Teichroeb, E-mail Communication, November 3, 2017), while in macaques (*Macaca*), both males and females display SSSB at various ages (Akers & Conaway, 1979; Chevalier-Skolnikoff, 1976). Loss of TRPC2 represents the first ultimate evolutionary explanation for both sexes expressing SSSB and predicts discovering such behaviors in both sexes of understudied catarrhines. For example, while preparing this report, an account of male-male mounting in golden snub-nosed monkeys (*Rhinopithecus roxellana*) was published (Fang, Dixson, Qi, & Li, 2018), while only female-female mounting had been noted earlier (Ren et al., 1995). These examples suggest selective pressures, working across more than 25 million years, led to many variations in the expression of SSSB in extant OWM and apes.

TRPC2 loss and ancestral primate VNO anatomy:

Extant OWMs and apes indicate that TRPC2 gene loss may have enabled SSSB, similar to loss of TRPC2 in KO mice (Dixson, 2012; Kimchi et al., 2007; Leypold et al., 2002) and examining a hard tissue fossilized VNO remnant, the VNO groove, also supports the idea of a waning VNO presence as catarrhine primates evolved. During the Eocene (56–34 mya), early primates like *Rooneyia* had a VNO groove not unlike extant strepsirrhines (Kirk, Daghighi, Macrini, Bhullar, & Rowe, 2014). By the beginning of the Oligocene (34-23 mya), *Aegyptopithecus* fossils from 29-30 mya indicate a similar, although shortened,

VNO groove, implying its accessory olfactory system was still functional (Garrett, 2015; Heritage, 2014; Rossie, 2005). It is around this period that TRPC2 loss may have led to the evolution of VNO anatomical reductions. Absence of a VNO groove in the later stem catarrhine, *Victoriapithecus* (Garrett, 2015), likely required loss of other VNO-related genes. Data from extant catarrhines and the fossil record suggest that VNO reduction is a derived feature of this group (Rossie, 2005) as both cercopithecoids and hominids have severely reduced VNOs (Garrett, 2015). While the soft tissue of the accessory olfactory bulb (AOB), which relays neuronal signals from the VNO to downstream brain regions (Keverne, 1998; Martinez-Marcos, 2009), is not available for analysis in the fossil record, a reduction in the VNO during catarrhine evolution is also reflected in the loss of the AOB in extant catarrhines, but not in NWMs and strepsirrhine (Meisami & Bhatnagar, 1998). Anatomical reduction of the VNO is also seen in TRPC2 KO mice, seemingly recapitulating the reduction of the VNO groove in ancestral catarrhine fossils (Bhatnagar & Meisami, 1998; Garrett, 2015; Rossie, 2005; Stowers et al., 2002). So, comparing the TRPC2 KO mice to WT controls may offer insight about how anatomy and physiology, both in the body and in the brain, was altered by the loss of TRPC2 in early catarrhine primates.

Behavior and physiology of TRPC2 KO mice recapitulate key components of primate evolution:

Looking at extant organisms, most strepsirrhine species are nocturnal, arboreal and solitary or live in pairs, while group living becomes more dominant in diurnal and ground dwelling primates (Crook & Gartlan, 1966; Dixson, 2012) see: (Galdikas, 1985; Sauther, Sussman, & Gould, 1999; Springer, Mellmann, Fichtel, & Kappeler, 2016 for some exceptions). Around 52 million years ago, some primate ancestors went from being solitary to social foragers by forming groups containing multiple members of both sexes (Shultz, Opie, & Atkinson, 2011). Shultz et al. (2011) suggests these multi-male/multi-female groups were well-stabilized before further social systems, like polygyny, developed. SSSB may have helped stabilize early groupings with multi-male/multi-female groups by providing a means to defuse tension (De Waal, 1987; Dixson, 1977; Fairbanks, McGuire, & Kerber, 1977; Oi, 1991; Yamagiwa, 1987), thereby facilitating group foraging and increased tolerance (De Ruiter, Weston, & Lyon, 2011). The ability for SSSB to reduce tension among what Shultz et al. (2011) calls “social foragers” likely extended to expression in all groups containing multiple members of the same sex. Extant primates that associate in single-sex groups appear more likely to express SSSB (Bagemihl, 1999; Vasey, 1995) suggesting a possible benefit for such social situations.

Disabling the TRPC2 gene in mice induces other changes in their behavior, beyond SSSB, that resemble inferred changes in the behavior of ancestral primates—specifically a reduction in aggression. Factors driving increased social behavior, like SSSB and decreased aggression, may have evolved as part of a series of steps in the evolutionary process in OWMs (Hare, Wobber, & Wrangham, 2012). Hare et al. (2012) describes this process as the self-domestication cascade, whereby decreases in aggressive behavior coincide with several other characteristics typically associated with domestication, including increases in social behaviors and delays in development. Interestingly, male-male aggression and maternal aggression (towards a strange male) are reduced in TRPC2 KO mice (Hasen & Gammie, 2009; Stowers et al., 2002). Because VNO removal in adulthood reduces male-male aggression in mice (Wysocki & Lepri, 1991) and in at least one prosimian, the grey mouse lemur (*Microcebus murinus*) (Aujard, 1997), TRPC2 loss in ancestral primates likely resulted in fewer instances of aggression as well. This change may have decreased the risks of social interactions among group-living species, and increased tolerance may allow for greater cooperation, allowing for the production of stable multi-male/multi-female groupings (Hare, Melis, Woods, Hastings, & Wrangham, 2007). This paradigm is taken to the extreme in female and male bonobo chimpanzees (*Pan paniscus*), where sociosexual behaviors, those which are sexual in form but related to other social functions, appear to have largely replaced aggression's social function (De Waal, 1990; Shultz, Opie, & Atkinson, 2011). Decreased male-male aggression could facilitate stability in groups containing multiple males by increasing tolerance and providing a benefit to such aggregations (Hare et al., 2007). It is possible that reductions in male-directed aggression from lactating females, decreases in infant-directed aggression by males, and increases in male parental behavior appeared after TRPC2 loss in primates, because those same shifts in behavior occur in mice when the gene is disabled (Hasen & Gammie, 2009; Orikasa et al., 2017). We propose that TRPC2 loss may have increased tolerance and stability in primate groups containing multiple males, females and progeny. We note that such increased sociality would also provide more opportunities for SSSB to arise and to be subject to selective pressures.

Some primate physiology may have been altered by loss of TRPC2 as well. Our research indicates KO mice show 1) a delay in pubertal development and 2) a loss or attenuation of sexually dimorphic organization in some brain regions (our unpublished data), both of which are part of the self-domestication cascade and well-known characteristics of primates (Hare et al., 2012; Shea, 1989). So, the cascading

effect of self-domestication may be the result of losing VNO-related genes, like TRPC2, that influence multiple aspects of behavior and physiology related to domestication. The brains of TRPC2 KO mice suggest that the first primates lacking TRPC2 did not conform to the previous sex-typical brain organization, or similar critical periods, for developing sex-typical behavior. Loss of TRPC2's influence during development in the first primates to lose the gene may also have allowed other sensory cues to organize brain circuitry. Catarrhine primates rely heavily on visual cues, and recent studies suggest the genetic ability for trichromacy developed well after any reduction in the main olfactory system (Garrett, 2015; Gonzales, Benefit, McCrossin, & Spoor, 2015). These increases in visual acuity are strongly associated with VNO functional loss specifically (Garrett, 2015). TRPC2 loss may have allowed visual information, an important source of reproductive cues in modern-day catarrhines, to replace some VNO influence on reproductive neural circuits. Thus, the loss of TRPC2-dependent development may have permitted alleles for greater visual acuity, as well as sex-specific responses to visual cues, to be subject to selection.

The specificity of TRPC2:

Reports of TRPC2 expression in the main olfactory epithelium (MOE) of mice (Omura & Mombaerts, 2014) imply the VNO is not the only source for behavioral changes in the KO mice. The main olfactory system is important for the detection of more general scents but also may detect specialized ligands as the VNO does (Baum & Cherry, 2015). MOE neurons send signals to the main olfactory bulb which, in similar fashion to the AOB, sends signals to downstream brain regions (de Olmos et al., 1978). These two systems work together to produce the behavioral and physiological effects of pheromones (Baum, 2012; Stowers & T. H. Kuo, 2015). TRPC2-positive neurons in the mouse MOE form two discrete populations (Omura & Mombaerts, 2015) with one specialized for detecting low oxygen environments (Bleymehl et al., 2016). Other TRPC2-expressing neurons co-express olfactory receptor proteins and adenylate cyclase 3, a cytoplasmic enzyme that assists in cell signaling (Omura & Mombaerts, 2014). These neurons form several glomeruli, clusters of nerve endings in the main or accessory olfactory bulb that receive input from a single sensory neuron, on the ventral aspect of the main olfactory bulb. Generally, ventral main olfactory bulb glomeruli are activated by urinary pheromones, and some glomeruli in this location send signals to the vomeronasal amygdala (Kang et al., 2009). So, while located in the MOE, these TRPC2 neurons may nevertheless be carrying pheromonal information. Interestingly, the Bruce effect,

which prevents embryo implantation in female mice exposed to a novel male scent, still occurs in TRPC2 KO mice (Kelliher et al., 2006). This physiological effect requires (1) recognition of individual conspecifics using the major histocompatibility complex class I peptide (MHC), a pheromone that carries discrete genetic information about a mouse, and (2) an intact AOB (Oboti et al., 2011). So, detection of MHC and signaling to the AOB is not entirely disrupted by the loss of the gene in mice. These findings indicate that TRPC2 loss specifically, rather than loss of pheromonal signaling overall or the entire VNO, increases SSSB in the KO mice. Indeed, while modern humans have no neural tissue in their vestigial VNO (Smith et al., 2002), there is growing evidence that humans can still detect and process some pheromonal information through the MOE (Berglund, Lindström, & Savic, 2006; Ivanka Savic, Berglund, & Lindström, 2005; Tan & Goldman, 2017; Wallrabenstein et al., 2015), including detection of the MHC (Milinski, Croy, Hummel, & Boehm, 2013). Loss of TRPC2 in our catarrhine ancestor may have disabled systems enforcing opposite-sex sex behavior despite the retention of some pheromonal signaling via the MOE.

Loss of pheromonal signaling likely alters many aspects of sexual behavior development in mammals (Wunsch, 2017) and at least some pheromones are still detected by both humans and TRPC2 KO mice. So we feel that SSSB may be uniquely tied to TRPC2 loss, not loss of all pheromonal signaling per se. While Kimchi et al. (2007) suggest VNO removal in adult female WT mice greatly increases mounting behavior, most groups have found VNO removal in adult mice blunts such behavior rather than expanding and enhancing it (Leypold et al., 2002; Martel & Baum, 2009; Pankevich et al., 2004; Wysocki & Lepri, 1991). One group specifically tested for increased SSSB after VNO removal in adult female mice and reported none (Martel & Baum, 2009). Other studies report that removal of the adult VNO in WT mice and an extant primate, the grey mouse lemur (*M. murinus*) reduced male-male aggression, but did not increase SSSB (Aujard, 1997; Pankevich et al., 2004; Pankevich et al., 2006; Wysocki & Lepri, 1991). These findings suggest that SSSB in the KO mice results from the reduced influence of TRPC2-specific VNO signals *during development*, rather than loss of VNO signals in adulthood, in both mice and primates.

The accessory olfactory system downstream of the VNO in the mouse brain is also influenced by loss of TRPC2 function. For example, the adult AOB of TRPC2 KO mice is reduced in volume compared to WT mice (Hasen & Gammie, 2009), as is the posterodorsal aspect of the medial amygdala (our unpublished data), suggesting that WT mice use TRPC2-dependent signals to organize downstream brain

regions prior to adulthood. We suggest that VNO influence on brain development in mice relies at least partly on TRPC2 function during the developmental period. Loss of such developmental influences on brain organization and function would be expected to alter behavior.

It is worth noting that KO of several other genes in mice that disrupts VNO or MOE signaling does not increase SSSB. For example, removal of $G_{\alpha}i2$, a g-protein that is expressed in VNO neurons, disrupts aggression but does not change sexual behavior (Norlin et al., 2003). Disruption of $G_{\alpha}o$, another g-protein crucial for VNO function, also reduces aggression without augmenting sexual behavior (Chamero et al., 2011). Mice lacking adenylate cyclase 3 exhibit *loss* of sexual behavior as well as aggression (Wang et al., 2006; Wang & Storm, 2011). Finally, knocking out CNGA2, which is expressed in both types of TRPC2-expressing MOE cells (Omura & Mombaerts, 2014), also *reduces* both sexual and aggressive behavior (Mandiyan, Coats, & Shah, 2005). Together, these data suggest that it is specifically the loss of TRPC2 signaling, probably during development, that drives the increase in SSSB in KO mice and so loss of this gene may have done so in ancestral catarrhine primates.

Non-catarrhine expression of SSSB:

The VNO systems of prosimians, and their role in physiological and social function, remain complex (Evans & Schilling, 1995; Garrett et al., 2013; Schilling, 1979). While SSSB has been reported in several of these species, i.e., brown lemurs (*Lemur fulvus*) and Verreaux's sifaka (*Propithecus verreauxi*) (Chandler, 1975; Richard, 1974), it appears less frequent than in catarrhines (Vasey, 2017). As mentioned previously, among NWMs that retain a functional TRPC2, there are varying degrees of VNO structure (e.g., size, number of sensory-neuron layers, presence of non-neuronal tissue etc.) (Bhatnagar & Meisami, 1998; Dennis et al., 2004; Hunter et al., 1984; Swaney & Keverne, 2009), and the expression of SSSB in these species (Busia, Denice, Aureli, & Schaffner, 2018; Ploog et al., 1963; Talmage-Riggs & Anschel, 1973) seems to vary depending on the prominence of the VNO (fig 20). As with prosimians, these instances of SSSB in NWM are less frequent than among OWMs and apes and expressed less frequently in limited social situations. Some NWMs have a complex VNO, such as Owl monkeys (*Aotus*) (Smith et al., 2011), which have not been documented displaying SSSB. Other NWM, like marmosets (*Callithrix*), have lost function of some VNO related genes, such as pheromone receptors (e.g., V2R genes) and g-protein second messengers (e.g., $G_{\alpha}o$) (Suárez, Fernández-Aburto, Manger, & Mpodozis, 2011; Young & Trask, 2007),

which partially rely on TRPC2 to propagate action potentials (Stowers & Spehr, 2015), and they express limited SSSB (Rothe, 1975). However, presence of these other VNO related genes among NWMs has not been extensively examined and $G_{\alpha}o$ KO mice don't show SSSB (Chamero et al., 2011), thus its loss and SSSB expression in marmosets may be coincidental. While no evidence has been presented to suggest VNO-related genes were lost in squirrel monkeys (*Saimiri sciureus*), another NWM that expresses SSSB (Schiml et al., 1996; Talmage-Riggs & Anschel, 1973), their VNO seems underdeveloped, containing only a single neuronal tissue layer (Smith & Bhatnagar, 2009; Smith et al., 2011), which might attenuate some TRPC2-dependent signals and allow limited SSSB expression. However, spider monkeys (*Ateles*) have no known disruptions to VNO-related genes and possess a complex VNO (Smith et al., 2011), yet still express SSSB, albeit at low levels (Busia et al., 2018). These instances make it clear that TRPC2 loss cannot account for all SSSB in primates, even if VNO function influences SSSB in some NWMs.

Interestingly, primates may not be the only animals with behavior affected by VNO/TRPC2 loss. Both whales and bats show a high frequency of SSSB (D'Agostino et al., 2017; Greenhall, 1965; Riccucci, 2010), and have a dysfunctional TRPC2 gene and/or show great variation in vomeronasal complexity (Bhatnagar & Meisami, 1998; Cooper & Bhatnagar, 1976; Yu et al., 2010; Zhao, Xu, Zhang, & Zhang, 2011). Perhaps loss of TRPC2 also allowed SSSB expression in these mammals. Additionally, just as loss of VNO influence may have led to the evolution of enhanced vision in primates, perhaps loss of VNO influences during ontogeny in bats and whales favored evolution of enhanced auditory location capacities. Importantly, primates, bats and whales are known to express SSSB more routinely than other animals that show SSSB (Bagemihl, 1999). As such, TRPC2 or the vomeronasal organ cannot be the only factor influencing SSSBs in all species. For example, knocking out the gene *fruitless* in *Drosophila* causes male flies to express SSSB (Ryner et al., 1996). Still, the possible contribution of TRPC2 to human SSSB makes this gene of special interest and provides a unique opportunity for future inquiries into human evolution.

Conclusions

Here, we hypothesize that pheromonal signals provided a barrier to expression of SSSB in the earliest primates, a barrier that is still present in most extant prosimians and some NWMs. We propose that these pheromonal signals had organized brain development to permit only opposite-sex sexual behavior.

Widespread and more frequent expression of SSSB in OWM and apes suggests the barrier was lost in their common catarrhine ancestor, which also carried a loss-of-function mutation in the TRPC2 gene (Liman & Innan, 2003) that is critical for pheromonal signaling. Because TRPC2 loss leads to SSSB in mice (Kimchi et al., 2007) and a reduction in sexual dimorphism in the brain (our unpublished data), we speculate that developmental signals carried by TRPC2 were responsible for constraining sexual behavior to opposite-sex interactions in present-day mice and the earliest primates. Only after the loss of this gene in catarrhines would it be possible for further phenotypes associated with SSSB, such as sociosexual behavior or a preference for SSSB, to develop. Put another way, the development of a sexual *preference* for same-sex partners or the involvement of SSSB in sociosexual interactions would be impossible if primate brains were organized to prevent any SSSB. Without the variation in our distant ancestors' sexual behavior, complex and diverse human sexual orientations would not exist today.

We are unaware of compelling evidence that any non-human catarrhine expresses a sexual orientation (heterosexual or otherwise) or that there exist preferences for a specific sex when engaging in sexual/sociosexual behaviors but note that such a preference would be difficult to document in species that cannot answer our surveys, for several reasons. First, a strong or exclusive preference for same-sex partners would presumably be rare in other species, as it is in our own, reducing the chances of such preferences being noted by human observers. This problem of rarity is compounded by the relatively small sizes of most primate groups, which would mean a rare individual with a strong preference for same-sex partners might never encounter a like-minded conspecific, and so never have the chance to display a same-sex preference for primatologists to observe. So, we remain agnostic about whether any Old World monkeys or other apes have sexual orientations. The absence of proof is not proof of absence.

The changes in sexual behavior caused by the loss in TRPC2 gene function in mice are associated with altered brain organization (Hasen & Gammie, 2009), including the reduction of sexual dimorphisms in adult brain anatomy (our unpublished data). These effects of TRPC2 KO in mice suggest that one step in decreasing sex differences in primate evolution was the loss of this gene, which might represent a step in a self-domestication cascade leading to further reductions in sex differences in brain and behavior (Hare et al., 2012). This notion of reduced brain sexual dimorphism in the course of primate evolution is reflected in papers asserting that modern human brains do not fit into male or female categories (Joel et al., 2015) [but

for a critique of this research see: (Del Giudice et al., 2016)]. Without getting into the contentious question of whether a brain dimorphism is important if there is considerable overlap between the sexes, we concede that the reduction of sex differences in the human brain and the interaction of sex and gender in our species (van Anders, 2015) limits application of our hypothesis to modern society. Humans have culturally-specific expectations about how people of different genders should behave, including whom they should choose as an appropriate sexual partner. Such human cultural attitudes could, arguably, overlay or obfuscate any evolved behavioral effects that arose following the loss of TRPC2 gene function.

In his seminal essay, *The Descent of Instinct,* Frank Beach explained how culture influenced ideas about so called 'instinctive' animal behaviors (Beach, 1955). Social and cultural assumptions regarding sexual behavior also influenced scientific discourse surrounding sex, gender and sexual minorities (Clucas, 2017; Hird, 2006; Serano, 2010; Spitzer, 2012). In this cultural milieu, SSSB became the target of so-called sexual orientation change efforts, therapies that pair aspects of SSSB with negative stimuli (Clucas, 2017) in an attempt to change the behavior. However, to the extent that we primates have expressed SSSB for millions of years it can hardly be considered "unnatural", so these "reparative" therapies (Cyphers, 2014) are irrational despite their persistence (Mallory, 2018). We propose that some experiences of the current queer community arose at least 25 mya when the loss of a functional TRPC2 gene allowed brain development that would support the possibility of SSSB, a requirement for the later appearance of a preference for same-sex partners in some individuals.

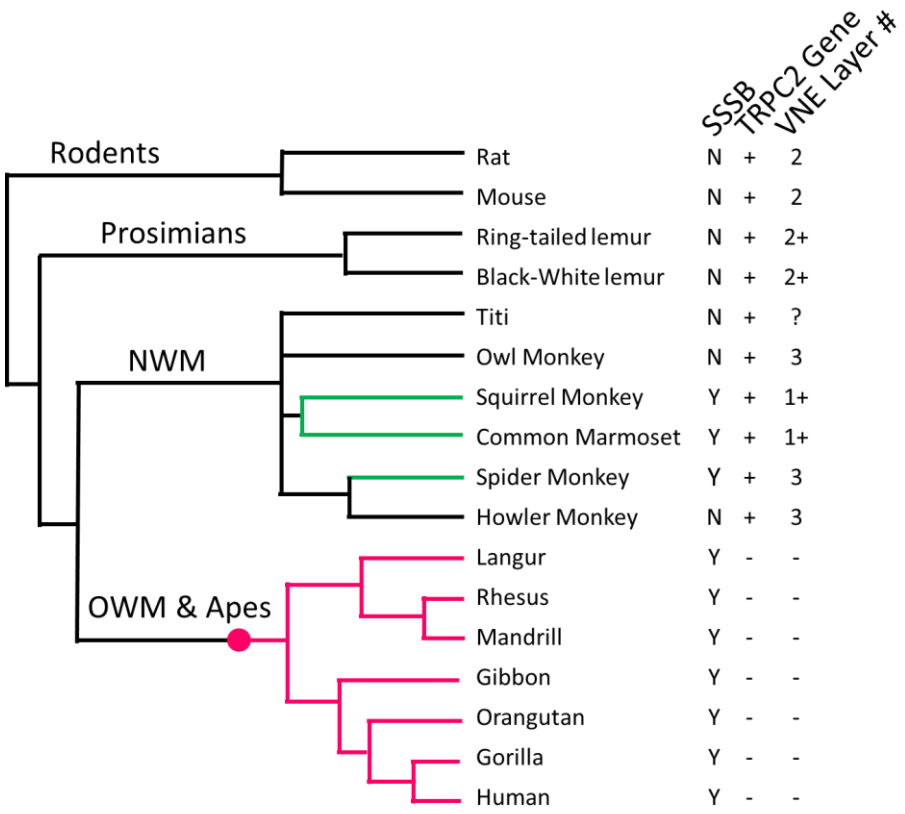

Figure 20: *TRPC2 loss led to SSSB in Old world Monkeys and Apes.* Same-sex sexual behavior is rare in the prosimians and many New World monkeys. However, nearly all Old World monkeys and Apes have been documented displaying same-sex sexual behavior in males or females (Bagemihl, 1999; Dixson, 2010; Vasey, 1995, 2017). The loss of the TRPC2 gene in the common ancestor of Old World monkeys and Apes (pink dot) coincides with the likely onset of same-sex sexual behavior 25 million years ago. TRPC2 may not influence SSSB in non-catarrhine primates as they retain the gene (Liman & Innan, 2003; J. Zhang & Webb, 2003). Ultimately, the vomeronasal organ sensory tissue is completely lost in all Old World monkeys and Apes (E. C. Garrett, 2015).Some New World monkeys display same-sex sexual behavior, such as Squirrel Monkeys and Marmosets, but they also show reductions to their vomeronasal neuroepithelial (VNE) layers with sensory tissue interspersed with non-sensory regions when compared with other new world monkeys (E. C. Garrett, 2015; Eva C Garrett et al., 2013; Hunter et al., 1984; T. D. Smith et al., 2002; T. D. Smith et al., 2011). Some prosimians and NWM, like the Spider Monkey, show SSSB (Vasey, 2017) despite a complex vomeronasal organ (Evans & Schilling, 1995; T. D. Smith et al., 2011).

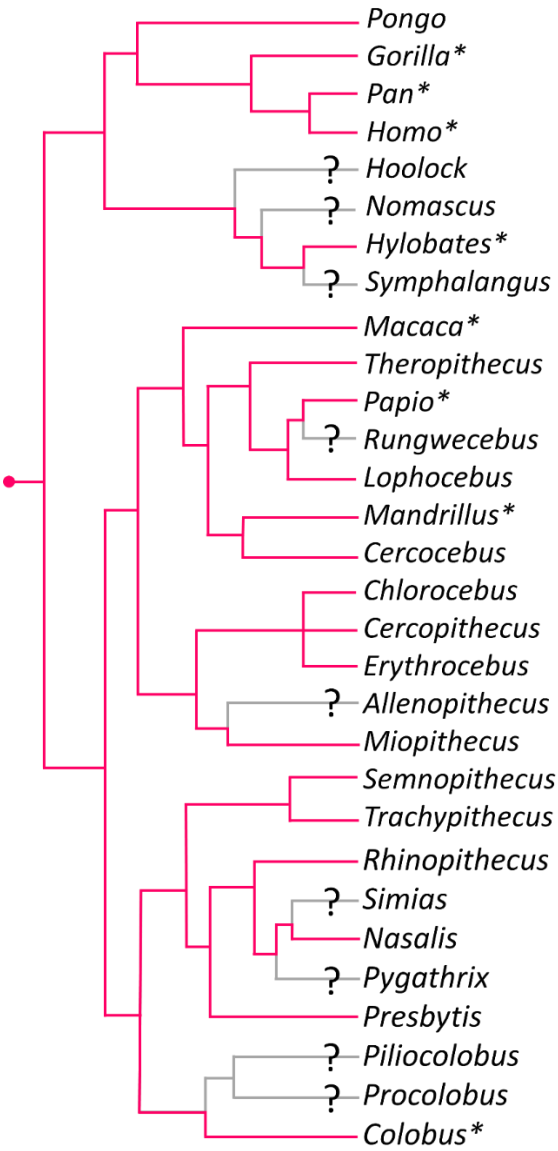

Figure 21: *Phylogeny of Old World Monkeys and Apes tracking same-sex sexual behavior (pink) to a common ancestor, a question mark indicates no same-sex sexual behavior has been documented* (Dixson, 2010, 2012; Vasey, 1995, 2017) (Personal communication; Teichroeb). Asterisks indicate a common premature stop codon in the TRPC2 gene used as evidence for the loss of gene function 25 million years ago (circle) in the catarrhine common ancestor (Liman & Innan, 2003; J. Zhang & Webb, 2003). All OWMs for which there is direct evidence of a nonfunctional TRPC2 gene show SSSB.

Overall conclusions and future directions

An important aspect of these projects is the detection of differences associated with higher testosterone (T) levels. Some, but not all, researchers have found higher testosterone levels in KO mice (Yu, 2015) which may play an important role in the measures I collected and in KO behavior (Martel & Baum, 2009). However, through-out these projects, I have accumulated data which suggests my findings are not the result of altered hormone levels. In chapter two I both directly and indirectly measured T levels. I found sex-typical levels of T and anogenital distance lengths, a T-sensitive developmental measure. Then, in chapter three I found that neuron soma size in the MePD, which is sensitive to T (Morris, Jordan, King, et al., 2008), was unaltered after TRPC2 KO. That T and T-sensitive measures are unaltered by TRPC2 KO suggests both brain and behavioral differences are unrelated to hormones. Although, in chapter four I discovered the VMHvl of TRPC2 KO males is not activated by female urine, which may prevent the pheromone-induced surge males receive after sensing a females urine (Coquelin et al., 1984). So, while circulating levels of T are likely similar between KOs and WTs, it is possible that the lifelong exposure received by KOs differs from WTs. Still, my findings support the idea that changes after TRPC2 KO are not a result of altered hormones in adulthood or development.

I originally argued that rather than simply reducing VNO function, the TRPC2 KO affected significant changes in the brain. In chapter three I show that TRPC2 KO influenced many regional and cellular measures in the MePD and VMHvl, two regions known to be involved with sex-typical behaviors altered in KO mice. Then in chapter four I found that female urine activates brain regions associated with sexual and aggressive behaviors in the male KO brain. Extended cohabitation with a receptive female caused the BNST of KO males to decrease activation in response to female urine. This is opposite to what has been previously found in male mice as sexual experience typically increases BNST activity in response to female urine, which produces the preference for female urine. However, the control strain for KO mice has not been tested using this paradigm and may yield different results. Still, the change in BNST activity after sexual experience in chapter four was accompanied by altered responses to the urinary status of intruders. This suggest the anatomical changes in the brain may be allowing functional changes as well. This is not so surprising considering many species have lost their TRPC2 gene, as I

describe in my final chapter. Future research with TRPC2 KO mouse may help us understand the brains and behavior of these organisms.

While 'altered sex differences' accurately describes changes to the KO MePD, it is important to consider how the sex-specific nature of cell loss fits with the idea of a "sex difference". One operational definition for sex difference is an "endpoint that exists on a continuum where the average is different between males and females." (McCarthy, Arnold, Ball, Blaustein, & De Vries, 2012) and MePD neuron number and size would certainly fit this description (Pfau et al., 2016). For example, high circulating testosterone maintains larger neuron soma such that removing testosterone makes male soma 'female-like', i.e. castrated male neurons are similar in size to unmanipulated females. Treating females with testosterone makes their soma larger, 'male-like'. So, testosterone levels control the average somal size of MePD neurons (Morris, Jordan, King, et al., 2008). In other words, these manipulations move the average size of neuron soma towards the opposite-sex. Neuron loss after TRPC2 KO happens in the *left* and *right* hemispheres of males and females respectively. One may conclude the TRPC2 KO removes a sex difference in the right hemisphere, as males become while adding one on the left. However, when you compare KO to WT of the same-sex it may be considered the loss of two distinct neuron subpopulations, a unique population on the *left* in males and the *right* in females. Such subpopulations would fit the definition of a sexual dimorphism, where the "Endpoint consists of two forms, one more prevalent in males and the other more prevalent in females. Endpoint may be present in one sex and absent in the other." (McCarthy et al., 2012). This question should guide future research determining if cell changes represent homogenous neuron loss or the loss of neuron subpopulations in separate hemispheres. The former suggesting 'sex difference' is the more accurate term and the latter supporting the use of 'sex dimorphism'. My findings add to literature that suggests some central nervous system sex differences rely on peripheral signals which may be unrelated to steroid hormones (Swift-Gallant et al., 2012). For example, sex differences in spinal neuron number rely on maternal behavior in rats. Dams lick the anogenital region of male pups more than females and this stimulation increases spinal neuron survival in males, producing a sexual dimorphism. These sexually dimorphic neurons control sexual behavior in males but not females (Moore, 1992). Similar to how rat pups experience different levels of licking, the way mice interact with and experience pheromones varies by sex. Sex differences in both the

behavioral interactions with pheromones and signaling within the AOC may provide a mechanism to produce brain sex dimorphisms, eventually leading to sex-typical behaviors in adulthood. TRPC2 may be critical for this process.

My work identifies several measures that may inform future inquiry. First, reproductive deficits and developmental differences in TRPC2 KO mice may result from extra-VNO expression of TRPC2. Examining unique population of TRPC2 expressing cells may uncover further explanations for the drastic changes seen after TRPC2 KO. For example, TRPC2 expression in the endometrium might be driving reproductive deficits *and* developmental differences regardless of TRPC2 expression elsewhere in the body. This is one drawback for global silencing of a gene but can be circumvented by tissue-specific genetic tools. It would be interesting to determine if silencing TRPC2 in the VNO alone could reproduce KO behaviors. Similarly, examining specific subpopulations of neurons in the MePD of both sexes and VMHvl of males might deepen our understanding of their function. Given the importance of ER, GABA and kisspeptin neurons within the MePD and VMHvl, these neuron subpopulations may be the most efficacious targets. One could also examine the MePD and VMHvl across the mouse lifespan to determine if differences in volume, neuron size and number and astrocyte number appear prior to adulthood, which would suggest TRPC2-dependent mechanisms are present during the development of these regions. Additional targets for regional analysis may be drawn from my study on urine-induced activation of the brain as the BNST is likely altered by TRPC2 KO. Manipulating neuron populations that are known to be lost, altered or inactive in TRPC2 KOs may help us understand how these regions produce the sex-typical behaviors and physiology of mice.

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