PUBERTAL CYTOGENESIS IN NEURAL CIRCUITS MEDIATING SEX-SPECIFIC SOCIAL BEHAVIORS

By

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ABSTRACT

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During puberty and adolescence, the brain experiences significant structural changes that mediate the maturation of sex-specific sociosexual behaviors, such as sexual behavior and intermale aggression. These sex-specific sociosexual behaviors are regulated by sexually dimorphic brain regions such as the posterodorsal medial amygdala (MePD) and ventromedial hypothalamus (VMH). The MePD in laboratory rodents is larger in males than females and is responsible for processing chemosensory signals to initiate context-appropriate social behaviors. The VMH is also larger in males than females in rodent species, which is primarily driven by sex differences in the ventrolateral subdivision (VMHvl). The VMHvl is involved in gating male sexual behavior and intermale aggression. Many of these sociosexual behaviors do not emerge until the onset of puberty, suggesting that morphological changes in the MePD and VMHvl during puberty may facilitate their development. Recent studies have discovered that new cells are added to the hypothalamus and amygdala during puberty and a subset of pubertally born cells in the MePD is activated in response to a social encounter. The current dissertation sets out to expand on these findings and examine pubertal cytogenesis in the MePD and VMH using the mouse model. In Chapter 1A, a sex difference in the number of pubertally born cells was revealed in the adult MePD, with males having more pubertally born cells than females. This increase in pubertally born cells in males was dependent on the presence of functional androgen receptors (ARs)

and pubertal and adult testosterone. In Chapter 1B, while I initially discovered a sex difference in pubertally born cells in the VMHvl, this finding was not replicated in subsequent studies. In addition, no sex differences were observed in the number of pubertally born cells in sexually monomorphic brain regions (i.e. dorsomedial and central subdivision of the VMH (VMHdm and VMHc, respectively) and dentate gyrus (DG)). These results suggest that the number of pubertally born cells in the adult VMH and DG may not be affected by gonadal hormones. In Chapter 2, I discovered that pubertal, but not adult, ARs promote the increase in pubertally born neurons, but not astrocytes, in the adult male MePD. However, pubertal and adult ARs did not influence the number of pubertally born neurons or astrocytes in the VMH or DG. In Chapter 3, I found that social interaction activated pubertally born cells in the adult male MePD, while social interaction with a male conspecific activated pubertally born cells in the adult male VMHvl and VMHdm. Social interaction did not activate pubertally born cells in the VMHc and DG. These studies demonstrate that gonadal hormones play an important role in regulating the proliferation and/or survival of pubertally born cells in the adult MePD, but not VMH or DG. Furthermore, I provide evidence that pubertally born cells in the MePD, VMHvl, and VMHdm are functionally incorporated into adult neural circuits. Thus, the addition and functional integration of pubertally born cells into neural circuits known to regulate social behavior may be a mechanism by which adult sociosexual behaviors emerge during puberty.

Dedicated to In Suk Kim.

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TABLE OF CONTENTS

LIST OF FIGURES

KEY TO ABBREVIATIONS

Avidin-biotin complex	ABC
analysis of variance	ANOVA
androgen receptor	AR
anteroventral periventricular nucleus	AVPV
5-bromo-2'-deoxyuridine	BrdU
3,3'-Diaminobenzidine tetrahydrochloride	DAB
dentate gyrus	DG
doublecortin	DCX
estrogen receptor alpha	ERα
glial fibrillary acidic protein	GFAP
immediate early gene	IEG
immunoreactive	ir
intraperitoneal	i.p.
induced testicular feminization mutation	*i*Tfm
luteinizing hormone	LH
posterodorsal medial amygdala	MePD
neural/glial antigen 2	NG2
neuronal nuclei	NeuN
postnatal day	P
principal nucleus of the bed nucleus of the stria terminalis	pBNST
progesterone receptor	PR

proliferating nuclear cell antigen	PCNA
sexually dimorphic nucleus of the preoptic area	SDN-POA
standard error of the mean	SEM
subgranular zone	SGZ
subventricular zone	SVZ
subcutaneous	s.c.
posterodorsal medial amygdala	MePD
Tris-buffered saline	TBS
ventromedial hypothalamus	VMH
ventromedial hypothalamus (ventrolateral subdivision)	VMHvl
ventromedial hypothalamus (dorsomedial subdivision)	VMHdm
ventromedial hypothalamus (central subdivision)	VMHc
wildtype	WT

INTRODUCTION

During puberty, the brain experiences substantial structural reorganization that, in turn, facilitates behavioral changes necessary to navigate the adult world. We've known for about 20-25 years that frontal and temporal cortical regions undergo significant remodeling during adolescence through synaptogenesis, synaptic pruning, apoptosis, and increased myelination (Barnea-Goraly et al., 2005; Blakemore et al., 2010; Giedd, 2004, 2008; Sisk et al., 2005). However, recent studies from our lab have revealed a new agent of change in the adolescent brain, namely the addition of new neurons and glial cells to subcortical regions such as the amygdala and hypothalamus, both of which are involved in regulating sex-specific social behaviors (Ahmed et al., 2008; Mohr et al., 2016; Mohr et al., 2013).

Postnatal Neuro- and Gliogenesis

Research on the generation of new neurons in the brain, or neurogenesis, outside of the early formative stages of brain development was a highly controversial topic for decades (Kaplan, 2001). In the 1960s, the popular opinion within the field of neuroscience was that the brain remained a static structure producing very little, if any, new neurons beyond the time of birth (Gross, 2000). However, in the 1980s and 90s, researchers began to reexamine the possibility of adult neurogenesis and the groundbreaking discovery was made that new neurons are, in fact, generated in the adult hippocampus and olfactory bulbs across several species (e.g. canaries, mice, rats, tree shrews, and primates) (Cameron et al., 1993; Goldman et al., 1983; Gould et al., 1997; Gould et al., 1999; Kempermann et al., 1997; Paton et al., 1984). These findings began to shift the view of neuroscience and reignited the field's interest in postnatal

neurogenesis. It is now widely recognized that new cells are continuously added to the hippocampus and olfactory bulbs throughout the lifespan.

Throughout the years, most of the efforts to investigate postnatal neurogenesis have been directed towards the hippocampus and olfactory bulbs. The hippocampus and olfactory bulbs gain a significant number of new neurons throughout the lifespan, continuously supplied with newborn neurons by the subgranular zone (SGZ) of the hippocampus and subventricular zone (SVZ) of the lateral ventricles, respectively (Alvarez-Buylla et al., 2004). The SGZ and SVZ have been characterized as neurogenic niches, giving rise to new neurons in the postnatal brain (Alvarez-Buylla et al., 2004). However, in recent years, it was discovered that new neurons are also added outside of these canonical neurogenic regions and investigators have come to realize that other limbic structures, such as the amygdala and hypothalamus, gain new cells during adolescence and adulthood (Ahmed et al., 2008; Fowler et al., 2003; Fowler et al., 2005; Mohr et al., 2016; Mohr et al., 2013; Saul et al., 2015).

Although research regarding adult neurogenesis continues to grow, research on pubertal neurogenesis remains relatively sparse. This lack of attention towards the pubertal brain is surprising, given the brain's increased plasticity during adolescence. Studies have determined that the adolescent brain gains significantly more new neurons than the adult brain (He et al., 2007; Ho et al., 2012; Saul et al., 2014). Moreover, it appears that pubertally born neurons are functionally distinct from adult born neurons. For instance, when using a transgenic mouse model to block neurogenesis in the SGZ and SVZ at different time points, researchers discovered that blocking pubertal neurogenesis in female mice reduced social exploration and increased social aversion

during female-female social interactions. In contrast, blocking adult neurogenesis did not have any significant effect on these social behaviors. However, pubertal and adult neurogenesis appear to have a similar function in other behaviors (i.e. anxiety and object-recognition memory), as blocking neurogenesis during both time points reduced anxiety-like behavior and impaired preference for novel objects (Wei et al., 2011). Interestingly, it was also discovered that reducing pubertal neurogenesis globally in mice using temozolomide, a chemotherapy drug, caused impaired spatial learning on the Morris water maze task. However, reducing adult or middle-aged neurogenesis in the same manner did not have a deleterious effect on spatial learning (Martinez-Canabal et al., 2013). Thus, neurons born at different developmental periods appear to perform specialized behavioral functions, with pubertally born neurons facilitating the acquisition of adaptive behaviors (e.g. appropriate social interactions and spatial navigation for mating and food foraging) required for adulthood.

Contrary to postnatal neurogenesis, the concept of postnatal gliogenesis, or the generation of new glial cells, has been readily accepted by neuroscientists for several decades (Kaplan, 2001). It was long believed that glial cells were merely supportive cells in the brain, or the glue that held the brain together, as astrocytes provided nutrients to neurons, microglia cleaned up debris from neuronal injury, and oligodendrocytes myelinated axons (Allen et al., 2009). In short, the major contribution of glial cells to neural functioning was largely undervalued and ignored and it was not until the late 1990s that scientists began looking past these passive roles originally assigned to glial cells to investigate the more active presence of glia. Today, we know that glial cells play an important role in maintaining a healthy neural environment.

Astrocytes regulate the formation, maintenance, and elimination of synapses in the brain (Clarke et al., 2013). Furthermore, astrocytes possess transporters that participate in the reuptake of neurotransmitters (e.g. glutamate) and can also release glutamate stores into the extracellular space to replenish the glutamate supplies of neighboring neurons (Clarke et al., 2013). In addition, not only do microglia clean up debris in the central nervous system, but they also mediate synaptic development by way of synaptic pruning (Harry et al., 2012) and oligodendrocytes have the capacity to release neurotrophic factors to aid in axonal repair (Alizadeh et al., 2015). Even though great strides have been taken to better understand the functional role of glial cells in the nervous system, little is known regarding their developmental trajectory in the postnatal brain. As with postnatal neurogenesis, the study of gliogenesis in the adult brain has dominated the field and research on pubertal gliogenesis remains scarce. The brain experiences synaptic reorganization and an increase in white matter volume during puberty and adolescence (Giedd et al., 1999), which are most likely mediated by glial cells. Therefore, it is essential that we gain a better understanding of pubertal gliogenesis to determine whether glial cells generated during puberty, rather than preexisting glial cells, contribute to structural and functional changes in the adolescent brain.

Because adaptive social functioning is critical in maintaining psychological health, it is important to understand the mechanisms by which neural circuits regulating social behaviors are remodeled in the pubertal brain. Currently, there is a paucity of research examining pubertal neurogenesis and gliogenesis, especially in regions outside of the traditional neurogenic regions such as the amygdala and hypothalamus,

which are known to regulate sociosexual behaviors (i.e. mating and aggression) that emerge during puberty (Cooke et al., 2003; Cooke et al., 2000; Kollack-Walker et al., 1992, 1995; Lee et al., 2014; Lin et al., 2011). Because the prevalence of mental health disorders that affect social behaviors increases during puberty(Paus et al., 2008), an improved understanding of the determinants and downstream effects of pubertal neurogenesis is clearly warranted. Given that some pubertally born neurons are functionally distinct from adult born neurons (Wei et al., 2011), not all findings regarding adult neurogenesis and gliogenesis can be extended to explain the regulation of these processes during puberty. It is essential that more attention be shifted onto pubertal neurogenesis and gliogenesis research in order to 1) further our understanding of the functional role of pubertally born cells in adulthood and 2) determine their contribution to adolescent structural brain changes.

The Social Neural Network

Rodents have complicated social networks and interact with one another based largely on chemosensory cues to determine appropriate social action that, in the wild, would increase their chances of survival and/or reproduction. For instance, adult male mice are strongly attracted to female mice, as they find their scent (e.g. urine or vaginal secretions) to be rewarding(Bell et al., 2013a; Brown, 1979). However, an adult male mouse can find the scent of another male mouse to be aversive, causing avoidance or aggressive behavior depending on the context of the social situation(Brown, 1979; Jones et al., 1989). These behaviors emerge and are refined throughout the adolescent period providing mice with the necessary skills to thrive as adults. The development of adult sociosexual behaviors (i.e. sexual behavior and aggression) has been attributed to

the maturation of the adolescent brain, which includes significant structural changes to the amygdala and hypothalamus(Sisk et al., 2005).

The posterodorsal medial amygdala (MePD) is a subregion in the amygdala that receives strong input from the accessory olfactory bulb and, in turn, sends afferent projections to hypothalamic regions known to govern reproductive and defensive behaviors(Pardo-Bellver et al., 2012). Thus, the MePD evaluates and categorizes chemosensory signals in order to initiate contextually-appropriate adult social behaviors (Samuelsen et al., 2009). In male mice, when the scent of a female (e.g. urine) is detected, the MePD becomes activated to initiate male sexual behavior (Samuelsen et al., 2009). Further, ablation of the MePD impairs opposite sex odor preference in male hamsters (Maras et al., 2006). The MePD of male rodents is also activated by social encounters with female and male conspecifics (Hong et al., 2014; Kollack-Walker et al., 1995; Taziaux et al., 2011). Sociosexual behaviors and opposite sex odor preference emerge around the time of puberty, coinciding with the sudden rise in pubertal gonadal hormones, primarily testosterone in males and estrogen in females (Cooke et al., 2003; Cooke et al., 2007; Cooke et al., 2005). The MePD is densely populated with androgen receptors (ARs) and estrogen receptors (ERs)(Simerly et al., 1990), and therefore, gonadal hormones may act via ARs and/or ERs in the MePD during puberty to mediate the maturation of sex-specific sociosexual behaviors in adulthood.

The adult MePD is a male-biased sexually dimorphic brain region. Males have a larger volume, more neurons and glial cells, larger soma size, and greater synapse number in the MePD than adult females (Cooke et al., 2003; Cooke et al., 2005; Morris et al., 2008a; Morris et al., 2008b). Manipulating adult hormones can eliminate some of

these sex differences. For example, castrating adult male rats can reduce MePD volume, neuronal soma size, and glial cell number, while treating adult females with testosterone for at least 4 weeks can increase MePD volume and neuronal soma size (Morris et al., 2008a). Although sex differences in the MePD exist prior to puberty, they become even more prominent during puberty (Johnson et al., 2013). Sex differences in pubertal hormone exposure (i.e. testosterone in males and estrogen in females) likely facilitate sex differences in the morphological changes observed in the MePD during puberty, which may mediate the emergence of sex-specific social behaviors (Juraska et al., 2013). In male rats, sex differences in MePD volume before puberty are independent of AR activity; however, this sex difference in MePD volume during puberty requires the presence of functional ARs (Johnson et al., 2013). Thus, the MePD is highly sensitive to circulating gonadal hormones, with males requiring pubertal ARs establish sex differences in the adult MePD. However, it still remains unclear how pubertal hormones, such as testosterone and estrogen, act via ARs and ERs to shape sex differences in the MePD during puberty to facilitate the acquisition of sex-specific sociosexual behaviors in adulthood.

The VMH is known regulate a wide variety of behaviors, such as feeding, female sexual behavior(King, 2006; McClellan et al., 2006; Pfaff et al., 1979), and male sociosexual behaviors (Choi et al., 2005; Lee et al., 2014; Lin et al., 2011). The ventrolateral subdivision of the VMH (VMHvl) and dorsomedial subdivision of the VMH (VMHdm) are the most well characterized subdivisions. Both the VMHvl and VMHdm are strongly innervated by the MePD and pheromonal cues are processed by the MePD (Pardo-Bellver et al., 2012) to then elicit reproductive or defensive behaviors,

respectively (Kunwar et al., 2015; Lin et al., 2011; Mongeau et al., 2003). In male mice, cell recordings revealed that VMHvl neurons are excited in response to aggressive interactions, while sexual encounters inhibited the same VMHvl neurons (Lin et al., 2011). In addition, optogenetic activation of VMHvl neurons evokes aggressive behaviors, whereas silencing these neurons inhibited aggressive behaviors(Lin et al., 2011). Further investigation revealed that estrogen receptor-α (ERα) neurons are responsible for controlling aggressive behavior in adult male mice, as activing ERα neurons in the VMHvl was able to initiate attack behaviors towards both male and female mice(Lee et al., 2014). Weak stimulation of these ERα neurons also promotes male sexual behavior(Lee et al., 2014). Another study showed that ERα and progesterone receptor ablation in the VMHvl reduces male sexual behavior and intermale aggression in adult male mice (Sano et al., 2013; Yang et al., 2013). Interestingly, when exposed to a predator, the VMHdm, but not VMHvl, shows a robust Fos response, a marker of neural activation, in adult male mice (Silva et al., 2013). Moreover, a predator scent alone (i.e cat collar) can also increase Fos expression in the VMHdm of adult male rats (Dielenberg et al., 2001). The VMHc has also been implicated in the control of defensive behaviors as stimulation of neurons in the VMHdm and VMHc induced avoidance behaviors, such as fleeing, jumping, and immobility, in adult male mice (Wang et al., 2015). Therefore, it is clear that, in adult male mice, the VMHvl is involved in gating male sexual behavior and intermale aggression, while the VMHdm and VMHc mediate defensive behaviors. As mentioned previously, male sexual behavior and intermale aggression begin to emerge at the onset of puberty; however, defensive behaviors are present prior to puberty. Interestingly, the VMHvl has a higher

density of ARs and ERs than the VMHdm and VMHc, suggesting that pubertal gonadal hormones may act on sex steroid receptors in the VMHvl to facilitate the emergence of these adult sociosexual behaviors.

Structurally, the VMH is a male-biased brain region, with males having a larger volume than females (Dugger et al., 2007; Matsumoto et al., 1983). Initial studies examining sex differences in the VMH established that males have a larger VMH than females early in development during the perinatal period. Castrating male rats on P1 eliminates sex differences in VMH volume in adulthood, while castrating male rats on P7 does not(Matsumoto et al., 1983). While early studies did not distinguish between VMH subdivisions, later studies discovered that the sex difference in VMH volume is largely accounted for by the VMHvl, while the VMHdm and VMHc are known to be monomorphic. Furthermore, the larger VMHvl volume in males can be accounted for by males having a larger neuronal soma size and a greater amount of neuropil than females, and not neuron number (Dugger et al., 2007; Flanagan-Cato et al., 2001; Madeira et al., 2001). While most of the structural VMHvl sex differences are established prior to puberty, sex differences in the number of spine synapses and shaft synapses do not emerge until puberty when males have significantly higher numbers compared to females (Pozzo Miller et al., 1991). In addition, treating females with testosterone eliminates this sex difference(Pozzo Miller et al., 1991). Therefore, synaptic reorganization in the VMHvl during puberty could contribute to the maturation of sex-specific sociosexual behaviors in adulthood.

The current literature indicates that the MePD and VMH play an integral role in the regulation of sociosexual behaviors in adulthood and that structural changes in

these brain regions during puberty may mediate the development of these behaviors to allow individuals to survive and thrive in the adult world. Recent reports indicate that new neurons and glial cells are added to the amygdala and hypothalamus during puberty(Ahmed et al., 2008; Mohr et al., 2016; Mohr et al., 2013), but it remains unknown whether or how these pubertally born cells may contribute to the gain of function observed during adolescent development. As previously mentioned, sociosexual behaviors such as opposite-sex odor preference and intermale aggression are not established until the pubertal period(Barkley et al., 1977; Bell et al., 2013a; Bell et al., 2013b; Brown, 1979; Jones et al., 1974, 1989; Maras et al., 2006; Tenk et al., 2009). Since these sociosexual behaviors are regulated by the MePD and VMH, it is possible that new neurons and glial cells born during puberty may alter existing neural circuits that underlie the development of these behaviors.

Defensive behaviors are present in early development, as rodents can display freezing behaviors at a young age(Wiedenmayer, 2009). The implementation of more adaptive defensive skills throughout development, such as avoidance behavior (Wiedenmayer, 2009), may result from the maturation of the VMHdm and VMHc. Given that many of these more advanced behaviors do not emerge until the pubertal period, it is of great interest to investigate how pubertal hormones shape the adolescent brain. Furthermore, a study has shown that pubertally born cells in limbic and hypothalamic regions of the male hamster are activated during a sexual encounter(Mohr et al., 2013). Thus, not only are new cells generated during puberty, they also mature into more permanent fixtures in the brain and likely participate in the expression of adult social behaviors. Since it has been established that the amygdala and hypothalamus are

capable of gaining new cells during puberty, it is of great interest to investigate how the addition of new cells during puberty contribute to sex differences in adulthood and whether these newly generated cells contribute to the maturation of these sociosexual behaviors.

Hormonal Regulation of Pubertal Neurogenesis and Gliogenesis

Neurogenesis and gliogenesis have not been well defined in the adolescent brain, and furthermore, there are very few studies that have examined this phenomenon outside of the canonical neurogenic zones. However, due to the strong influence of pubertal hormones on MePD and VMH, it is of great interest to examine how manipulation of gonadal hormones affects proliferation and/or survival of pubertally born neurons and glia into the adult brain and also, how these pubertally born cells contribute to the gain of function (e.g. sociosexual behaviors) during adolescent development.

In one of the initial studies examining pubertal cytogenesis, sex differences in the number of pubertally born cells added to sexually dimorphic brain regions in rats were examined(Ahmed et al., 2008). The study focused on three sexually dimorphic brain regions: the medial amygdala, the sexually dimorphic nucleus in the preoptic area (SDN-POA), and the anteroventral paraventricular nucleus (AVPV). The medial amygdala and SDN-POA are both male-biased brain regions, as these regions are volumetrically larger in males than females and are known to mediate male sex behavior(Morris et al., 2004). In contrast, the AVPV is a female-biased brain region known to be involved in controlling the luteinizing hormone (LH)-surge in adult females(Mohr et al., 2016; Morris et al., 2004). The study found that, during puberty, the medial amygdala and SDN-POA had more pubertally born cells surviving to adulthood

in males than in females; however, in the AVPV females had more pubertally born cells than males in adulthood(Ahmed et al., 2008). Furthermore, it appeared that these sex differences were driven by gonadal hormones, as pre-pubertal gonadectomy reduced the number of pubertally born cells in medial amygdala and SDN-POA in males, but not females, and the number of pubertally born cells in AVPV in females, but not males(Ahmed et al., 2008). These results suggest that one role pubertal sex hormones play in achieving fully mature adult neural circuits is to promote the addition and/or survival of new cells in specific sexually dimorphic regions of the brain.

Despite these compelling results, very little work has been done to follow-up these findings and examine the effects of gonadal hormones on the proliferation or survival of pubertally born cells. However, there is evidence that demonstrates the importance of gonadal hormones in modulating the addition of new cells in the adult brain. In adult male rats, androgens increased cell survival in the DG (Hamson et al., 2013; Spritzer et al., 2007). These results were supported by a follow-up study that demonstrated functional ARs were necessary to promote the survival of adult born neurons (Hamson et al., 2013). Although, testosterone did not influence cell proliferation in the DG or VMH of adult male meadow voles (Fowler et al., 2003), estrogen enhanced the cell survival in the DG (Ormerod et al., 2004). Additionally, estrogen enhanced cell proliferation in the DG of adult female rats (Tanapat et al., 1999), but not adult female meadow voles (Fowler et al., 2005). In adult male meadow voles and hamsters, testosterone did increase cell proliferation (Antzoulatos et al., 2008; Fowler et al., 2003) in the medial amygdala; in male meadow voles, this increase in proliferation was largely due to the effects of estrogen rather than androgens. Furthermore, testosterone did not

enhance cell survival in the medial amygdala of adult male hamster (Antzoulatos et al., 2008). However, testosterone appears to have a different effect on the pubertal brain, as testosterone promoted the number of pubertally born cells in the adult male rat medial amygdala, suggesting that testosterone enhance the proliferation and/or survival of these pubertally born cells. Interestingly, a study also demonstrated that, in male rats, the addition of pubertally born astrocytes was dependent on the presence of functional ARs. Based on this information, it appears that gonadal hormones have a species-, brain region-, age-, and sex-specific effect on the proliferation and survival of newly born cells in the postnatal brain.

Studies of pubertal cytogenesis have also confirmed that a subset of pubertally born cells in the medial amygdala, SDN-POA, and AVPV of adult male and female rats differentiate into mature neurons or astrocytes (Ahmed et al., 2008; Mohr et al., 2016). In adult male hamsters pubertally born cells in the medial amygdala, medial preoptic area, and arcuate nucleus also differentiate into mature neurons and astrocytes, with the arcuate nucleus and MePD showing the highest percentage of pubertally born cells expressing a mature neuronal marker (30% and 23% respectively) compared to the medial preoptic area (9%) (Mohr et al., 2013). Interestingly, a study in male rats discovered that 56-day old pubertally born cells did not differentiate into mature neurons in the adult amygdala, but discovered that 10-day old pubertally born cells did express doublecortin (~30%), an immature neuron marker, and NG2 (54%), an oligodendrocyte precursor cell marker (Saul et al., 2015). The latter study only administered BrdU during P30-31(Saul et al., 2015), while a previous study examining the medial amygdala administered BrdU for 3 weeks during puberty (P28-49)(Mohr et al., 2013). Therefore,

differences in the number of pubertally born neurons in the amygdala may be due to differences in the age of these cells. It is possible that pubertally born cells born outside of P30-31 are more likely to differentiate into mature neurons. Overall, these studies demonstrate that a subset of pubertally born cells in the amygdala and hypothalamus survive into adulthood and differentiate into mature neuronal and glial phenotypes. However, the proportion of cell phenotypes (i.e. neurons vs. glia) may be site-specific and age-specific.

While it is likely that gonadal hormones play a key role in regulating pubertal cytogenesis, further investigation is required to determine how hormones, such as testosterone and estrogen, mediate the addition of new neurons and glial cells in the different regions of the pubertal brain. It is unknown whether ARs or ERs modulate the survival of pubertally born cells into adulthood. In order to ask more in-depth questions regarding the hormone regulation of pubertal cytogenesis, it will be in the best interest for researchers to utilize the mouse model because of the wide array of genetic tools currently available for dissecting and understanding the relevant neurobiological mechanisms. At the moment, it is unknown whether, in mice, there are sex differences in pubertally born cells in sexually dimorphic brain regions, such as the MePD and VMH. Thus, further investigation is required to determine if and how gonadal hormones influence the survival of pubertally born cells in the amygdala and hypothalamus and how these pubertally born cells contribute to adult sociosexual behaviors in mice.

Dissertation Overview

Previous studies in rats and hamsters have shown that new cells are added to the amygdala and hypothalamus during puberty, and some of these cells differentiate

into mature neurons or glial cells (Mohr et al., 2013). There is also evidence that a subset of these pubertally born cells become functionally integrated into adult neural circuits. However, it remains unclear how pubertal gonadal hormones may contribute to the proliferation and survival of these pubertally born cells or how these cells contribute to the gain of function that characterizes adolescent development. To investigate postnatal neurogenesis, many researchers utilize 5-bromo-2'-deoxyuridine (BrdU), a thymidine analog that is incorporated into DNA during the S-phase of the cell cycle. Today, it remains a popular tool and is commonly referred to as a cell birth date marker. Some skeptics in the field argue that BrdU labels postmitotic cells undergoing DNA repair, rather than new neurons (Taupin, 2007); however, studies have shown that BrdU-immunoreactive (ir) cells co-express proliferative markers such as PCNA or Ki67, indicating that these BrdU-ir cells are actually new proliferating cells and not older, preexisting neurons undergoing DNA repair(Lagace et al., 2010; Mohr et al., 2016). More specifically, our lab has demonstrated that about 95% of pubertally born cells in the anteroventral paraventricular nucleus of the hypothalamus (AVPV) co-express PCNA (Mohr et al., 2016). Therefore, we will use BrdU to label pubertally born cells in the current dissertation.

Since pubertal cytogenesis has not yet been examined in the mouse brain, the experiments in the current dissertation focus its attention on pubertal cytogenesis in mice. In chapter 1 of this dissertation, I first examine whether pubertal cytogenesis occurs in the mouse brain and whether there is a sex difference in the survival of pubertally born cells in sexually dimorphic brain regions in adulthood. For this dissertation, I examine the following brain regions of interest (ROIs): MePD, VMHvl,

VMHdm, VMHc, and DG. Again, the MePD and VMHvl are male-biased brain regions, having a larger volume in males than females(Dugger et al., 2007; Morris et al., 2008a; Morris et al., 2008b; Pfau et al., 2016), while the VMHdm, VMHc, and DG are known to be monomorphic brain regions, showing no sex differences in volume in adulthood(Dugger et al., 2007; Tabibnia et al., 1999). The results of chapter 1 show that new cells are added to the pubertal brain in mice, and there is a sex difference in the survival of these pubertally born cells with males having a greater number of pubertally born cells than females in the MePD and VMHvl (both male-biased brain regions). No sex differences in pubertally born cells were detected in the VMHdm, VMHc, and DG. The results of chapter 1 also demonstrate that functional ARs are necessary to enhance the survival of pubertally born cells in the adult MePD, but not VMHvl, VMHdm, VMHc, or DG, using iTfm male mice, a transgenic mouse model where AR is rendered non-functional throughout the lifespan. Furthermore, the presence of both pubertal and adult testosterone increases the number of pubertally born cells in the adult MePD of male mice.

Following the results of Chapter 1, Chapter 2 of this dissertation investigates whether AR specifically during puberty or adulthood are necessary to enhance the survival of pubertally born cells. We discover that AR during puberty, but not adulthood, is required to increase the number of pubertally born cells in the adult MePD. Disabling AR during puberty or adulthood does not affect the density of pubertally born cells in the VMHvl, VMHdm, VMHc, or DG. We also find that blocking AR during puberty reduces the survival of pubertally born neurons, but not pubertally born astrocytes, in the adult MePD.

In Chapter 3 of this dissertation, I examine whether pubertally born cells are functionally incorporated in response to male-male or male-female social interactions. The results of chapter 3 show that pubertally born cells in the MePD and VMH are functionally incorporated into existing neural circuits and are activated (i.e., express fos) in response to social interactions in adulthood. More specifically, we find that male-male and male-female social interactions activate pubertally born cells in the MePD to equivalent levels, whereas male-male social interactions, in particular, activate pubertally born cells in the VMH.

This dissertation is a first step towards understanding the role of pubertal cytogenesis in the amygdala and hypothalamus of the mouse brain, a phenomenon that is virtually unexplored. While we establish that pubertal testosterone act via ARs in the male mouse brain to increase the number of pubertally born cells in the adult MePD and that a subset of pubertally born cells are functionally integrated into the adult brain, there is still much to learn about the significance of pubertally born cells in adult brain functions. In the conclusions chapter of this dissertation, I further discuss the implications of the dissertation findings and consider future studies to expand on these results to better understand the mechanisms underlying pubertal cytogenesis and its contribution to the function of the adult brain.

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CHAPTER 1A

Sex differences in pubertally born cells in the posterodorsal medial amygdala in adult mice

ABSTRACT

The posterodorsal medial amygdala (MePD) regulates many sex-specific social behaviors, such as sexual behavior and aggression, and is sexually dimorphic, with males having a larger volume than females in adult rats and mice. While structural sex differences in the rodent MePD exist prior to puberty, they become even more exaggerated during pubertal development and are maintained into adulthood. Recently, sex differences in the addition of new cells during puberty have been reported in the MePD of rats, with males displaying more pubertally born cells in the adult MePD than females. This result suggests neuro- and gliogenesis as a possible mechanism by which sex differences in MePD structure are shaped during puberty. However, it is currently unknown whether sex differences in pubertal cytogenesis also exist in the mouse MePD or whether gonadal hormones influence the proliferation and/or survival of pubertally born cells in the mouse brain. To investigate this, we conducted four experiments in which all mice were administered a daily i.p. injection of 5-bromo-2'-deoxyuridine (BrdU) during puberty (postnatal day (P)28-49). First, we examined sex differences in the survival of pubertally born cells in the adult MePD. Then, we used an androgen receptor (AR)-null transgenic mouse model (iTfm) along with male and female wildtype littermates to investigate the role of AR on generating sex differences in pubertally born cells in the adult MePD. Next, we manipulated pubertal testosterone in male, female, and iTfm males to determine whether pubertal testosterone contributed to

sex differences in pubertally born MePD cell numbers in adulthood. Lastly, we explored the differential effects of pubertal and adult testosterone on the addition of pubertally born cells in the adult MePD by treating male mice with testosterone during puberty and/or adulthood. Overall, we discovered a sex difference in pubertally born cell number and density in the adult MePD with male mice having more pubertally born cells than females. In addition, we found that functional ARs and pubertal testosterone are necessary to establish sex differences in pubertally born cell number in the adult MePD. Finally, we concluded that both pubertal and adult testosterone enhance the number of pubertally born cells in adult male mice. These results indicate that pubertal testosterone and ARs play an important role in establishing sex differences in the number of pubertally born cells in the adult MePD of mice. Since many of the adult social behaviors controlled by the MePD emerge at the onset of puberty and require the presence of gonadal hormones, these findings suggest that cells added during puberty may potentially contribute to the emergence of sex-specific adult behaviors in mice.

INTRODUCTION

Puberty and adolescence represent a developmental period during which the brain undergoes significant changes in both structure and function. Well documented structural changes in the adolescent brain include synaptic pruning and growth, cell death, and myelination, which likely promote the acquisition of mature behaviors (e.g. social and reproductive behaviors) necessary to navigate the adult world (Barnea-Goraly et al., 2005; Blakemore et al., 2010; Blakemore et al., 2006; Giedd, 2004; Giedd et al., 1999; Zehr et al., 2006). However, recently, researchers have identified a new

and surprising agent of change in the adolescent brain: neurogenesis and gliogenesis in the amygdala (Ahmed et al., 2008; Mohr et al., 2013; Saul et al., 2015).

Research on adolescent neurogenesis and gliogenesis traditionally focus on two brain regions, the hippocampus and the olfactory bulb – both long recognized to gain new cells during adolescence and even adulthood. The subventricular zone and the subgranular zone of the hippocampus are characterized as neurogenic niches, giving rise to new neurons throughout the lifespan (Alvarez-Buylla et al., 2004). However, in recent years, the focus on neuro- and gliogenesis has turned to regions outside of these canonical neurogenic zones, as studies have discovered other limbic regions, like the medial amygdala, also gain new cells in adolescence and adulthood (Ahmed et al., 2008; Fowler et al., 2003; Fowler et al., 2002; Mohr et al., 2013; Saul et al., 2015).

The posterodorsal medial amygdala (MePD) is a sexually dimorphic brain region known to regulate sociosexual behaviors, such as sexual behavior (Cooke et al., 2003; Cooke et al., 2000). The adult male rodent MePD has a larger volume, more neurons and glial cells, and larger neuronal soma size than the adult female MePD (Johnson et al., 2008; Morris et al., 2008a; Morris et al., 2008b; Pfau et al., 2016). Many structural sex differences in the adult MePD can be altered by manipulating circulating gonadal hormones (Cooke et al., 2003; Johnson et al., 2013; Morris et al., 2008a; Morris et al., 2008b), which is not surprising given that the MePD is densely populated with sex steroid receptors (Simerly et al., 1990). For example, castration of adult males leads to a reduction of MePD volume, smaller soma size, and fewer glial cells (Morris et al., 2008b), and conversely, treating adult females with testosterone increases MePD volume and soma size (Morris et al., 2008a). However, the manipulation of gonadal

hormones in adulthood does not have a significant effect on the number of MePD neurons (Morris et al., 2008a), suggesting the sex difference in MePD neuron number is organized before adulthood.

Studies examining pubertal cytogenesis in the amygdala of rats and hamsters reveal a sex difference in the addition of new cells in the medial amygdala, including the MePD, with adult males having more pubertally born cells in the medial amygdala than adult female rats (Ahmed et al., 2008). Furthermore, pre-pubertal castration of male rats eliminates this sex difference, with the number of pubertally born cells decreasing to levels comparable to that of gonadally intact adult females (Ahmed et al., 2008). The evidence, thus far, suggests that the addition of new cells during puberty may be a key cellular event in the development of sex differences in the MePD. However, while gonadal hormones are known to affect the number of pubertally born cells in the adult MePD, the mechanisms through which gonadal hormones act to influence pubertal cytogenesis requires further investigation.

Sex differences in the MePD exist prior to puberty, but these sex differences are amplified in adulthood. This process appears to depend on the presence of functional androgen receptors (AR) in rats (Johnson et al., 2013). Using male rats insensitive to circulating androgens due to testicular feminization mutation (Tfm), researchers showed wildtype male rats have a significantly larger volume and more astrocytes in the adult MePD than either wildtype female or Tfm male rats (Johnson et al., 2013). However, prior to puberty, Tfm male rats have a MePD volume and astrocyte numbers more comparable to wildtype males than females (Johnson et al., 2013). These findings indicate that the pubertal increase in MePD volume and astrocyte number in males

requires functional ARs, and prompted investigation of whether ARs also influence pubertal cytogenesis in mice.

Given that mouse models enable the use of a wide array of genetic tools to dissect and understand pubertal cytogenesis, the current study investigates pubertal cytogenesis in the MePD of mice. First, we examine sex differences in the number of pubertally born cells in the adult MePD. Next, we look at how AR and pubertal testosterone influence sex differences in pubertally born cells in the adult MePD using Cre-Lox technology to disable AR in male mice. Lastly, we examine the differential effects of pubertal and adult testosterone on the number of pubertally born cells in the adult MePD.

MATERIALS AND METHODS

Animal Housing

All mice were group housed (2-3 same-sex mice/cage) in clear plexiglass cages (29x18x13cm) in a 12:12 light:dark cycle (lights on: 7:00 AM; lights off: 7:00 PM). All subjects were provided ad libitum rodent chow (Harlan Teklad 8640 Rodent Diet, Madison, WI) and water. All housing and experimental procedures were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by Michigan State University's Institutional Animal Care and Use Committee.

Experiment 1: Are there sex differences in the number of pubertally born cells in the adult MePD?

Experiment 1 examined whether cells are added to the mouse MePD during puberty and whether there are sex differences in the number of pubertally born MePD

cells that survive into adulthood. Male (n=16) and female (n=16) wild-type (WT) C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, Maine) on postnatal day 21 (P21). During puberty (P28-49), all mice received a daily i.p. injection of the cell birth date marker 5-bromo-2'-deoxyuridine (BrdU; 200 mg/kg body weight; Prod. No. B5002; Sigma-Aldrich) during the light period between 10:00 AM-12:00 PM. In adulthood, half of the males and females were perfused on P60; the remaining half were perfused on P90. To analyze group differences in total number of BrdU-immunoreactive (ir) cells and BrdU-ir cell density, a two-way ANOVA was used with sex and age at sacrifice as independent factors. For all analyses, $p<0.05$ was considered statistically significant, and Cohen's d was calculated to determine effect sizes.

Experiment 2: Are functional androgen receptors necessary to establish sex differences in the number of pubertally born cells in adulthood?

Experiment 2 investigated the role of ARs on the number and density of pubertally born MePD cells, using male mice with induced testicular feminization mutation (*i*Tfm). *i*Tfm males are an AR-null model that is induced by cre/lox technology as previously described (Chen et al., 2014). WT male (n=10), WT female (n=10), and *i*Tfm males (n=10) were obtained from an in-house *i*Tfm colony. All animals were weaned and group housed on P24. During puberty (P28-49), all mice received a daily i.p. injection of BrdU (200 mg/kg body weight) during the light period between 8:00 AM-10:00 AM. To standardize the levels of circulating hormones in the three genotypes in adulthood, all mice were gonadectomized on P60 and received a subcutaneous Silastic capsule implant filled with testosterone (1.6 mm – inner diameter and 3.2 mm – outer diameter; 6 mm – effective release length, 1.6 cm – total capsule length). All animals

were perfused on P90. A one-way ANOVA was used to determine group differences in the total number of BrdU-ir cells, BrdU-ir cell density, and volume by genotype. For all analyses, $p<0.05$ was considered statistically significant, and Cohen's d was calculated to determine effect sizes.

Experiment 3: Is pubertal testosterone required to establish sex differences in the number of pubertally born MePD cells in adulthood?

Experiment 3 examined the effects of pubertal testosterone and the role of ARs on the survival of pubertally born MePD cells in adult mice. WT male (n=20), WT female (n=20), and *i*Tfm males (n=20) were obtained from the same *i*Tfm colony as in Experiment 2. All animals were weaned and group housed on P24. On P26, all animals were gonadectomized and, for each genotype, half the animals were subcutaneously implanted with a blank Silastic capsule (No T@P) and the other half received a testosterone-filled Silastic capsule (T@P). During puberty (P28-49), all animals received a daily i.p. injection of BrdU (200 mg/kg body weight) during the light period between 8:00 AM-10:00 AM. On P60, subcutaneously implanted capsules were removed from all animals and replaced with a testosterone-filled Silastic capsule to produce equivalent levels of circulating testosterone in all groups during adulthood. All animals were perfused on P90. A two-way ANOVA was used to analyze group differences in the total number of BrdU-ir cells, BrdU-ir cell density, and volume, using genotype and pubertal testosterone treatment as independent factors. For all analyses, $p<0.05$ was considered statistically significant, and Cohen's d was calculated to determine effect sizes.

Experiment 4: Do pubertal and adult testosterone have differential effects on the number of pubertally born cells in the adult MePD?

The purpose of Experiment 4 was to tease apart the effects of pubertal vs adult testosterone on the number of pubertally born MePD cells in male mice. WT male mice (n=32) were obtained from a WT C57BL/6 in-house colony. All animals were weaned and group housed on P24. On P26, all mice were gonadectomized and half the mice were subcutaneously implanted with a blank Silastic capsule and the remaining half received a testosterone-filled Silastic capsule. During puberty (P28-49), all animals received a daily i.p. injection of BrdU (200 mg/kg body weight) during the light period between 12:00 PM-2:00 PM. On P60, subcutaneously implanted capsules were removed from all subjects. Within each group, half the mice then received a blank Silastic capsule, while the other half received a testosterone-filled Silastic capsule. Thus, four experimental groups were created: 1) no testosterone after gonadectomy on P26 (T-/-); 2) testosterone during puberty only (T+/-); 3) testosterone in adulthood only (T-/+); and 4) testosterone during both puberty and adulthood (T+/+). All animals were perfused on P90. Group differences in the total number of BrdU-ir cells, BrdU-ir cell density, and volume were examined using a one-way ANOVA comparing four groups: T-/-, T+/-, T-/+, and T+/+. For all analyses, $p<0.05$ was considered statistically significant, and Cohen's d was calculated to determine effect sizes.

Tissue Collection and Processing

All mice were given an overdose of sodium pentobarbital (150 mg/kg) by i.p. injection and intracardially perfused with 0.9% saline followed by cold 4% paraformaldehyde in 0.1 M phosphate buffered saline (pH 7.4). Brains were removed

and postfixed overnight in 4% paraformaldehyde, then stored in 30% sucrose at 4 °C until sectioning. Brains were cut on a freezing microtome into 30 µm coronal sections, and collected as either 3 (Experiment 1) or 4 alternate series of sections (Experiments 2-4). For each experiment, the entire first series was Nissl stained with thionin, dehydrated, and coverslipped.

Immunohistochemistry

In each experiment, the entire second series of brain sections was processed for immunohistochemistry to label BrdU-ir cells. Free-floating sections were rinsed in Tris-buffered saline (TBS; 0.05 M; pH 7.6) for 30 minutes and then incubated in 0.6% H_2O_2 for 10 minutes. After a 15-minute rinse in TBS, the sections were incubated in 0.93N HCl for 30 minutes at 37°C (except in experiment 4 – the sections were incubated in 2N HCl for 1 hour at 37°C). Sections were next neutralized in 0.1M borate buffer (pH 8.5) for 10 minutes. Following a 30-minute rinse in TBS, the sections were blocked in TBS containing 0.01% Triton-X-100 and 3% donkey serum, and then incubated in monoclonal rat anti-BrdU (catalog no. MCA2060; Serotec) at a working concentration of 1 µg/ml at 4°C for 48 hours. Sections were then rinsed in TBS for 30 minutes and incubated in biotinylated donkey anti-rat secondary antibody (catalog no. 712-065-150; Jackson Immunoresearch) at a working concentration of 2 µg/ml for 2 hours at room temperature. Following a 30-minute rinse in TBS, the sections were incubated in avidin/biotinylated enzyme complex (ABC Elite Kit, Vector Laboratories, Burlingame, CA) for an hour and then reacted in 3,3'-Diaminobenzidine tetrahydrochloride (DAB; Sigma; working concentration 0.25 mg/ml) for about 2 minutes. Following this reaction, all sections were mounted onto gelatin-subbed slides and coverslipped.

Microscopic Analysis

All microscopic analyses were performed by an observer blind to treatment group using an Olympus BX51 microscope under brightfield illumination using Neurolucida version 10 (MBF Bioscience). The regions of interest were traced using the Nissl-stained series and contours were superimposed onto adjacent sections of the single-label BrdU series. The boundaries of the MePD on the Nissl series were determined using a standard mouse brain atlas (Paxinos et al., 2004). To consistently sample anatomically-matched sections across all animals, the following landmarks were used to trace the MePD: the shape of the optic tract, the size and shape of the lateral ventricles, the size and shape of the intercalated amygdalar nucleus, and the size and shape of the stria terminalis. BrdU-ir cells were identified by the presence of a dark brown nuclear stain.

Single-label BrdU Analysis

In experiment 1, only five anatomically-matched bilateral sections of the MePD were available for analysis due to poor tissue quality of the more posterior sections. Animals that did not have five anatomically-matched sections were excluded from analysis. Final sample sizes were: P60 females (n=7), P60 males (n=5), P90 females (n=5), and P90 males (n=6). In experiments 2-4, six sections throughout the full rostral-caudal extent of the MePD were available for analysis. Animals that did not have six anatomically-matched sections were excluded from analysis. The final sample size for each experiment were: Experiment 2 – males (n=9), females (n=9), and iTfm (n=9); Experiment 3 – No T@P males (n=7), No T@P female (n=7), No T@P iTfm (n=9); T@P

males (n=7), T@P females (n=7), and T@P iTfm (n=9); Experiment 4 – T-/- males (n=10), T-/+ males (n=10), T+/- males (n=8), and T+/+ males (n=9).

For all experiments, the total number of BrdU-ir cells was determined by summing the number of BrdU-ir cells across all sections analyzed. The density of BrdU-ir cells in each animal was calculated by dividing the total number of BrdU-ir cells (summed across sections) by the total area (summed across sections). For experiments 2-4, MePD volume was estimated by summing the area of all sections analyzed and multiplying by the number of series (4) and section thickness (30 μm).

RESULTS

Experiment 1: Adult male mice have more pubertally born MePD cells than adult female mice.

We found BrdU-ir cells in several regions of the adult mouse brain, including the MePD (Fig 1.1), indicating that new cells are generated in the mouse brain during puberty. Two-way ANOVA was performed to examine sex differences in the number of pubertally born MePD cells in adulthood at P60 and P90. A main effect of sex on total number of BrdU-ir cells in the MePD was observed (F (1,19)=20.84, p=0.000). Male mice had ~1.8 times more pubertally born MePD cells than female mice (Fig 1.2A). There was not a main effect of age at sacrifice, indicating that pubertally born cells survive from P60 to P90 with very little attrition of cells. Thus, if new cells born during puberty survive until P60, they will likely survive until P90. There was no significant interaction between sex and age of sacrifice. Thus, at P60, males have more BrdU-ir cells in the MePD than females, and this sex difference is stably maintained until at least P90. A similar pattern of results was obtained when BrdU-ir cell density was

analyzed. Two-way ANOVA revealed a significant main effect of sex ($F(1,19)=13.05$, $p=0.002$), with no main effect of age at sacrifice and no significant interaction. Male mice had ~1.4 times the density of BrdU-ir cells in the MePD compared to female mice (Fig 1.2B).

Experiment 2: Dysfunctional androgen receptors are sufficient to eliminate sex differences in the number of pubertally born cells in the adult MePD.

One-way ANOVA revealed a significant difference among genotypes on the total number of BrdU-ir cells in the adult MePD ($F(2,24)=9.27$, $p=0.001$). WT male mice had 1.5 times the number of BrdU-ir cells in the MePD compared to WT female ($p=0.011$, $d=1.62$) and 1.7 times the number of BrdU-ir cells than *i*Tfm male mice ($p=0.001$, $d=1.84$) (Fig 1.3A). No significant difference was observed between WT female and *i*Tfm male groups. Quantitative analysis also found a significant difference between genotypes on the density of BrdU-ir cells in the adult MePD ($F(2,24)=10.25$, $p=0.001$), with WT males having 1.5 and 1.6 times the density of pubertally born MePD cells compared to WT female ($p=0.006$, $d=1.87$) and *i*Tfm mice ($p=0.001$, $d=1.78$), respectively (Fig 1.3B). There was no significant difference between the WT female and *i*Tfm male mice in the density of pubertally born cells in the MePD. There was no significant difference between genotypes on MePD volume ($F(2,24)=0.90$, $p=0.42$) (Fig 1.3C).

Experiment 3: Pubertal testosterone and functional androgen receptors enhance the number of pubertally born MePD cells.

Two-way ANOVA found a significant main effect of genotype ($F(2,40)=9.13$, $p=0.001$) and pubertal testosterone treatment ($F(1,40)=23.22$, $p=0.000$) on the total

number of pubertally born MePD cells in adulthood, as well as a significant interaction ($F(2,40)=4.61$, $p=0.016$) (Fig 1.4A). Bonferroni post-hoc tests revealed no significant differences in the number of BrdU-ir cells between genotype in the No T@P groups. However, significant differences among genotypes were observed amongst the T@P groups. Female mice treated with pubertal testosterone had the most pubertally born MePD cells, with 1.3 times the number of BrdU-ir cells compared to WT male mice ($p=0.026$, $d=0.88$), and 2 times the number of cells compared to iTfm mice ($p=0.000$, $d=2.03$). In addition, male WT mice had 1.5 times the number of BrdU-ir cells compared to iTfm mice ($p=0.011$, $d=1.33$).

A similar pattern of results was obtained when BrdU-ir cell density was analyzed, i.e., significant main effects of genotype ($F(2,40)=7.81$, $p=0.001$) and pubertal testosterone treatment ($F(1,40)=15.63$, $p=0.000$), as well as a significant interaction ($F(2,40)=4.87$, $p=0.013$) (Fig 1.4B). Bonferroni post-hoc tests revealed no significant differences between genotype within the no T@P groups on the density of BrdU-ir cells in the MePD. However, there were significant differences amongst all genotypes within the T@P groups on the density of BrdU-ir cells. Female mice treated with pubertal testosterone had 1.3 times and 2.1 times higher density of pubertally born cells in the MePD than males ($p=0.034$, $d=0.89$) and iTfm mice ($p=0.000$, $d=2.08$), respectively. Male mice also had a 1.6 times higher density of BrdU-ir cells than iTfm mice ($p=0.012$, $d=1.19$). Two-way ANOVA of MePD volume found no significant main effect of genotype ($F(2,40)=0.24$, $p=0.790$); however, there was a significant main effect of pubertal testosterone treatment on MePD volume ($F(1,40)=4.95$, $p=0.032$, $d=0.71$), with mice that received pubertal testosterone having an MePD volume that is ~10% larger

than the No T@P groups (Fig 1.4C). No significant interaction was found ($F(2,40)=1.22$, $p=0.307$).

Experiment 4: The presence of both pubertal and adult testosterone increases the number of pubertally born MePD cells.

One-way ANOVA revealed significant group differences on the total number of BrdU-ir cells in the MePD ($F(3,33)=13.10$, $p=0.000$) (Fig 1.5A). Bonferroni post hoc analyses showed that T+/+ males had significantly more pubertally born MePD cells than T-/- ($p=0.000$, $d=2.39$), T-/+ ($p=0.004$, $d=1.76$), and T+/- ($p=0.038$, $d=1.36$) males. In addition, T+/- males had more BrdU-ir cells in the MePD than T-/- males ($p=0.028$, $d=1.43$). Similarly, T-/+ males had a larger number of BrdU-ir cells compared with T-/- males (non-significant trend, $p=0.10$, $d=1.17$). Again, an ANOVA showed a similar pattern of results with significant group differences on the density of pubertally born cells in the MePD ($F(3,33)=10.69$, $p=0.000$) (Fig 1.5B). Bonferroni post hoc analyses showed that T+/+ males had significantly a higher density of BrdU-ir cells than T-/- ($p=0.000$, $d=2.42$) and T-/+ ($p=0.024$, $d=1.43$) males, but not T+/- males ($p=0.420$). In addition, T+/- males had a higher density of pubertally born cells in the MePD than T-/- males ($p=0.009$, $d=1.60$). Similar to total BrdU-ir cell number, T-/+ males had a higher density of BrdU-ir cells compared with NoT@P/NoT@A males (non-significant trend, $p=0.10$, $d=1.21$).

Significant group differences in MePD volume were also observed ($F(3,33)=3.18$, $p=0.037$) (Fig 1.5C). Bonferroni post hoc analysis found that T+/+ male mice had a significantly larger MePD volume than T-/- male mice ($p=0.031$, $d=1.40$). No significant differences in MePD volume were observed between all other groups.

DISCUSSION

Sex differences in pubertally born cells in the adult MePD

In the current study, we discovered a sex difference in pubertally born cells in the adult MePD, with males having a greater number and higher density of pubertally born cells than females, and this sex difference persists from P60 to P90. Because male mice typically have a larger MePD volume than females (Morris et al., 2008b; Pfau et al., 2016), this sex difference in the density of pubertally born cells shows that the gain of BrdU-ir cells is greater per mm^2 in males. This indicates that the greater number of BrdU-ir cells in males cannot be accounted for by sex differences in MePD size. These results corroborate previous findings on pubertal cytogenesis in rats, which also found that males had a higher number of pubertally born cells than females in the adult medial amygdala (Ahmed et al., 2008).

Interestingly, past studies have also shown that a subset of pubertally born cells in the rat and hamster MePD differentiate into mature neurons or astrocytes (Ahmed et al., 2008; Mohr et al., 2013); however, sex differences in the phenotype of pubertally born cells have not yet been examined. It has been established that male rodents have more neurons in the adult MePD than females (Morris et al., 2008a), while another study discovered no sex differences in neuron number prior to puberty (Cooke et al., 2005). These results suggest that sex differences in the number of neurons in the MePD develop during puberty, thus, the addition of new cells during puberty may be a potential mechanism by which adult sex differences in MePD neuron number are shaped. Further investigation regarding the phenotype of pubertally born cells will

provide more insight regarding when sex differences in MePD neuron number are established.

It should also be noted that neurons are not the only cell-type to increase in number in brain regions such as the amygdala and hypothalamus from adolescence to adulthood as several studies have shown that glial cells (i.e. astrocytes, microglia, and oligodendrocytes) also increase in number during puberty (Chareyron et al., 2012; Johnson et al., 2013; Mohr et al., 2016; Mohr et al., 2013; Saul et al., 2015). While sex differences in the addition of microglia and oligodendrocytes in the MePD during puberty have not been examined, it has been shown that male rats gain significantly more astrocytes in the MePD than females during puberty (Johnson et al., 2013). Therefore, the sex difference in the number of pubertally born cells in the adult MePD could also be accounted for by the addition of new glial cells.

Sex differences in the adult MePD: The role of androgen receptors and pubertal testosterone

The current findings also demonstrate that the presence of functional ARs and pubertal testosterone are necessary to establish sex differences in pubertally born MePD cells in adulthood as *i*Tfm males exhibit a more feminine number and density of pubertally born cells in the adult MePD. It should be noted that for this experiment, male, female, and *i*Tfm males remained gonadally intact during puberty, but received testosterone treatment in adulthood to standardize circulating levels of testosterone. Despite adult testosterone treatment, a sex difference in pubertally born cells was still detected, indicating adult testosterone treatment in female mice did not enhance the number of pubertally born cells to masculine levels. These results indicate that pubertal

testosterone plays an important role in establishing sex differences in the adult MePD. To confirm this, we manipulated pubertal testosterone and, again, standardized circulating levels of testosterone in adulthood. We found that male and female mice given pubertal testosterone had more pubertally born cells that *i*Tfm male mice with pubertal testosterone as well as those not given pubertal testosterone across all genotypes. These results demonstrate that both functional androgen receptors and pubertal testosterone are required to generate sex differences in pubertally born cells in the adult MePD. Similarly, a previous study in rats showed that pre-pubertal gonadectomy eliminated sex differences in the number of pubertally born cells in the adult medial amygdala (Ahmed et al., 2008). Furthermore, sex differences in the addition of astrocytes during puberty in the adult MePD requires functional AR (Johnson et al., 2013). These results suggest that pubertal testosterone may be activating ARs to facilitate the proliferation, migration, and/or survival of pubertally born cells in the MePD.

Confirming previous studies, we found that remaining gonadally intact during puberty and receiving testosterone in adulthood increased female MePD volume comparable to the size of males; however, we also discovered that the adult MePD volume was not significantly different between wildtype male and *i*Tfm male mice with adult testosterone. This novel finding suggests that adult testosterone acts via estrogen receptors (ERs) to increase MePD volume. The adult MePD volume of Tfm males, without hormone manipulation, is partially demasculinized, having an intermediate volume between wildtype males and females (Morris et al., 2005). The volumetric growth of MePD volume in *i*Tfm males in response to aromatized testosterone is feasible, as previous studies have demonstrated that both ERs and ARs contribute to

generating sex differences in MePD volume and neuronal soma size (Cooke et al., 2003).

Furthermore, we discovered that pubertal testosterone masculinized adult MePD volume, regardless of genotype. Subjects that received pubertal testosterone had a larger MePD volume than those that did not receive pubertal testosterone. Since the absence of pubertal testosterone reduced MePD volume in both male and female mice, despite adult testosterone, we can conclude that pubertal testosterone organizes the MePD to allow for adult testosterone to increase its volume in adulthood. Pubertal testosterone could be acting via ARs or ERs to organize the MePD, but since *i*Tfm males also had larger MePD volume when treated with pubertal testosterone, it seems that an ER-dependent mechanism is a likely candidate. *i*Tfm males have non-functional ARs, leading them to have small, undescended testes that secrete lower amounts of testosterone compared to wildtype males (Chen et al., 2014); however since we previously showed that adult testosterone can increase MePD volume in *i*Tfm male mice that are gonadally intact during puberty, it is possible that this low level of testosterone in *i*Tfm males may be aromatized to produce levels of estrogen that are sufficient to activate ERs and organize the MePD during puberty to enable adult testosterone to increase MePD volume. Furthermore, adult testosterone increases female MePD volume (Morris et al., 2008a; Morris et al., 2008b) even though female rodents do not typically experience high levels of androgens during puberty, indicating that pubertal estrogen activity in the MePD is sufficient to organize the MePD to allow for masculinizing effects of adult testosterone on MePD volume.

Pubertal and adult testosterone enhance the number of pubertally born cells in the adult male MePD

Results from the current report establish that pubertal and adult testosterone work together to increase the number of pubertally born cells in the adult male MePD. As predicted, male mice had more pubertally born cells in the adult MePD when treated with testosterone during puberty and adulthood than male mice that received no hormone treatment. In addition, pubertal testosterone alone increased the number of pubertally born cells compared to those that did not receive pubertal and adult testosterone, but without adult testosterone, cell counts were lower than males that received testosterone throughout the lifespan. Furthermore, adult testosterone alone resulted in fewer pubertally born cells in the adult MePD than those that received testosterone throughout the lifespan. These findings suggest that testosterone plays an important role in enhancing either the proliferation, migration, and/or survival of pubertally born cells during the pubertal period, but adult testosterone works to maximize the number of pubertally born cells in the adult MePD.

Moreover, testosterone treatment during puberty and adulthood increased MePD volume compared to males that received no hormone treatment during these time points. However, male mice given only pubertal or adult testosterone maintained an intermediary MePD volume. We previously established that the presence of pubertal testosterone is necessary for adult male mice to maintain a larger MePD volume relative to females. Furthermore, in previous findings in this study discussed above, this activational effect of adult testosterone in males was only seen when males also experienced pubertal testosterone which presumably organized the MePD in a

masculine fashion. Without the presence of pubertal testosterone, adult testosterone did not increase MePD volume. Thus, the discovery of this intermediate volume in males given testosterone only during puberty or adulthood was unexpected. A previous study in male hamsters did find that pubertal testosterone increased MePD volume independent of the presence of adult testosterone, suggesting that pubertal testosterone, at least in male hamsters, is sufficient to induce a long-term increase in MePD volume (De Lorme et al., 2012). However, since we detected a substantial increase in MePD volume in males that received testosterone during both puberty and adulthood compared to males that only received testosterone during puberty, we believe this not to be the case in male mice. These differences between mice and hamsters could be a result of differing testosterone treatments (i.e. hamster: 2 weeks of testosterone in adulthood; mice: 4 weeks of testosterone in adulthood); however, it is also possible that testosterone may organize the pubertal brain differently between species.

To our knowledge, there are currently no other studies that have examined the influence of gonadal hormones on the number of pubertally born cells into adulthood, specifically in the MePD; however, a few studies regarding adult neurogenesis may help elucidate the effects of gonadal hormones on proliferation and/or survival of newly born cells in the brain. A study in adult male rats demonstrated that while, testosterone did not affect cell proliferation in the DG, it does enhance cell survival (Spritzer et al., 2007). Interestingly, this enhancement in cell survival is due to actions of androgens, and not estrogen. Treatment with DHT, a potent androgen, but not estrogen, enhanced cell survival in the DG (Spritzer et al., 2007) and moreover, adult male rats treated with DHT

and flutamide, an AR antagonist, as well as Tfm male rats, displayed reduced levels of cell survival in the DG (Hamson et al., 2013). Surprisingly, testosterone appears to differentially effect cell proliferation in the medial amygdala compared to the DG with testosterone increasing cell proliferation in the medial amygdala of adult male meadow voles (Fowler et al., 2003), but these effects were mediated by estrogens and not androgens (Fowler et al., 2003). Furthermore, estrogen treatment has been shown in increase cell proliferation in medial amygdala of adult female meadow voles (Fowler et al., 2005) as well as the DG of adult female rat (Tanapat et al., 1999). Taken together, these results indicate that, in adulthood androgens and estrogens have a brain region-specific and sex-specific effect on adult neurogenesis. In males, androgens regulate cell survival, at least in the hippocampus, while estrogens promote cell proliferation in the medial amygdala. In females, estrogen promotes cell proliferation in both the DG and medial amygdala. Further investigation is required to determine whether pubertal estrogens and androgens can act in a similar manner to influence cell proliferation, migration, and/or survival to contribute to sex differences in pubertally born cell number in the adult MePD.

Significance of pubertally born cells in the MePD

The MePD is a key regulator of sex-specific sociosexual behaviors, such as male sexual behavior (Kollack-Walker et al., 1992, 1995). Many of these social behaviors emerge around the time of puberty, suggesting that structural changes that occur during the pubertal period may facilitate the development of behaviors required to survive and thrive in the adult world. Morphological changes in the pubertal brain, including the addition of new cells, are largely influenced by gonadal hormones, which increase at the

onset of puberty. Interestingly, manipulation of gonadal hormones significantly alters the development of social behaviors. For instance, testosterone treatment before and during puberty, but not after, promotes the development of male sexual behavior in hamsters(Schulz et al., 2009). In addition, opposite-sex odor preference, a behavior integral to male sexual behavior, does not develop until puberty(Bell et al., 2013). A study in male hamsters found that pubertally born cells in the MePD express Fos, a marker of neural activation, in response to a sexual encounter(Mohr et al., 2013). Therefore, the acquisition and incorporation of pubertally born cells into existing neural circuits may critically mediate the emergence of sociosexual behaviors and it is through the hormonal control of this cellular process that sex-specific sociosexual behaviors emerge.

Conclusions

The overall aim of this study was to examine sex differences in pubertal cytogenesis and determine the role of gonadal hormones on pubertally born cells in the adult mouse MePD. We discovered that there is a sex difference in the number of pubertally born cells in the adult MePD with males having more cells than females. We also revealed that androgen receptors are required to establish sex differences in pubertally born cell number in the adult MePD. Moreover, we found that pubertal and adult testosterone work together to enhance the number of pubertally born cells in the adult MePD. Our results establish a novel mechanism by which the pubertal brain is shaped by pubertal gonadal hormones to potentially contribute to sex differences in MePD morphology seen in adulthood. It is clear that pubertal androgens act via ARs to stimulate proliferation and/or enhance survival of pubertally born cells during puberty,

while adult testosterone increases the number of pubertally born cells; however, at this time, it is currently unknown whether adult testosterone acts through an AR or ER dependent mechanism to promote this increase in cell number. Follow up experiments will involve an analysis of the phenotype of pubertally born cells, which may differentiate into neurons or glia, and exploration of the potential functions pubertally born cells may serve in mediating sex-specific behaviors in adulthood.

Figure 1.1. Posterodorsal medial amygdala (MePD) in adult male and female mice. Representative photomicrographs displaying traces of the MePD on Nissl-stained coronal sections in the (A) male and (B) female adult mice and pubertally born cells in the MePD of (C) male and (D) female adult mice. The small inlaid boxes show BrdU-labeled cells at a higher magnification. OT = optic tract; MePV = posteroventral medial amygdala; BLA = basolateral amygdala. Low mag scale bar = 200 μm; high mag scale bar = 10 μm.

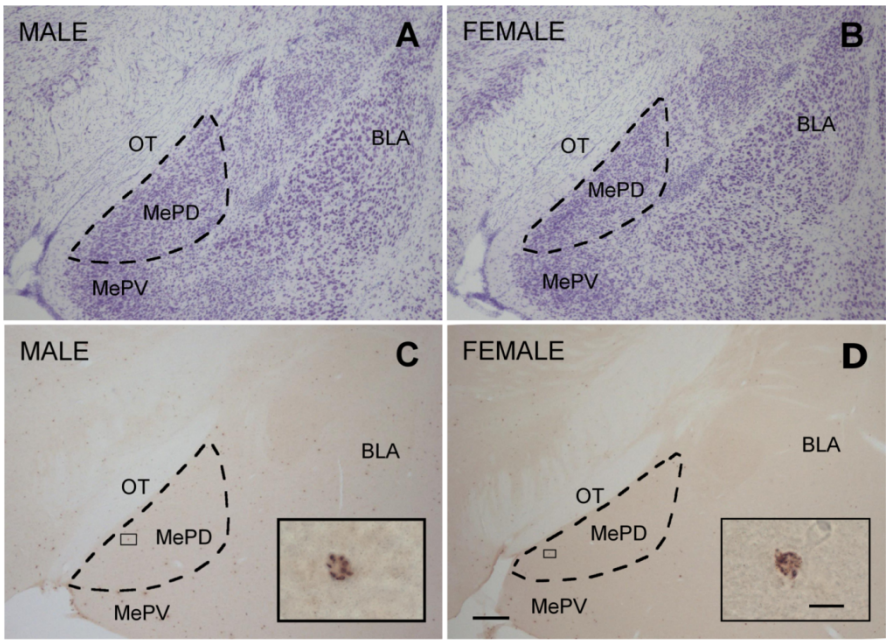


Figure 1.2. Sex differences in the total number and cell density of BrdU-ir cells in the posterodorsal medial amygdala (MePD) in adult mice. Quantitative analysis revealed that, in the MePD, (A) male mice have more pubertally born cells than female mice at P60 and P90 with no significant attrition of cells between the two time points. (B) The density of BrdU-ir cells was also significantly higher in the male MePD when compared to female mice. Data are represented as mean ± SEM.

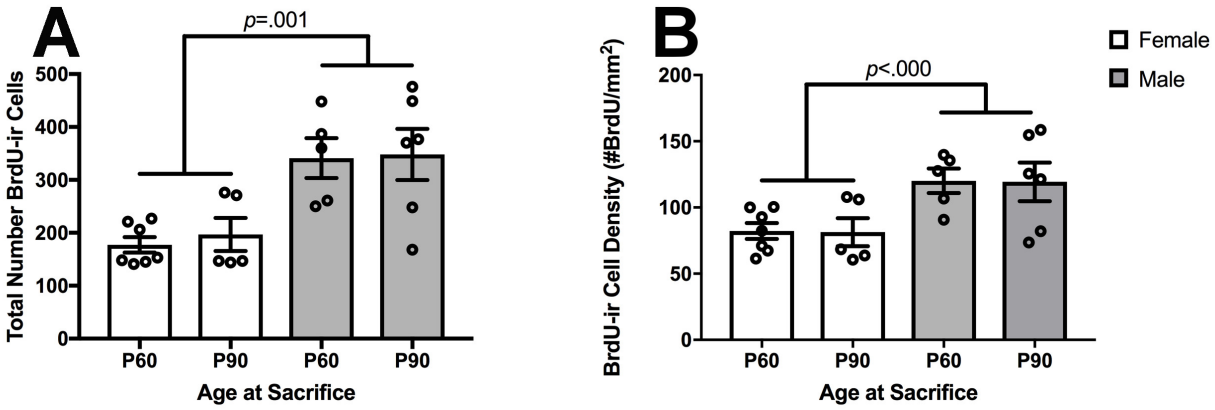


Figure 1.3. Sex differences in the total number and density of BrdU-ir cells in the MePD are dependent on functional androgen receptors and independent of adult testosterone. Quantitative analysis revealed that (A) male mice have significantly more pubertally born cells in the MePD when compared to female and iTfm mice treated with testosterone in adulthood. (B) The density of BrdU-ir cells in the adult MePD is higher in males than female and iTfm mice treated with adult testosterone. (C) No sex differences were observed in MePD volume between male, female, and iTfm mice when treated with testosterone. Data are represented as mean ± SEM.

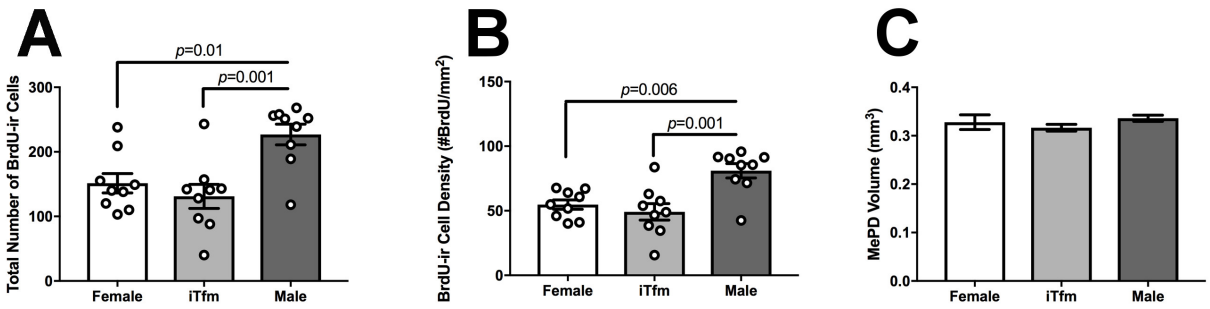


Figure 1.4. Sex differences in the total number and density of BrdU-ir cells in the MePD are dependent on pubertal testosterone and functional androgen receptors. Quantitative analysis revealed that (A) T@P female mice have significantly more BrdU-ir cells than all other groups and T@P male mice had more pubertally born cells than no T@P groups and T@P iTfm mice. (B) The density of BrdU-ir cells in the adult MePD is significantly higher in T@P female mice when compared to all other groups. (C) No sex difference is observed in MePD volume between male, female, and iTfm mice; however, animals that received testosterone during puberty have a larger MePD volume than groups that did not receive testosterone during puberty. Data are represented as mean ± SEM. *ps<0.04. #ps<0.02.

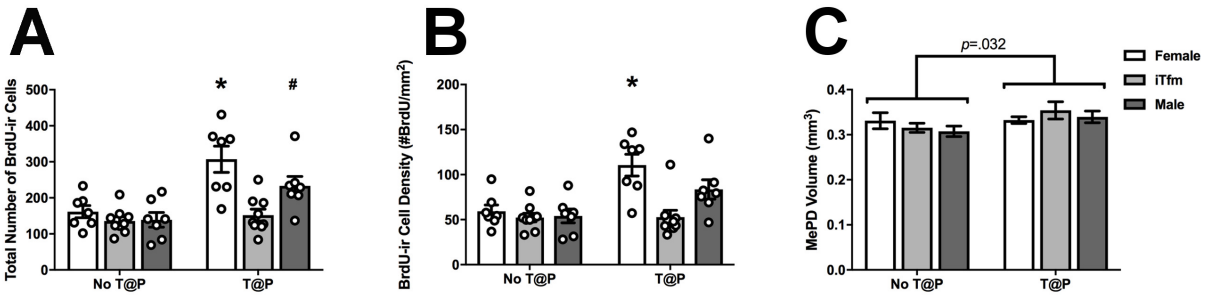
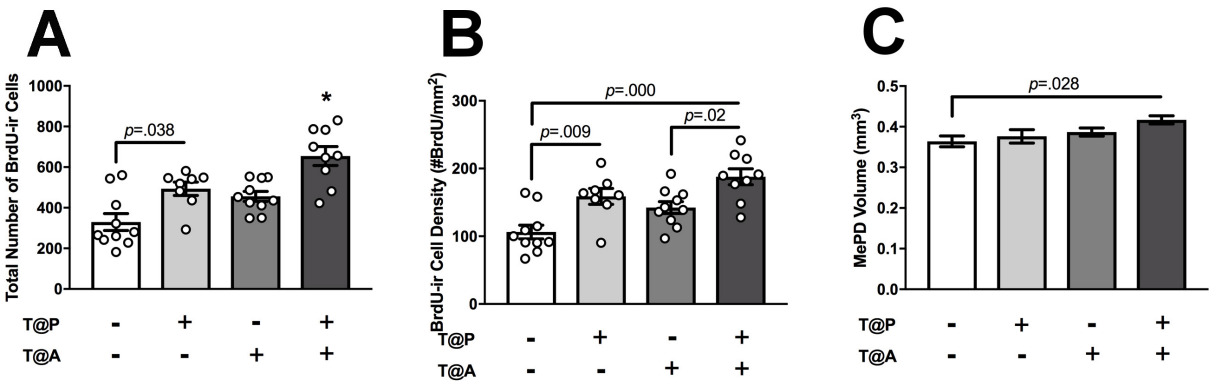


Figure 1.5. Pubertal and adult testosterone are necessary to increase the number of pubertally born cells in the adult male MePD. Quantitative analysis revealed that (A) T+/+ male mice had significantly more BrdU-ir cells in the MePD than all other groups. In addition, T+/- male mice had significantly more pubertally born cells in the MePD than the T-/- group. (B) T+/+ male mice had a higher density of pubertally born cells in the MePD compared to the T-/- and T-/+ groups. T+/- male mice also had a significantly higher density of BrdU-ir cells than the T-/- group. (C) T+/+ male mice had a significantly larger MePD volume than the T-/- male mice. Data are represented as mean ± SEM. T@P: testosterone during puberty; T@A: testosterone during adulthood.



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CHAPTER 1B

Pubertal cytogenesis in the mouse ventromedial hypothalamus and dentate gyrus

ABSTRACT

Many sex-specific sociosexual behaviors, such as sexual behavior and aggression, emerge during puberty and are regulated by sexually dimorphic structures in the brain like the ventromedial hypothalamus (VMH). The VMH is a male-biased neural structure and its volume is larger in males than females with the ventrolateral subdivision of the VMH (VMHvl) mainly driving this sex difference. The dorsomedial and central subdivisions of the VMH (VMHdm and VMHc, respectively) do not display a volumetric sex difference. Recent studies in rats and hamsters have found that new cells are added to the amygdala and hypothalamus in the pubertal brain, which may facilitate the acquisition of adult sociosexual behaviors. However, it is currently unknown whether mice experience pubertal cytogenesis in the VMH to potentially contribute to sex differences observed in the adult structure. In the current study, we examined whether there was a sex difference in the number of pubertally born cells in the VMHvl. To investigate this, we administered 5-bromo-2'-deoxyuridine (BrdU), a cell birth date marker, to male and female mice during puberty and examined the total number and density of pubertally born cells in adulthood at two time points, postnatal day (P)60 and P90. We also analyzed the VMHdm, VMHc. and dentate gyrus (DG), another monomorphic structure, as a control regions. Furthermore, we examined the role of androgen receptors (ARs) and pubertal testosterone on pubertal cytogenesis in the VMH and DG using an AR-null mouse model (iTfm). The current study detected a sex difference in the VMHvl, with males having significantly more pubertally born cells than

females and this sex difference was stably maintained from P60 to P90. No sex differences were discovered in pubertally born cell density in the VMHdm, VMHc, and DG. Furthermore, we discovered that ARs and pubertal testosterone do not promote the increase of pubertally born cells in all subdivisions of the VMH and DG. These findings demonstrate that sex differences in pubertal cytogenesis of the VMHvl are generated, independent of ARs and pubertal testosterone, to potentially contribute to structural sex differences observed in adulthood that may mediate the emergence of sex-specific adult behaviors in mice.

INTRODUCTION

Puberty and adolescence represent a critical period during which the brain undergoes significant changes in both its structure and function. Well documented structural changes in the adolescent brain include synaptic pruning and growth, cell death, and myelination, which likely promote the acquisition of mature behaviors necessary to navigate in the adult world, including social and reproductive behaviors (Barnea-Goraly et al., 2005; Blakemore et al., 2010; Giedd, 2004, 2008; Sisk et al., 2004). However, more recent studies have identified yet another cellular mechanism by which the adolescent brain undergoes change, namely, cytogenesis (Ahmed et al., 2008; He et al., 2007; Mohr et al., 2016; Mohr et al., 2013; Saul et al., 2015; Staffend et al., 2014).

Research on cytogenesis after the early formative stages of brain development has focused on two brain regions, the hippocampus and the olfactory bulb, long recognized to gain new cells throughout the lifespan. However, in recent years, neurogenesis and gliogenesis outside of these canonical neurogenic regions have

gained attention as investigators come to realize that other brain regions, such as the amygdala and hypothalamus, also gain new cells in adulthood (Fowler et al., 2003; Kokoeva et al., 2005; Ruscio et al., 2015). While the literature regarding cytogenesis in the adult brain continues to grow, studies investigating cytogenesis in the pubertal brain remain sparse even though evidence indicates that cytogenesis may be a key cellular event of brain development during puberty, given its wide spread occurrence in many different brain regions of several different rodent species, including rats and hamsters (Mohr et al., 2016; Mohr et al., 2013; Saul et al., 2015; Staffend et al., 2014). In Chapter 1A of this dissertation, we discovered that new cells are added to the posterodorsal medial amygdala (MePD) during puberty in mice, and in the current study, we turn our attention to the ventromedial hypothalamus (VMH).

The VMH is a male-biased sexually dimorphic brain region known to have a larger volume in males than females, with the ventrolateral subdivision of the VMH (VMHvl), in particular, driving this sex difference. The dorsomedial VMH (VMHdm) and the central VMH (VMHc) are sexually monomorphic in structure, having no significant volumetric differences detected in adulthood (Dugger et al., 2007). Recent evidence indicates that the murine VMHvl receives input from the MePD to gate the expression of sexual behavior or aggression as appropriate to the social context (Choi et al., 2005; Lin et al., 2011). These adult sociosexual behaviors emerge during the pubertal period, a time when rodents experience a significant increase in pubertal hormones, primarily testosterone in males and estrogen in females. While it has been established that gonadal hormones regulate synaptic organization in the VMH during puberty (Pozzo

Miller et al., 1991), it remains unknown how gonadal hormones influence the addition of new cells to the pubertal brain, particularly the VMH.

In the initial studies examining pubertal cytogenesis using 5-bromo-2'-deoxyuridine (BrdU), a cell birth date marker, researchers observed the addition of new cells in amygdala and hypothalamus, both regions involved in social behaviors in rats and hamsters. More specifically, there was a sex difference in the number of pubertally born cells in sexually dimorphic regions of the brain: the medial amygdala and the sexually dimorphic nucleus of the preoptic area (SDN-POA). The medial amygdala and SDN-POA of adult male rats had more BrdU-immunoreactive (ir) cells than female rats. Likewise, a brain region that is larger in adult females, the anteroventral paraventricular nucleus (AVPV), contained more BrdU-ir cells in adult females than in adult males (Ahmed et al., 2008). Pubertally born cells have also been found to differentiate into mature neurons or glia. Pubertally born cells in the adult hamster are even activated by a social encounter (Mohr et al., 2013). Thus, not only are new cells generated during puberty, they also appear to mature into more permanent fixtures in the brain and become functionally integrated into existing neural circuits. Therefore, in an effort to gain better understanding of the possible functions pubertally born cells may have, the current study first aimed to determine whether new cells were added to the VMH during puberty, and whether gonadal hormones led to an increase in their number in a sex-dependent manner.

In the current study, we administered BrdU to mice during puberty (P28-49) to label new born cells. First, we assessed sex differences in the number and density of pubertally born cells in the VMHvl, VMHdm, and VMHc at two time points: P60 and P90.

We also included the dentate gyrus (DG) in our analyses, as this brain region is also known to be sexually monomorphic in structure, and therefore, may serve as a control region. Next, we investigated the role of androgen receptors (ARs) and pubertal testosterone on pubertal cytogenesis in all regions of interest using an AR-null mouse model (iTfm) and comparing them to wildtype male and female mice.

MATERIALS AND METHODS

Animal Housing

In each experiment, all subjects were group housed (2-3 same-sex mice/cage) in clear plexiglass cages (29x18x13cm) in a 12:12 light:dark cycle (lights on: 7:00 AM; lights off: 7:00 PM) and provided ad libitum rodent chow (Harlan Teklad 8640 Rodent Diet, Madison, WI) and water. All housing and experimental procedures were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by Michigan State University's Institutional Animal Care and Use Committee.

Experiment 1: Are there sex differences in pubertally born cells in the adult VMH and DG?

In experiment 1, we assessed the number and density of pubertally born cells in the adult mouse VMHvl, VMHdm, VMHc, and DG to determine whether there was a sex difference in each region of interest. Male (n=16) and female (n=16) wild-type (WT) C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, Maine) on postnatal day 21 (P21). 5-bromo-2'-deoxyuridine (BrdU; 200 mg/kg body weight; Prod. No. B5002; Sigma-Aldrich) was administered daily (i.p.) during puberty (P28-49) during the light period between 10:00 AM-12:00 PM. Then, in adulthood, half of each sex were

perfused on P60 and the remaining half were perfused on P90. A two-way ANOVA was used to examine group differences in the total number of BrdU-ir cells and BrdU-ir cell density using sex and age at sacrifice as independent factors.

Experiment 2: Does the masculinization of pubertal born cells in the VMHvl require functional androgen receptors?

In experiment 2, we investigated the role of androgen receptors (AR) on the density of pubertally born cells in the VMHvl, VMHdm, VMHc, and DG, using male mice with induced testicular feminization mutation (*i*Tfm). As described in a recent study, iTfm males are an AR-null mouse model induced by cre/lox technology and are phenotypically female, but genetically male(Chen et al., 2014). WT male (n=10), WT female (n=10), and *i*Tfm males (n=10) were obtained from an in-house *i*Tfm colony. All animals were weaned and group housed on beginning on P24. All subjects received a daily i.p. injection of BrdU (200 mg/kg body weight) during puberty (P28-49) at the lights on period between 8:00 AM-10:00 AM. In order to standardize circulating levels of testosterone in adulthood, on P60, all mice were gonadectomized and subcutaneously implanted with a Silastic capsule filled with testosterone (1.6 mm – inner diameter and 3.2 mm – outer diameter; 6 mm – effective release length, 1.6 cm – total capsule length) and then perfused on P90. A separate one-way ANOVA was used to analyze group differences in the density of BrdU-ir cells by genotype for each region of interest.

Experiment 3: Is pubertal testosterone required to generate sex differences in the density of pubertally born cells in the adult VMHvl?

In experiment 3, we examined the role of pubertal testosterone and androgen receptors on the number of pubertally born cells in the VMHvl, VMHdm, VMHc, and DG

of adult mice. WT male (n=20), WT female (n=20), and *i*Tfm males (n=20) were obtained from the same *i*Tfm colony discussed in Experiment 2. All animals were weaned and group housed on P24. On P26, all animals were gonadectomized and, for each genotype, half the animals were subcutaneously implanted with a blank Silastic capsule (No T@P) and the other half received a testosterone-filled Silastic capsule (T@P). During puberty (P28-49), all animals received a daily i.p. injection of BrdU (200 mg/kg body weight) during the light period between 8:00 AM-10:00 AM. On P60, subcutaneously implanted capsules were removed in all animals and replaced with a testosterone-filled Silastic capsule to produce equivalent levels of circulating testosterone in all groups in adulthood. All animals were perfused on P90. For each region of interest, a separate two-way ANOVA was used to analyze group differences in the density of BrdU-ir cells using genotype and pubertal testosterone treatment as independent factors.

Tissue Collection and Processing

All mice were given an overdose of sodium pentobarbital (150 mg/kg) by i.p. injection and intracardially perfused with 0.9% saline followed by cold 4% paraformaldehyde in 0.1 M phosphate buffered saline (pH 7.4). Brains were removed and postfixed overnight in 4% paraformaldehyde, then stored in 30% sucrose at 4 °C until sectioning. Brains were cut on a freezing microtome into 30 µm coronal sections, and collected as either 3 (Experiment 1) or 4 alternate series of sections (Experiments 2-3). For each experiment, the first series was Nissl stained with thionin, dehydrated, and coverslipped.

Immunohistochemistry

In each experiment, the second series of brain sections was processed for immunohistochemistry to label BrdU-immunoreactive (ir) cells. Free-floating sections were rinsed in Tris-buffered saline (TBS; 0.05 M; pH 7.6) for 30 minutes and then incubated in 0.6% H_2O_2 for 10 minutes. After a 15-minute rinse in TBS, the sections were incubated in 0.93N HCl for 30 minutes at 37°C (except in experiment 4 – the sections were incubated in 2N HCl for 1 hour at 37°C). Sections were next neutralized in 0.1M borate buffer (pH 8.5) for 10 minutes. Following a 30-minute rinse in TBS, the sections were blocked in TBS containing 0.01% Triton-X-100 and 3% donkey serum, and then incubated in monoclonal rat anti-BrdU (catalog no. MCA2060; Serotec) at a working concentration of 1 µg/ml at 4°C for 48 hours. Sections were then rinsed in TBS for 30 minutes and incubated in biotinylated donkey anti-rat secondary antibody (catalog no. 712-065-150; Jackson Immunoresearch) at a working concentration of 2 µg/ml for 2 hours at room temperature. Following a 30-minute rinse in TBS, the sections were incubated in avidin/biotinylated enzyme complex (ABC Elite Kit, Vector Laboratories, Burlingame, CA) for an hour and then reacted in 3,3'-Diaminobenzidine tetrahydrochloride (DAB; Sigma; working concentration 0.25 mg/ml) for about 2 minutes. Following this reaction, all sections were mounted onto gelatin-subbed slides and coverslipped.

Microscopic Analysis

All microscopic analyses were performed by an observer blind to treatment group using an Olympus BX51 microscope under brightfield illumination using Neurolucida version 10 (MBF Bioscience). The regions of interest were traced using the Nissl-

stained series (Fig 1.6A-B) and contours were superimposed onto adjacent sections of the single-label BrdU series (Fig 1.6C-D). The boundaries of the VMH on the Nissl series were determined using a standard mouse brain atlas (Paxinos et al., 2004). The following landmarks were used to trace the VMH: the size, shape, and staining intensity of the nucleus itself, as well as the size and shape of the third ventricle and the size and shape of the optic tract. BrdU-ir cells were identified by the presence of a dark brown nuclear stain.

Single-label BrdU Analysis

In experiment 1, six sections of the VMH were bilaterally analyzed, which encompassed the rostral-caudal extent of the ventrolateral, dorsomedial, and central subdivisions. Animals that did not have six anatomically-matched VMH sections due to poor tissue quality or missing sections were excluded from final analysis. The final sample sizes were as follows: P60 females (n=7), P60 males (n=5), P90 females (n=8), and P90 males (n=8). In addition, the 2 most rostral bilateral sections of the DG were analyzed (Fig 1.8A-D). Due to poor tissue quality, 2 males from the P60 cohort were removed from the final analysis. For each region of interest, the total number of BrdU-ir cells was determined by summing the number of BrdU-ir cells across all sections analyzed. The density of BrdU-ir cells in each animal was calculated by dividing the total number of BrdU-ir cells (summed across sections) by the total area (summed across sections).

For experiments 2 and 3, three – four sections of the VMH were bilaterally analyzed in the ventrolateral, dorsomedial, and central subdivisions. Animals that did not have at least 3 anatomically-matched VMH sections were excluded from final

analysis. The final sample sizes were as follows: Experiment 2 – males (n=10), females (n=10), and iTfm (n=10); Experiment 3 – No T@P males (n=9), No T@P female (n=7), No T@P iTfm (n=9); T@P males (n=9), T@P females (n=5), and T@P iTfm (n=8). The 2 most rostral bilateral sections of the DG were analyzed. Animals that did not have at least 2 anatomically-matched DG sections were excluded from final analysis. The final sample sizes were as follows: Experiment 2 – males (n=10), females (n=10), and iTfm (n=10); Experiment 3 – No T@P males (n=9), No T@P female (n=8), No T@P iTfm (n=8); T@P males (n=9), T@P females (n=8), and T@P iTfm (n=10). For each region of interest, the density of BrdU-ir cells in each animal was calculated by dividing the total number of BrdU-ir cells (summed across sections) by the total area (summed across sections).

RESULTS

Experiment 1: Male mice have more pubertally born cells than females in the VMHvl, but not the VMHdm, VMHc, or DG.

In the VMHvl, there was a main effect of sex ($F(1,24)=12.96$, $p=0.001$), with male mice having a higher number and density of pubertally born cells than female mice in adulthood (Fig 1.5E-F). There was no main effect of age at sacrifice and no significant interaction on total number of BrdU-ir cells in the VMHvl. Also, there were no main effects of sex or age at sacrifice and no interaction on the density or number of BrdU-ir cells in the VMHdm (Fig 1.7A-B), VMHc (Fig 1.7C-D), and DG (Fig 1.8E).

Experiment 2: Androgen receptors are not necessary to increase the number of pubertally born cells in the VMH and DG.

In the VMHvl, there was no significant different in the density of pubertally born cells between genotype (Fig 1.9A). There was also no significant difference in the density of pubertally born cells in the VMHdm (Fig 1.9B), VMHc (Fig 1.9C), and DG (Fig 1.9D) between genotype.

Experiment 3: Pubertal testosterone does not increase the number of pubertally born cells in the VMH or DG.

In the VMHvl, there was no significant main effect of genotype or pubertal testosterone treatment on the density of pubertally born cells (Fig 1.10A). There was also no significant interaction of genotype and pubertal testosterone treatment on the density of pubertally born cells in the VMHvl. There was also no significant main effect of genotype on the VMHdm (Fig 1.10B), VMHc (Fig 1.10C), and DG (Fig 1.10D) or pubertal testosterone treatment in the VMHdm, VMHc, and DG. There was also no significant interaction of genotype and pubertal testosterone treatment on the density of pubertally born cells in the VMHdm, VMHc, and DG.

DISCUSSION

The current study discovered that new cells are born in the VMH during puberty with no significant attrition of cells seen from P60 to P90. In our initial study, we observed a sex difference in the number and density of pubertally born cells in the VMHvl with males having more pubertally born cells than females. However, in our follow-up studies, we failed to replicate these findings, as no significant group differences in pubertally born cell density were observed between male, female, and

*i*Tfm male mice. *i*Tfm male mice have non-functional ARs, thus it appears that functional ARs are not required to promote the increase of pubertally born cells in the VMHvl. Pubertal testosterone is also not necessary to increase pubertally born cell density in the VMHvl. A study in adult male meadow voles found that hormone treatment (i.e. testosterone, estrogen, and DHT, a potent androgen) did not affect cell proliferation in the VMH (Fowler et al., 2003). It is unknown how gonadal hormones affect cell proliferation in the VMHvl during puberty, but based on these results, it is possible that gonadal hormones do not play a role in the proliferation or survival of pubertally born cells in the VMHvl.

Methodical differences between experiments could account for the inconsistent findings on sex differences in pubertally born cells in the VMHvl shown in the present study. For instance, the initial sex differences study, we used gonadally intact males and females, while the subjects in the latter studies underwent 1-2 surgeries. There is evidence that shows stress can decrease cell proliferation and cell survival in the brain (Mirescu et al., 2006) and exposure to anesthetic, such as isoflurane, can cause cell death in the brain (Zhu et al., 2010). While no signs of distress were observed in the animals used in this study, beyond the discomforts of surgery, it is possible that these surgical procedures dampened sex differences initially detected between males and females in the VMHvl. However, if this is the case, it would appear that the posterodorsal medial amygdala (MePD) is somehow immune to the effects of surgery, as the sex difference in the density of pubertally born MePD cells was maintained following surgeries. Alternatively, the sex differences in pubertally born cells within the VMHvl in the initial experiment may have resulted from type 1 error. However, the fact

that statistical significance for these findings survived a rather conservative Bonferroni correction for multiple comparisons makes this possibility less likely. It is clear that further investigation is required to understand the role of gonadal hormones on cell proliferation and survival in the mouse VMHvl.

Sex differences in VMH volume are established early in development around the perinatal period (Matsumoto et al., 1983). Castration of male rats at P1 feminized VMH volume in adulthood, while castration at P7 did not reduce adult VMH volume (Matsumoto et al., 1983). In addition, there is no sex difference in neuron number in the adult VMH (Madeira et al., 2001); however, sex differences in the number of synapses are established in the VMH during puberty with males having more synapses than females. In addition, treating females with testosterone can masculinize the number of synapse in the VMH (Pozzo Miller et al., 1991). Interestingly, recent studies have discovered that glial cells, such as astrocytes, oligodendrocytes, and microglia, play a critical role in the formation, maintenance, and elimination of synapses(Aguzzi et al., 2013; Allen et al., 2009; Clarke et al., 2013). Therefore, while we do not always observe a sex difference in pubertally born cell density, gonadal hormones may affect the differentiation of pubertally born cells into specific cell phenotypes. For instance, testosterone could promote the genesis and/or survival of glial cells in the VMH to facilitate synaptic reorganization. Follow-up studies will be necessary to explore whether pubertally born cells participate in the synaptogenesis in the VMHvl.

No sex differences in the number and density of pubertally born cells were detected in the VMHdm, VMHc, and DG, brain regions that are sexually monomorphic in volume(Dugger et al., 2007; Tabibnia et al., 1999). These results corroborate findings

from a previous study, which showed no sex differences in the DG (Ahmed et al., 2008). We also discovered that ARs and pubertal testosterone did not increase the number of pubertally born cells in the VMHdm, VMHc, and DG. Male, female, and *i*Tfm male mice had a similar density of pubertally born cells in the VMHdm, VMHc, and DG, regardless of pubertal testosterone treatment. While there are currently no previous studies examining the effects of gonadal hormones on pubertal cytogenesis in the VMH, a recent study did find that there were no sex differences in the total number of pubertally born cells in the adult DG and furthermore, pre-pubertal gonadectomy did not affect pubertally born cells in the DG (Ahmed et al., 2008). Although no significant effects of hormonal manipulations were detected in the number of pubertally born cells in the DG, several studies have shown that androgens and estrogen play an important role in the addition of new cells in the DG during adulthood. When adult male rats are castrated, the level of cell proliferation in the dentate gyrus does not change significantly from of gonadally intact male rats(Spritzer et al., 2007). Similarly, in adult male meadow voles, the administration of testosterone, estrogen, or DHT did not alter levels of cell proliferation in the DG(Fowler et al., 2003). However, it appears that androgens play an important role in cell survival in the adult DG, as gonadally intact male rats had significantly more BrdU-ir cells in the DG than castrated males one month after BrdU treatment (Spritzer et al., 2007) and treating adult male rats with DHT and flutamide reduced the survival of adult born cells in the DG compared to males treated with only DHT (Hamson et al., 2013). Furthermore, Tfm male rats treated with testosterone had fewer adult born cells survive in the adult DG compared to testosterone-treated males (Hamson et al., 2013). On the other hand, in female rats, estrogen seems to have a

strong effect on cell proliferation, but not cell survival in the DG (Barker et al., 2008; Ormerod et al., 2003; Tanapat et al., 1999). Interestingly, it has been reported that 2 days after BrdU treatment, adult female rats have more BrdU-ir cells in the DG than males, but 2 weeks after BrdU administration, there is no longer a sex difference (Tanapat et al., 1999). Taken together, these studies suggest that development of the male and female brains may each follow unique trajectories, which are driven by sex differences in hormone level with cell proliferation and cell death working together to shape sexually dimorphic neural circuits. Therefore, even though no sex differences were observed in the number of pubertally born cells in the adult DG, there is still a possibility that gonadal hormones may have distinct effects on cell proliferation and survival depending on sex. Thus, in order to better understand the role of pubertal hormones on pubertal cytogenesis, more research must be done to examine the differential effects of gonadal hormones on the proliferation and survival of pubertally born cells in the mouse brain.

In the current study, we initially discovered a sex difference in the number and density of pubertally born cells in the VMHvl, with males having more BrdU-ir cells than females. However, we were unable to detect this sex difference in two follow-up experiments. Moreover, we did not observe any sex differences in the number and density of pubertally born cells in the VMHdm, VMHc, and DG. In addition, the current findings provide evidence that ARs and pubertal testosterone are not necessary to increase the number of pubertally born cells in the VMHvl, VMHdm, VMHc, and DG. Based on these results, it is clear that the role of gonadal hormones on pubertal cytogenesis the mouse VMHvl requires further investigation. Follow up experiments

should examine the role of estrogen on pubertal cytogenesis. Furthermore, future studies should address whether gonadal hormones influence differentiation of pubertally born cells into specific cell phenotypes, allowing us to explore the function of such pubertally born cells in mediating sex-specific behaviors in adulthood.

Figure 1.6. Total number and cell density of BrdU-immunoreactive cells in the ventrolateral subdivision of the ventromedial hypothalamus (VMHvl) in adult male and female mice. Representative photomicrographs displaying traces of the VMH on Nissl-stained coronal sections in the (A) male and (B) female adult mice and pubertally born cells in the VMH of (C) male and (D) female adult mice. The small inlaid boxes show BrdU-labeled cells in the VMHvl at a higher magnification. (E) ANOVA revealed that adult male mice have significantly more pubertally born cells than female mice (p=0.001). (F) ANOVA revealed that male mice have a higher density of BrdU-ir cells in the VMHvl than female mice in adulthood (p=0.01). VMHdm = dorsomedial subdivision of the VMH; VMHc = central subdivision of the VMH. Low magnification scale bar indicates 200 μm; high magnification scale bar indicates 10 μm.

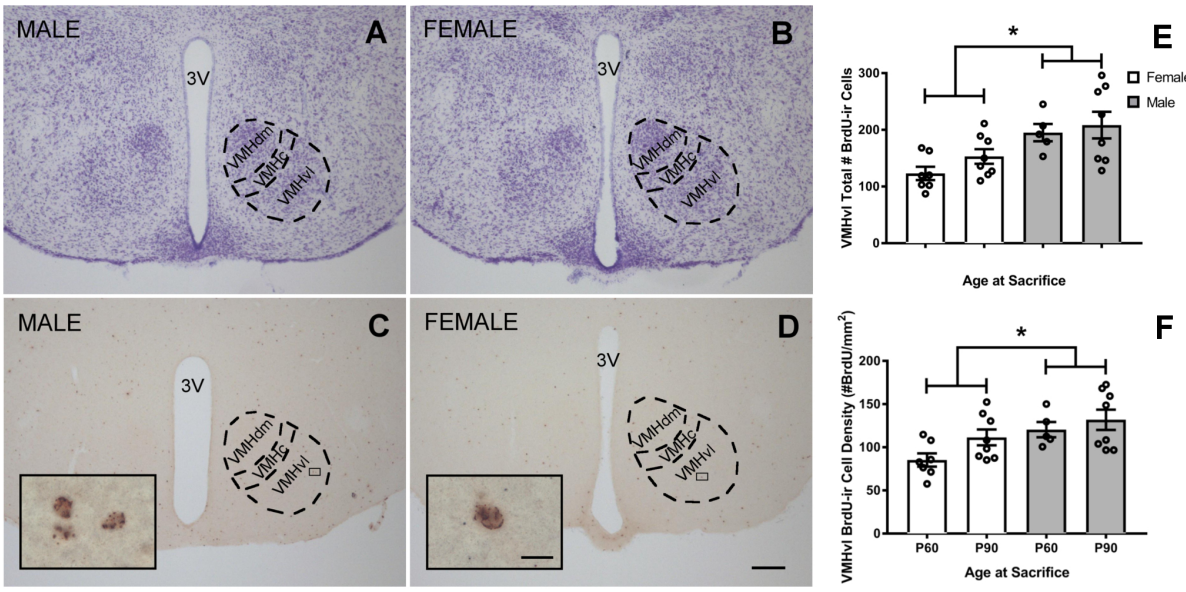


Figure 1.7. Total number and cell density of BrdU-ir cells in the dorsomedial and central subdivisions of the ventromedial hypothalamus in adult male and female mice. (A) Quantitative analysis revealed no sex difference in (A) BrdU-ir cell density and no sex difference in (B) total number of pubertally born cells in the VMHdm. (C) ANOVA found no significant differences in BrdU-ir cell density in the VMHc between male and female mice, and (D) comparable numbers of BrdU-ir cells in the VMHc.

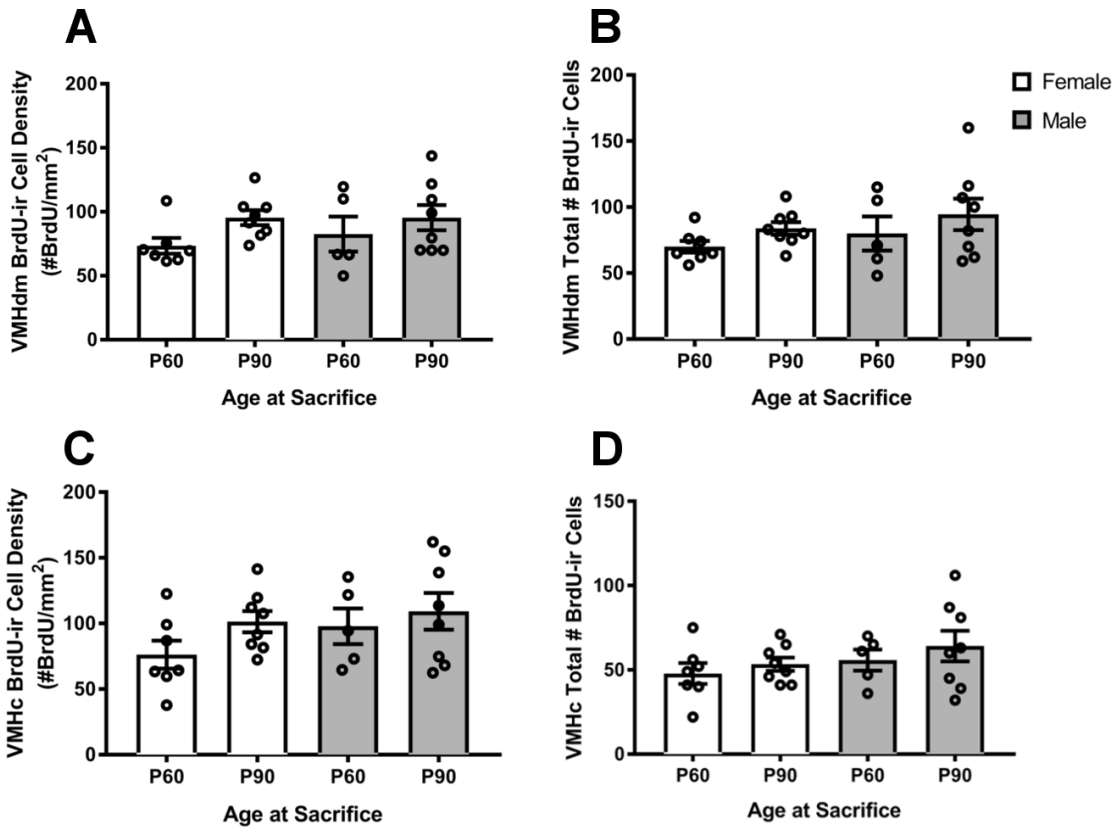


Figure 1.8. BrdU-ir cell density in the dentate gyrus of the hippocampus in adult male and female mice. Representative photomicrographs showing coronal sections of the DG stained for BrdU-ir cells in (A) male and (B) female mice sacrificed on P60 and (C) male and (D) female mice sacrificed on P90. (E) Two-way ANOVA (Sex x Age at Sacrifice) revealed a comparable number of pubertally born cells between all groups. Low magnification scale bar indicates 200 μm; high magnification scale bar indicates 10 μm.

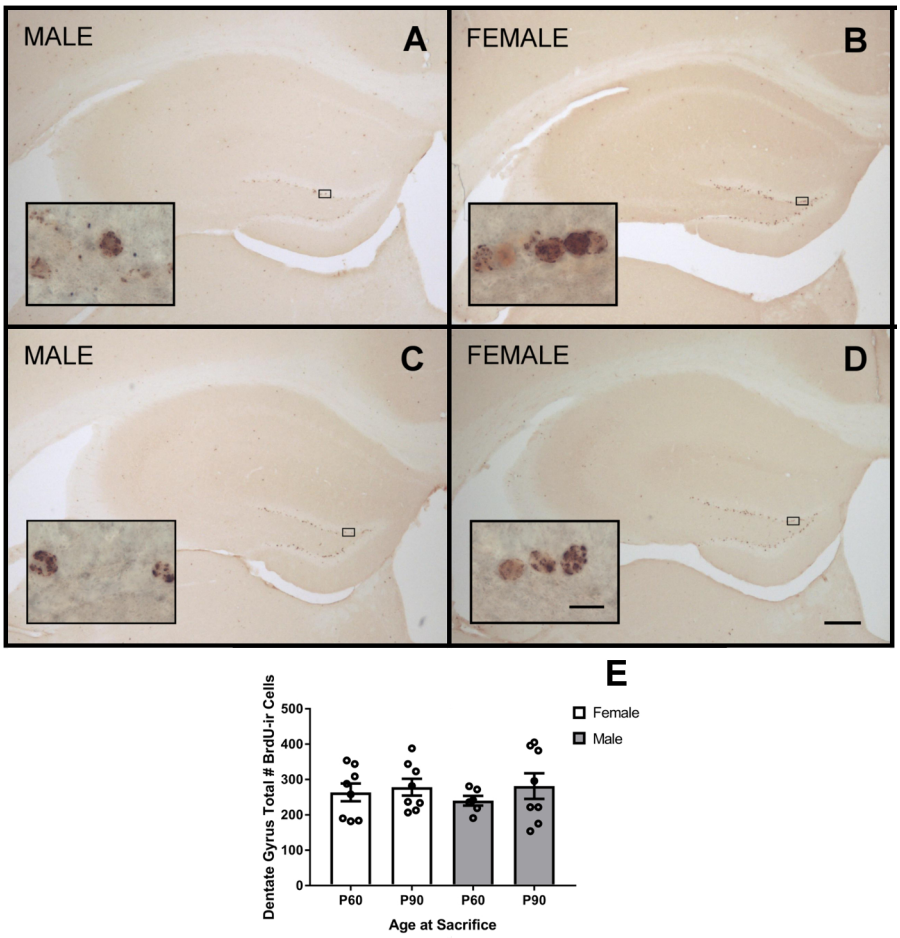


Figure 1.9. Androgen receptors are not required to enhance the density of pubertally born cells in the VMH and DG. A one-way ANOVA revealed no significant differences in the density of pubertally born cells between male, female, and iTfm male mice in the (A) VMHvl, (B) VMHdm, (C) VMHc, and (D) DG.

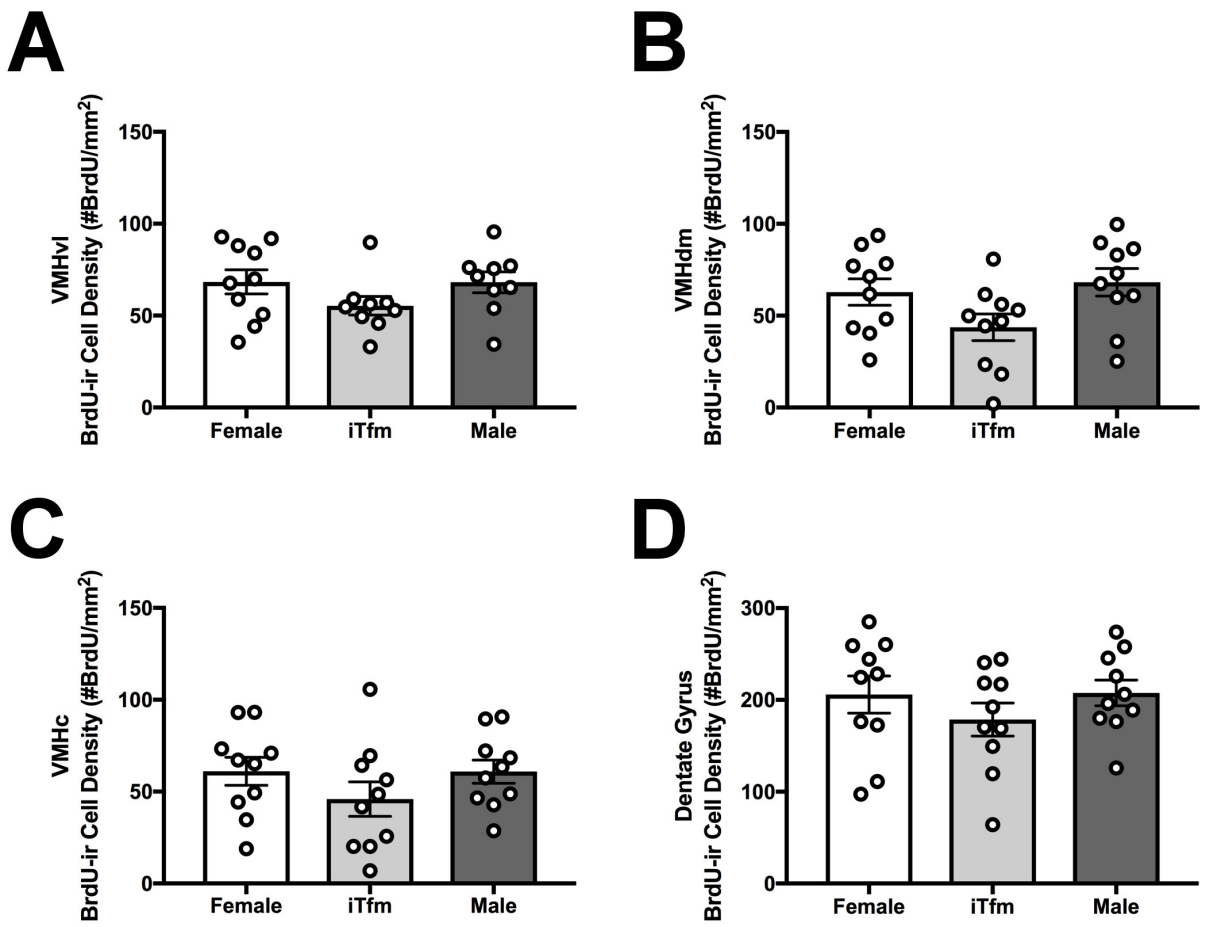
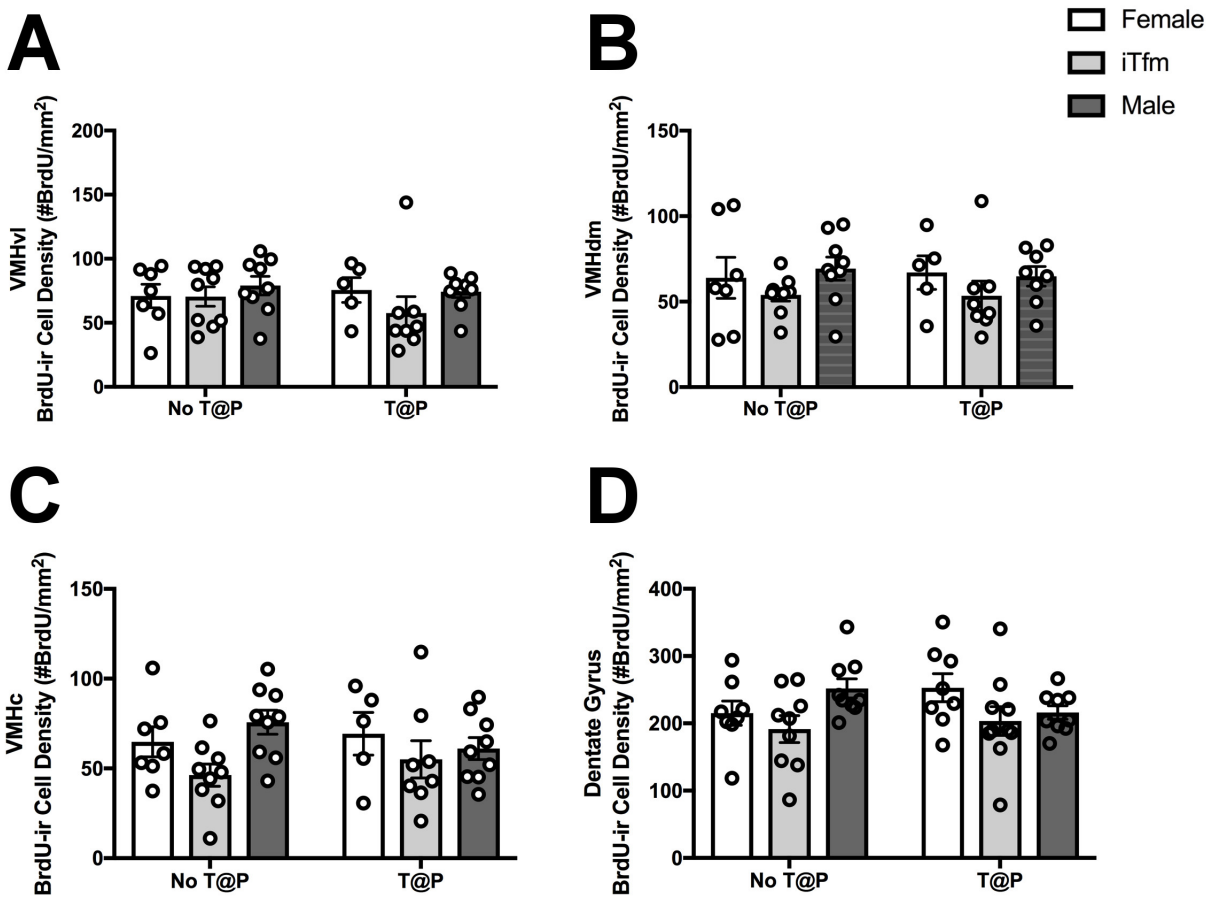


Figure 1.10. Pubertal testosterone is not necessary to increase the number of pubertally born cells in the male, female, and iTfm male mice in the VMH and DG. Quantitative analysis using a two-way ANOVA (Genotype x pubertal testosterone treatment) revealed a comparable density of pubertally born cells between all groups in the (A) VMHvl, (B) VMHdm, (C) VMHc, and (DG).



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CHAPTER 2

Androgen receptors during puberty, but not adulthood, increase pubertally born neurons in the posterodorsal medial amygdala in adult male mice

ABSTRACT

The pubertal brain undergoes significant structural changes that facilitate the development of adult sociosexual behaviors. Recent studies in rats and hamsters have demonstrated that new neurons and glial cells are added to the brain during puberty and become functionally integrated into brain regions, like the medial amygdala, which is known to regulate social behaviors in rodents. In Chapter 1 of this dissertation, we discovered that new cells are also added to the mouse posterodorsal medial amygdala (MePD) and the ventromedial hypothalamus (VMH) during puberty. Furthermore, there was a sex difference favoring males in the number of pubertally born cells in the adult MePD and ventrolateral subdivision of the VMH (VMHvl). In addition, using an AR-null mouse model (*i*Tfm), we discovered that ARs as well as pubertal testosterone promote the number of pubertally born cells in the adult male MePD, but not VMHvl. However, since *i*Tfm male mice have non-functional ARs throughout the lifespan, it is currently unknown when ARs are necessary to masculinize the number pubertally born cells in the adult MePD. Furthermore, it is unclear whether androgens play a role in influencing the genesis and/or differentiation of specific cell phenotypes of pubertally born cells in the MePD and VMH. Therefore, in the current study, we examined the role of ARs specifically during puberty and adulthood, by treating male mice with flutamide, an AR antagonist. Male mice were castrated (P26), implanted with a testosterone capsule, and then treated with a time-release flutamide pellet during puberty (P26-50) or adulthood

(P50-74). Control mice were castrated at P26 but implanted with only a testosterone capsule or received a sham surgery and remained gonadally intact. All mice were administered BrdU during the first week of puberty (P28-35) and sacrificed in adulthood at P74. We discovered that blocking pubertal, but not adult, ARs reduces the number of pubertally born cells in the adult male MePD. In addition, blocking pubertal ARs also reduces the number of pubertally born neurons, but not astrocytes, in the adult male MePD. Neither pubertal nor adult ARs were to necessary increase the number of pubertally born cells, including pubertally born neurons and astrocytes, in the VMH and DG. Thus, ARs, specifically during puberty, are critical for the gain of pubertally generated neurons in the adult male MePD.

INTRODUCTION

The pubertal brain endures extensive remodeling, which facilitates the acquisition of sociosexual behaviors required for survival and successful reproduction in adulthood. These adult sociosexual behaviors are regulated by sexually dimorphic brain regions, such as the posterodorsal medial amygdala (MePD) and the ventromedial hypothalamus (VMH) (Cooke et al., 2003; Cooke et al., 2000; Kollack-Walker et al., 1995; Lin et al., 2011; Samuelsen et al., 2009; Yang et al., 2013). During puberty, males and females experience a surge in gonadal hormones, primarily testosterone and estrogen, respectively(Sisk et al., 2004), and since the MePD and VMH are densely populated with sex steroid receptors, such as androgen receptors (ARs) and estrogen receptors (ERs)(Simerly et al., 1990), it is not surprising that these pubertal gonadal hormones induce significant structural changes to these brain regions. In the MePD and VMH, pubertal increase in gonadal hormones can alter volume, neuronal soma size,

number of glial cells, and synaptogenesis (Cooke et al., 2007; Cooke et al., 2009; Johnson et al., 2013; Pozzo Miller et al., 1991). More recently, studies have discovered that pubertal gonadal hormones also influence the addition of new cells in the adolescent brain, including the MePD and VMH(Ahmed et al., 2008).

A previous pubertal cytogenesis study in rats detected a sex difference in the number of pubertally born cells in the adult medial amygdala. Adult male rats have more pubertally born cells than females in the medial amygdala and a subset of these cells differentiate into mature neurons or astrocytes (Ahmed et al., 2008). The accumulation of pubertally born cells in the adult medial amygdala depends on the presence of gonadal hormones, as pre-pubertal castration reduces the total number of pubertally born cells (Ahmed et al., 2008). In Chapter 1 of this dissertation, a sex difference in pubertally born cells was also discovered in the MePD and VMHvl of mice. Male mice have more pubertally born cells than females in the MePD and ventrolateral subdivision of the VMH (VMHvl). No sex differences were detected in monomorphic brain regions, such as the dorsomedial and central subdivisions of the VMH (VMHdm and VMHc, respectively) and the dentate gyrus of the hippocampus (DG). Using androgen-insensitive *i*Tfm male mice, it was established that sex differences in pubertally born cell number in the adult mouse MePD, but not VMHvl, depend on the presence of ARs. However, since *i*Tfm males have non-functional ARs throughout the lifespan, it is currently unknown *when* AR activity is critical to mediate the masculinization of the number of pubertally born cells in the adult male MePD.

In the current study, we examined whether AR activity specifically during puberty or adulthood was required for the masculine development of pubertally born cells in the

adult MePD. Regions of interest also included the VMHvl, VMHdm, VMHc, and DG in an effort to replicate previous findings to ensure ARs did not play a critical role in the proliferation and/or survival of pubertally born cells. Furthermore, we analyzed the percent colocalization of pubertal born cells with NeuN, a mature neuron marker, and GFAP, an astrocytic marker, to determine whether ARs during puberty or adulthood promote the differentiation and/or survival of pubertally born neurons or astrocytes in the adult MePD, VMH, and DG.

MATERIALS AND METHODS

Animal Subjects and Experimental Design

48 male mice were obtained from a wildtype C57BL/6 in-house mouse colony and were weaned at P24. All animals were group housed (4-5 mice/cage) in clear plexiglass cages (29x18x13cm) in a 12:12 light:dark cycle (lights on: 7:00 AM, lights off: 7:00 PM). All subjects were provided ad libitum rodent chow (Harlan Teklad 8640 Rodent Diet, Madison, WI) and water. All housing and experimental procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Michigan State University's Institutional Animal Care and Use Committee.

Mice were randomly assigned to one of the following four experimental groups (n=12/group): Group 1 (Flut@Puberty): On P26, mice were castrated and implanted subcutaneously (s.c.) with a testosterone capsule (1.6 mm – inner diameter and 3.2 mm – outer diameter; 6 mm – effective release length, 1.6 cm – total capsule length) and flutamide pellet (100 mg flutamide/pellet – 5 mg dose/day, 21-day release, Innovative Research of America, Saratoga, FL). On P50, all implants were removed and replaced

with only a testosterone capsule. Group 2 (Flut@Adulthood): On P26, male mice were castrated and received a testosterone capsule implant (s.c.). On P50, the testosterone capsule was removed and replaced with a new testosterone capsule and a flutamide pellet. Group 3 (T Only): On P26, male mice were castrated and received a testosterone capsule implant (s.c.). On P50, the testosterone capsule was removed and replaced with a new testosterone capsule. Group 4 (Sham): On P26, male mice received a sham surgery, remaining gonadally intact throughout the duration of the study, and received a blank capsule implant (s.c.). On P50, the blank capsule was removed and replaced with a new blank capsule.

BrdU administration

During the first week of puberty (P28-P35), all subjects were administered BrdU (1.0 mg/ml, Sigma-Aldrich) dissolved in the drinking water (Snyder et al., 2011). BrdU drinking water was prepared fresh every 3 days. During BrdU administration, all mice were housed in Innocage mouse cages (37 x 23 x 14 cm) with Aquavive amber water bottles (Innovive, San Diego, CA). The use of the Aquavive amber water bottles allowed us to administer BrdU via drinking water in smaller volumes (about 100 ml of water/bottle) to avoid excess BrdU waste. The Aquavive amber water bottles also had minimal leakage and since BrdU is light sensitive, the amber bottles provided adequate protection from light. In addition, the use of disposable Innocage mouse cages avoided BrdU contamination of reusable animal cages.

Tissue Collection and Processing

On P74, all mice were given an overdose of sodium pentobarbital (150 mg/kg) by i.p. injection and intracardially perfused with 0.9% saline followed by cold 4%

paraformaldehyde in 0.1 M phosphate buffered saline (pH 7.4). Brains were removed and postfixed overnight in 4% paraformaldehyde, then switched to 30% sucrose solution at 4 °C until sectioning. All brains were sectioned within one week of perfusion. Brains were cut on a freezing microtome into 30 µm coronal sections (from about Bregma -0.82 to Bregma -2.74) and collected as 4 alternate series. For each experiment, the first series was Nissl stained with thionin, dehydrated, and coverslipped.

Single-Label BrdU Immunohistochemistry

The entire second series of brain sections underwent immunohistochemistry to label BrdU-immunoreactive (ir) cells. Free-floating sections were rinsed in Tris-buffered saline (TBS; 0.05 M; pH 7.6) for 30 minutes and then incubated in 0.6% H_2O_2 for 10 minutes. After a 15-minute rinse in TBS, the sections were incubated in 2N HCl for 60 minutes at 37°C. Sections were then neutralized in 0.1M borate buffer (pH 8.5) for 10 minutes. Following a 30-minute rinse in TBS, the sections were blocked in TBS containing 0.01% Triton-X-100 and 3% donkey serum, and then incubated in monoclonal rat anti-BrdU (catalog no. MCA2060; Serotec) at a working concentration of 1 µg/ml at 4°C for 48 hours. Sections were then rinsed in TBS for 30 minutes and incubated in biotinylated donkey anti-rat secondary antibody (catalog no. 712-065-150; Jackson Immunoresearch) at a working concentration of 2 µg/ml for 2 hours at room temperature. Following a 30-minute rinse in TBS, the sections were incubated in avidin/biotinylated enzyme complex (ABC Elite Kit, Vector Laboratories, Burlingame, CA) for an hour and then reacted in 3,3'-Diaminobenzidine tetrahydrochloride (DAB; Sigma; working concentration 0.25 mg/ml) for about 3 minutes. Following this reaction, all sections were mounted onto gelatin-subbed slides, dehydrated, and coverslipped.

Triple-Label BrdU/GFAP/NeuN Immunofluorescence

The entire third series of brain sections underwent immunofluorescence to label for BrdU, GFAP (a marker of astrocytes), and NeuN (a marker of mature neurons). Free-floating sections were rinsed in Tris-buffered saline (TBS; 0.05 M; pH 7.6) for 30 minutes and then incubated in 0.1% sodium borohydride for 10 minutes. After a 15-minute rinse in TBS, the sections were incubated in 2N HCl for 60 minutes at 37°C. Afterward, the sections were neutralized in 0.1M borate buffer (pH 8.5) for 10 minutes. Following a 30-minute rinse in TBS, the sections were blocked in TBS containing 0.01% Triton-X-100 and 3% donkey serum for 1 hour, and then incubated in a primary antibody cocktail at 4°C for 48 hours, which contained monoclonal rat anti-BrdU (catalog no. MCA2060; Serotec) at a working concentration of 1 µg/ml, polyclonal rabbit anti-GFAP (catalog no. Z0334; Dako) at a working concentration of 0.58 µg/ml, and monoclonal mouse anti-NeuN (catalog no. MAB377, Chemicon) at a working concentration of 1 µg/ml. Sections were then rinsed in TBS for 30 minutes and incubated in a secondary antibody cocktail for 2 hours at room temperature, which contained Cy2 AffiniPure donkey anti-rat secondary antibody (catalog no. 712-225-153; Jackson Immunoresearch), Cy3 AffiniPure donkey anti-rabbit (catalog no. 711-165-152, Jackson Immunoresearch), and Cy5 AffiniPure donkey anti-mouse (catalog no. 715-175-151, Jackson Immunoresearch). Following a 30-minute rinse in TBS, all sections were mounted onto gelatin-subbed slides, dehydrated, and coverslipped.

Microscopic Analysis

All microscopic analyses were performed by a blind observer. The regions of interest for all microscopic analyses included the posterodorsal medial amygdala

(MePD), the ventrolateral, dorsomedial, and central subdivisions of the ventromedial hypothalamus (VMHvl, VMHdm, and VMHc, respectively), and the dentate gyrus (DG). In the single-label BrdU analysis, the regions of interest were traced using the Nissl-stained series and contours were superimposed onto adjacent sections of the single-label BrdU series using. The boundaries of the MePD (Fig 2.1A-H) and VMH (Fig 2.2A-H) were determined using a standard mouse brain atlas. In the triple-label BrdU/GFAP/NeuN analysis, the regions of interest were traced using the NeuN stain to determine MePD (Fig 2.4A), VMH (Fig 2.5A), and DG (Fig 2.6A) boundaries. To consistently sample anatomically-matched sections across animals, the following landmarks were used to trace the MePD: the shape of the optic tract, the size and shape of the lateral ventricles, the size and shape of the intercalated amygdalar nucleus, and the size and shape of the stria terminalis. For the VMH, the size, shape, and staining intensity of the nucleus itself, as well as the size and shape of the third ventricle were used to consistently sample anatomically-matched sections across animals. For the DG, the two most rostral sections were analyzed (Fig 2.3A-D).

Single-label BrdU Analysis

The single-label BrdU series was analyzed using an Olympus BX51 microscope under brightfield illumination using Neurolucida version 10 (MBF Bioscience). These analyses examined new cells born during the first week of puberty (P28-35). The BrdU-ir cells analyzed were approximately 39-46 days old. The full rostral-caudal extent of the MePD, VMHvl, VMHdm, and VMHc were analyzed, which included a total of six MePD sections and five VMH sections. Animals that did not have all anatomically-matched sections were excluded from analysis. The final sample sizes were as follows:

Flut@Puberty (n=12), Flut@Adulthood (n=11), T Only (n=11), and Sham (n=12). The total number of BrdU-ir cells was determined by summing the number of BrdU-ir cells across all sections analyzed. The density of BrdU-ir cells in each animal was calculated by dividing the total number of BrdU-ir cells (summed across sections) by the total area (summed across sections). The volumetric estimates of the MePD, VMHvl, VMHdm, and VMHc were calculated by summing the area of all sections analyzed in each region of interest and multiplying by the number of series (4) and section thickness (30 μm).

Triple-Label BrdU/GFAP/NeuN Analysis

The triple-label BrdU/GFAP/NeuN series was analyzed using an Olympus FluoView FV1000 confocal laser-scanning microscope. A z-stack orthogonal viewer was utilized to verify colocalization of BrdU/GFAP and BrdU/NeuN. All regions of interest were analyzed using the multi-line Argon gas laser (488 nm), green Helium Neon laser (543 nm), and red Helium Neon laser (633 nm) to detect BrdU-, GFAP-, and NeuN-positive cells, respectively. Again, the full rostral-caudal extent of the MePD, VMHvl, VMHdm, and VMHc were analyzed. For this confocal analysis, a total of 6 mice/group were used to analyze the MePD and VMH and a total of 2 mice/group was used to analyze the DG. The total number of BrdU-, GFAP-, and NeuN-positive cells was determined by summing the number of BrdU-, GFAP-, and NeuN-positive cells across all sections analyzed. The % colocalization of BrdU/NeuN cells were calculated by dividing the total number of BrdU/NeuN-positive cells by the total number of BrdU-positive cells multiplied by 100. The % colocalization of BrdU/GFAP cells were calculated by dividing the total number of BrdU/ GFAP-positive cells by the total number of BrdU-positive cells multiplied by 100. The following represents the average number of

BrdU-ir cells analyzed per animal in each brain region of interest: MePD – 585 BrdU-ir cells, VMHvl – 182 BrdU-ir cells, VMHdm – 100 BrdU-ir cells, VMHc – 55 BrdU-ir cells, and DG – 341 BrdU-ir cells.

Statistical Analysis

For the single-label BrdU analysis, group differences in total number of BrdU-ir cells, BrdU-ir cell density, and volume were each analyzed using a one-way ANOVA to compare all four treatment groups. For the triple-label BrdU/GFAP/NeuN analysis, a one-way ANOVA was used to determine whether the % colocalization of BrdU/GFAP and BrdU/NeuN differed between treatment groups. For all analyses, a significance value of $p < 0.05$ was used and significant results were followed up with a Bonferroni post-hoc test. Cohen's d was used to calculate effect sizes.

RESULTS

Functional androgen receptors during puberty, but not adulthood, promote the number of pubertally born cells in the adult male MePD.

ANOVA revealed a significant difference between treatment groups on the total number of BrdU-ir cells in the adult MePD ($F(3,42)=6.42$, $p=0.001$)(Fig 2.1I). Males that received testosterone throughout the lifespan ($p=0.001$, $d=1.52$) and males that received flutamide during adulthood ($p=0.015$, $d=1.90$) had more BrdU-ir cells in the adult MePD than male mice that received flutamide during puberty. Males in the T Only group had approximately 43% more pubertally born cells in the MePD than males in the Flut@Puberty group, while males in the Flut@Adulthood group had about 34% more BrdU-ir cells than males in the Flut@Puberty group. Sham males were not significantly different from all other groups and instead, had an intermediate number of MePD BrdU-

ir cells, with 18% more BrdU-ir cells than the Flut@Puberty group (*d*=0.96), and 18% and 12% fewer BrdU-ir cells than the T Only (*d*=0.79) and Flut@Adulthood (*d*=0.71) groups, respectively. Furthermore, an ANOVA found that the density of BrdU-ir cells in the adult MePD was significantly different between groups ($F(3,42)=7.24$, $p=0.001$)(Fig 2.1J). Similar to the total number of BrdU-ir cells, the T Only ($p=0.000$, $d=1.69$) and Flut@Adulthood ($p=0.014$, $d=1.60$) groups had a higher density of BrdU-ir cells in the adult MePD than the Flut@Puberty groups. Again, the sham males were not significantly different from all other groups. There were no significant differences in MePD volume among the groups ($F(3,42)=0.264$, $p=0.851$)(Fig 2.1K).

Functional androgen receptors, either during puberty or in adulthood, do not promote the number of pubertally born cells in the VMH and DG.

ANOVA revealed no significant differences among groups in the total number of BrdU-ir cells in the adult VMHvl ($F(3,41)=1.264$, $p=0.299$) (Fig 2.2I), VMHdm ($F(3,41)=1.952$, $p=0.136$) (Fig 2.2L), or VMHc($F(3,41)=0.568$, $p=0.639$) (Fig 2.2O). There were also no significant differences between groups in the density of pubertally born cells in the adult VMHvl ($F(3,41)=1.586$, $p=0.208$) (Fig 2.2J), or VMHc ($F(3,41)=0.974$, $p=0.414$) (Fig 2.2P). There was a significant difference between groups in the VMHdm ($F(3,41)=2.905$, $p=0.046$) (Fig 2.2M) with sham males having a higher density of pubertally born cells than the Flut@Puberty group ($p=0.033$, $d=1.19$). There were also no significant differences between groups in the volume of the adult VMHvl ($F(3,41)=1.347$, $p=0.272$) (Fig 2.2K), VMHdm ($F(3,41)=1.362$, $p=0.268$) (Fig 2.2N), or VMHc ($F(3,41)=1.28$, $p=0.294$) (Fig 2.2Q). In addition, no significant differences were

observed between groups on the total number of BrdU-ir cells ($F(3,42)=2.091$, $p=0.116$) in the dentate gyrus (Fig 2.3E).

Androgen receptors during puberty, but not adulthood, enhances the differentiation of pubertally born cells into mature neurons in the MePD.

An ANOVA found a significant difference between groups in the percent colocalization of BrdU/NeuN-positive cells in the adult MePD (F(3,20)=12.799, p=.000) (Fig 2.4B, 2.4D). Bonferroni post-hoc analysis revealed that the Flut@Puberty male mice had a significantly lower proportion of pubertally born cells that colocalized with the mature neuron marker, NeuN, than Flut@Adulthood (p=0.002, d=2.92), T Only (p=0.003, d=2.89), and sham male mice (p=0.000, d=2.88). However, no significant group differences were observed in the percent colocalization of BrdU/GFAP-positive cells in the adult MePD (F(3,20)=2.003, p=0.146) (Fig 2.4C, 2.4E), suggesting that androgen receptors play an important role in the differentiation or survival of pubertally born neurons, but not pubertally born astrocytes.

Androgen receptors during puberty or adulthood are not required to promote the differentiation of pubertally born cells into mature neurons or astrocytes in the VMH and DG.

An ANOVA revealed no significant differences between all groups in the percent colocalization of BrdU/NeuN-positive cells in the VMHvl (F(3,20)=0.190, p=0.902) (Fig 2.5B), VMHdm (F(3,20)=2.304, p=0.108) (Fig 2.5D), and VMHc (F(3,20)=1.189, p=0.339) (Fig 2.5F). An ANOVA revealed no significant differences between all groups in the percent colocalization of BrdU/GFAP-expressing cells in the VMHvl (F(3,20)=0.419, p=0.741) (Fig 2.5C), VMHdm (F(3,20)=1.078, p=0.381) (Fig 2.5E), and

VMHc ($F(3,20)=0.729$, $p=0.547$) (Fig 2.5G). No significant differences group differences were observed in the percent colocalization of BrdU/NeuN-positive cells in the DG ($F(3,8)=1.429$, $p=0.359$) (Fig 2.6B) or in the percent colocalization of BrdU/GFAP-positive cells in the DG ($F(3,8)=0.987$, $p=0.483$) (Fig 2.6C).

DISCUSSION

The differential effects of pubertal and adult androgen receptors on pubertally born cells in the MePD

In the current study, we discovered that androgen receptor (AR) activity during puberty, but not adulthood, is necessary to increase the accumulation of BrdU-ir cells in the adult male MePD. Treatment with flutamide, an AR antagonist, during puberty significantly reduced the total number and density of pubertally born cells in the adult MePD compared to males that received flutamide only during adulthood or males that received only testosterone. Interestingly, gonadally intact sham males had an intermediate number and density of pubertally born cells in the MePD. Gonadally intact males normally display a pulsatile pattern of testosterone release (Nyby, 2008), while exogenous testosterone treatment provides a steady level of circulating testosterone. Therefore, it is possible that exogenous testosterone treatment may increase the number of BrdU-ir cells compared to endogenous testosterone. From Chapter 1A of this dissertation, we discovered that both pubertal and adult testosterone are necessary to enhance the number of pubertally born cells in the MePD of adult mice. Together with the results from the current experiment, we have identified that pubertal testosterone acts via ARs to increase the number of pubertally born cells in the adult male MePD. Since removing adult testosterone reduces the number of BrdU-ir cells (Chapter 1A),

but treating with flutamide in adulthood does not (this study), it is likely that adult testosterone is aromatized into estrogen to act on MePD estrogen receptors (ERs), and not ARs, to maintain masculine numbers of pubertally born cells in the adulthood.

Few studies have examined the role of gonadal hormones on cell proliferation and survival in the male medial amygdala. In male voles, testosterone increased cell proliferation in the medial amygdala and this effect was largely due to estrogen, and not androgens (Fowler et al., 2003). In adult hamsters, testosterone increased cell proliferation, but not cell survival, in the posterior medial amygdala (Antzoulatos et al., 2008). So far, all the studies that have examined the effects of gonadal hormones on cytogenesis in the medial amygdala have been in adults and have not specifically analyzed the MePD (Antzoulatos et al., 2008; Fowler et al., 2003; Fowler et al., 2005). It is possible that testosterone may have a different effect on cell proliferation and survival during puberty than adulthood. Further investigation will be required to examine the role on testosterone on the proliferation and survival of new generated cells during puberty.

In the current study, pubertal ARs can increase the number of pubertally born cells in the adult MePD by influencing cell proliferation, cell survival, or cell migration. Activation of ARs during puberty could promote cell proliferation in the MePD to increase pubertally born cells; although, it is also possible that pubertal ARs promote the migration of new cells into the MePD from neurogenic niches. Alternatively, the absence of ARs may promote the death of pubertally born cells, ultimately leading to fewer BrdU-ir cells in the adult MePD with the flutamide treatment. Unfortunately, due to the experimental design of the current study, we are unable to determine how pubertal ARs stimulate an increase in pubertally born cells in the adult MePD. Future studies will

need to investigate whether pubertal ARs play a role in cell proliferation, survival, or migration in the MePD.

In the current report, blocking pubertal ARs also reduced the number of pubertally born neurons in the adult male MePD. These results are the first to demonstrate that pubertal androgens affect the survival of a specific cell phenotype in the male MePD. In adulthood, males have more neurons in the MePD than females (Morris et al., 2008b), and a study has shown that this sex difference in neuron number does not exist pre-pubertally (Cooke et al., 2009), suggesting that more neurons are added to the male MePD during puberty. The current findings suggest that androgens may facilitate the addition of pubertal neurons to the male MePD. Although, the mechanisms by which ARs act to enhance the number of pubertally born neurons is currently unknown. It is possible that amygdalar progenitor cells or immature neurons express ARs, by which androgens directly act on pubertally born cells to alter the transcription of genes that regulate differentiation or androgens may act on ARs in other neurons or glial cells, which then indirectly promote the differentiation of pubertally born cells. At the moment, it is unknown whether pubertally born cells, themselves, express ARs. It was discovered that immature neurons in the adult DG of rats did not express ARs (Hamson et al., 2013); however, we've already established that androgen actions can be different between species, ages, and brain regions, thus, even though immature neurons in the adult DG do not express ARs, it is still possible that immature pubertally born cells in the male MePD do express ARs. Several studies have shown that astrocytes in the MePD of adult rats express ARs (Johnson et al., 2012). Thus, androgens may act on astrocytes to influence pubertally born cell differentiation

potentially through the release neurotrophic factors, as there is evidence that astrocytes can release neurotrophic factors like brain-derived neurotrophic factor (BDNF) (Dougherty et al., 2000; Fulmer et al., 2014).

Blocking ARs during puberty may also promote neuronal death in the MePD. Studies have demonstrated that cell death is a mechanism by which sex differences in the adult brain are established in regions such as the principle nucleus of the bed nucleus of the stria terminalis (pBNST) and anteroventral periventricular nucleus (AVPV) (Forger, 2006; Forger et al., 2004; Gotsiridze et al., 2007). Testosterone decreases cell death in the pBNST and increases cell death in the AVPV (Chung et al., 2000; Forger, 2006; Sumida et al., 1993). Therefore, in the MePD, it could be that ARs do not affect the differentiation of pubertally born cells into neurons, but promote their survival into adulthood. While we have confirmed that the number of pubertally born neurons is greater when pubertal ARs are present, it still remains to be determined whether the same number of neurons are born in all groups and AR enhances survival of these neurons, whether AR works to enhances the differentiation of pubertally born neurons or a combination of these mechanisms.

Although blocking ARs during puberty reduced the number of pubertally born neurons, blocking ARs during puberty or adulthood did not have an effect on the number of pubertally born astrocytes in the adult male MePD. These results were quite surprising, as a previous study discovered that ARs regulated the increase in the number of astrocytes during puberty in the MePD of male rats (Johnson et al., 2012). In the current study, we only administered BrdU during the first week of puberty, therefore it is possible that AR may affect the addition of pubertally born astrocytes during mid to

late puberty. We can, however, conclude that androgens during early puberty do not affect the number of pubertally born astrocytes in the adult male MePD.

We also discovered that blocking ARs during puberty or adulthood did not affect male adult MePD volume as there were no significant differences in MePD volume at P74 between males treated with flutamide only during puberty, males treated with flutamide only during adulthood, males treated only with testosterone, and sham males. Several studies have shown that testosterone has an activational effect on the volume of the adult MePD (Cooke et al., 2003; Morris et al., 2008a; Morris et al., 2008b). Testosterone treatment in gonadectomized adult male or female rodents for at least 4 weeks can increase the MePD volume to a masculine size, whereas castrating a male rodent can decrease MePD volume to be comparable to the female MePD volume (Morris et al., 2008b). Even though testosterone is traditionally viewed as the dominant male gonadal hormone acting via ARs, testosterone can also be aromatized into estrogen to act via ERs. In fact, a study demonstrated that testosterone acts via both ARs and ERs to maintain the adult male MePD volume (Cooke et al., 2003). Thus, in the current experiment it is possible that testosterone can increase the volume of the MePD to a masculine size by acting via ERs.

Contributions of pubertal and adult androgens on the VMH and DG

The current findings show that ARs are not required during puberty or adulthood to increase the total number or density of pubertally born cells in the adult VMHvl, VMHdm, and VMHc. Similarly, in Chapter 1B of this dissertation, we discovered comparable numbers of pubertally born cells in the VMHvl, VMHdm, and VMHc between androgen-insensitive *i*Tfm male and wildtype male mice. In adult male voles,

testosterone treatment did not alter cell proliferation in the VMH, furthermore, treatment with estradiol and DHT did not have any effect on cell proliferation as well(Fowler et al., 2003). Taken together, these results indicate that gonadal hormones may not play a role in pubertal cytogenesis in the male VMH. Although, we cannot rule out the possibility that testosterone may be acting via ERs rather than ARs in the VMH to promote the addition of new cells during puberty, as the VMHvl is densely populated with both ARs and ERs(Simerly et al., 1990). A study in female mice found that estrogen increased the number of adult born cells in the VMH 34 days post-BrdU administration, indicating that estrogen treatment may influence cell survival in the VMH (Bless et al., 2016). Thus, it is possible that gonadal hormones do not influence cell proliferation in the VMH during puberty; however, adult testosterone, aromatized into estrogen, act via ERs to influence the survival of pubertally born cells.

The current study also discovered that ARs during puberty or adulthood did not influence cell differentiation in the VMH, as all groups had the same proportion of BrdU-ir colocalize with NeuN or GFAP in all VMH subdivisions. A study in female mice discovered that estrogen treatment enhanced the survival of adult-neurons in the VMH (Bless et al., 2016). Interestingly, in female meadow voles, estrogen treatment did not influence number of BrdU cells colocalized with TuJ1, a neuronal marker, in the adult VMH 24 hours post-BrdU treatment (Fowler et al., 2005). These results suggest that estrogen does not affect the genesis of new neurons in the VMH, but long-term estrogen treatment could influence the survival of newborn VMH neurons. However, since these studies were conducted in adult female rodents, further investigation is

needed to examine the role of estrogen on cell differentiation of pubertally born cells in the VMH of male mice.

We also found that pubertal and adult androgens did not influence adult volumes of the VMHvl, VMHc, and VMHdm. In a previous study, it was discovered that ARs were required to fully masculinize the VMHvl volume in adult rats, as Tfm rats had a more feminine VMHvl volume compared to wildtype male (Dugger et al., 2007). However, in the current study, we do not observe any volumetric changes as a result of blocking ARs during puberty or adulthood. This result suggests that androgens work to masculinize VMH volume prior to puberty. A previous study in adult male rats found that castrating males on P1 reduced VMH volume in adulthood; however, castrating male rats at P7 did not have any effect on volume(Matsumoto et al., 1983). Therefore, it seems that perinatal androgens have a long-term organizational effect on VMH volume in males, increasing VMH volume in adulthood independent of androgen manipulation during puberty or adulthood.

In this study, pubertal and adult androgens did not appear to influence the number of pubertally born cells in the DG of adult male mice. A previous study in adult male rats demonstrated testosterone enhanced cell survival in the DG, and it appears this increase in cell survival was driven by the presence of androgens, as treatment with DHT, but not estrogen, increased the number of surviving BrdU-ir cells (Spritzer et al., 2007). Furthermore, adult Tfm male rats and adult wild-type male rats treated with flutamide also showed a reduction in cell survival in the dentate gyrus(Hamson et al., 2013). However, in male meadow voles, gonadal hormones (i.e. testosterone, estrogen, and DHT) did not have any effect on cell proliferation in the DG (Fowler et al., 2003) and

estrogen treatment in castrated adult male rats did not increase cell proliferation compared to oil-treated male rats (Barker et al., 2008). Therefore, in adulthood, androgens promote the survival of new cells, while estrogen enhances cell proliferation, which is inconsistent with our current findings. There are several methodological factors that could account for these differences. For instance, the studies in male rats examining the effects of gonadal hormones on cell survival single-housed their subjects after weaning. Several studies have shown that social isolation can have a negative impact on hippocampal neurogenesis(Ibi et al., 2008; Leasure et al., 2009; Lieberwirth et al., 2012; Spritzer et al., 2011) and more recently, a study showed that while castration reduced the number of BrdU-ir cells in the DG of adult male rats 16 days post-BrdU treatment, socially isolated male rats had even fewer BrdU-ir cells than pair-housed males (Spritzer et al., 2011). Therefore, in the current study, the absence of androgens during puberty or adulthood could have led to a reduction in cell survival, but social housing (4-5 mice/cage) may provide a compensatory mechanism to ultimately enhance cell survival. In addition, the age difference of when BrdU was administered could also explain these inconsistencies. Significantly more cells are born during puberty than adulthood, and more pubertally born cells are likely to survive than adult born cells (Curlik et al., 2014; Ho et al., 2012; Staffend et al., 2014). There is evidence that cells born during puberty are functionally distinct from adult-born neurons(Kirshenbaum et al., 2014; Martinez-Canabal et al., 2013; Wei et al., 2011), therefore, the regulation of the survival and functional integration of new born cells by gonadal hormones may be different during adolescence and adulthood. While the differential effects of gonadal hormones on pubertal and adult cytogenesis have not

been examined, a study has established that fluoxetine, an antidepressant drug, increases cell proliferation in male adults compared to controls, but does not affect cell proliferation in male adolescents(Hodes et al., 2009). Therefore, although gonadal hormones may influence cell proliferation and survival in the adult DG, it is possible that they do not have this same effect in the pubertal DG.

Furthermore, we discovered that pubertal and adult androgens did not alter the proportion of pubertally born cells co-expressing NeuN or GFAP in the adult DG. A previous study in adult male rats discovered that androgens enhanced neurogenesis in the DG as DHT increased the number of adult-born neurons in the DG than male rats treated with DHT and flutamide 30 days after BrdU administration (Hamson et al., 2013). Although, another study found no differences in the number of cells showing BrdU-ir and NeuN-ir colocalization in the DG between castrated and gonadally intact male rats 16 days after BrdU treatment (Spritzer et al., 2011), indicating that long-term, but not short-term, androgen treatment enhances neurogenesis in the DG. There is also evidence that estrogen enhances cell survival in the adult male meadow vole dentate gyrus, but only when estrogen is administered 6-10 days post-BrdU administration (Ormerod et al., 2004).. Based on these findings, androgens and estrogens could be working together to enhance the number of pubertally born neurons in the DG, therefore, estrogenic effects may compensate for non-functional ARs during puberty or adulthood to enhance survival of pubertally born neurons in the adult DG. In regard to astrogliogenesis, it has been shown that astrocytes can express ARs and ERs (Azcoitia et al., 1999; Finley et al., 1999; Garcia-Ovejero et al., 2002; Johnson et al., 2012), but very few studies have examined the role of gonadal hormones on the addition of new

astrocytes to the dentate gyrus under non-pathological conditions, as the recruitment of reactive astrocytes and microglia to sites of neuronal injury have been a primary focus in this field of study(Rajkowska et al., 2007; Rodriguez et al., 2009; Sofroniew et al., 2010). While it remains unknown at what stage of astrogliogenesis that astrocytes begin to express ARs and ERs, but it is possible that gonadal hormones may act directly on newly born astrocytes, themselves, to enhance survival. In the MePD, the addition of pubertal astrocytes in males was dependent on the presence of functional androgen receptors. While this is the case in the MePD, the survival of pubertally born astrocytes in the DG does not appear to exclusively depend on androgens, thus, androgens and estrogens may work together at different stages of development to promote the survival of pubertally born astrocytes.

Conclusions

In the current study, we discovered that androgens during puberty, but not adulthood, facilitate an increase in the number of pubertally born cells in the adult male MePD. Furthermore, pubertal androgens enhance the number of pubertally born neurons, but not astrocytes, in the adult male MePD. However, we also determined that neither pubertal nor adult androgens influence the number of pubertally born VMH and DG cells, including pubertally born neurons and astrocytes. These findings demonstrate that the actions of androgens have an age-specific and site-specific effect on the survival of pubertally born cells with pubertal androgens enhancing the survival of pubertally born neurons in the MePD, but not affecting other brain regions such as the VMH or DG. This study is the first to determine that pubertal ARs influence a specific cell phenotype in the MePD. In future studies, it will be important to examine the role of

ARs on generating certain cell phenotypes in cells born during mid to late puberty. Furthermore, it will be critical to determine whether pubertally born cells become functionally integrated into adult neural circuits and then determine whether neurons and glia have different roles in mediating the emergence of male sociosexual behaviors.

Figure 2.1. Blocking androgen receptors during puberty in male mice reduces the number of pubertally born cells in the adult posterodorsal medial amygdala (MePD). Representative photomicrographs displaying the traces of the MePD on Nissl-stained coronal sections and pubertally born cells in the adult MePD, respectively, in (A-B) Flut@Puberty male mice, (C-D) Flut@Adulthood male mice, (E-F) T Only male mice, and (G-H) sham male mice. Quantitative analysis revealed that (I) Flut@Puberty male mice have fewer pubertally born cells and (J) a lower density of BrdU-ir cells than Flut@Adulthood and T Only male mice. (K) No volumetric differences were detected in the adult MePD between all groups. Data are represented as mean ± SEM. The small inlaid boxes show BrdU-labeled cells at a higher magnification. Low mag scale bar = 200 μm; high mag scale bar = 10 μm.

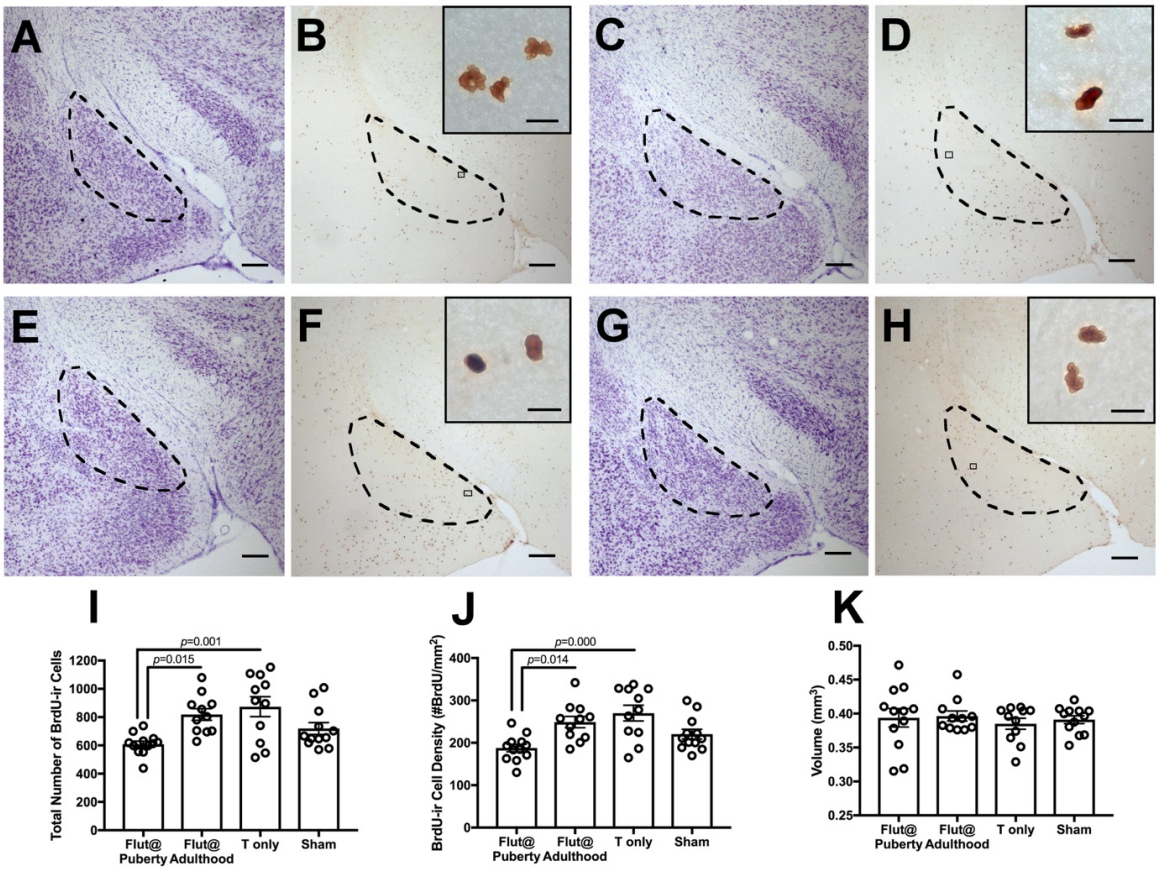


Figure 2.2. Androgen receptors are not required to increase the number of pubertally born cells in the adult ventromedial hypothalamus (VMH). Representative photomicrographs displaying the traces of the VMHvl, VMHdm, and VMHc on Nissl-stained coronal sections and pubertally born cells in the adult VMH, respectively, in (A-B) Flut@Puberty male mice, (C-D) Flut@Adulthood male mice (E-F) T Only male mice, and (G-H) sham male mice. An ANOVA revealed no significant differences in (I, L, O) total number of pubertally born cells, (J, M, P) density of BrdU-ir cells, and (K, N, Q) volume of the adult VMHvl, VMHdm, and VMHc between all groups. Data are represented as mean ± SEM. The small inlaid boxes show BrdU-labeled cells at a higher magnification. Low mag scale bar = 200 μm; high mag scale bar = 10 μm.

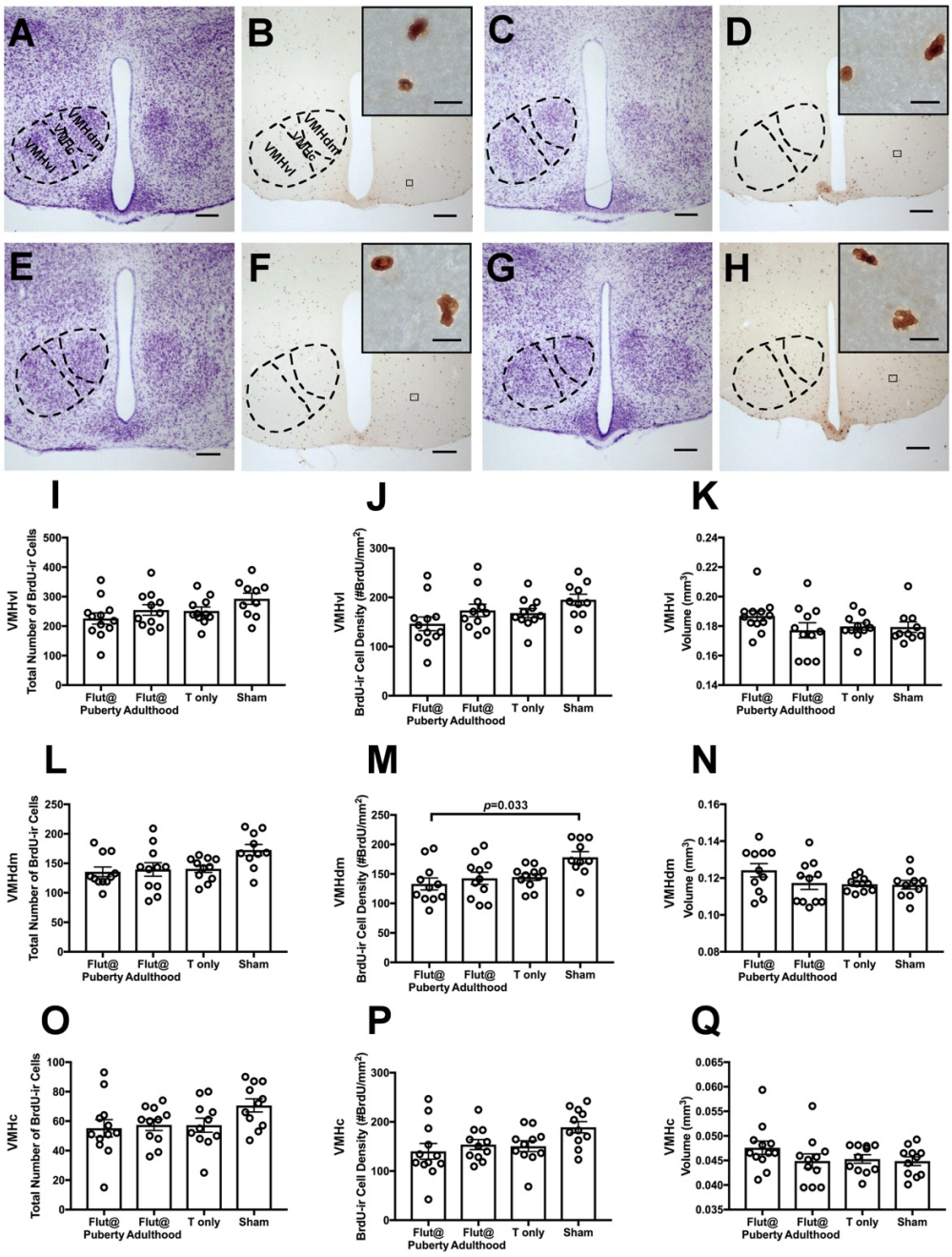


Figure 2.3. Androgen receptors are not required to increase the number of pubertally born cells in the adult dentate gyrus of male mice. Representative photomicrographs displaying pubertally born cells in coronal sections of the adult dentate gyrus in (A) Flut@Puberty male mice, (B) Flut@Adulthood male mice, (C) T Only male mice, and (D) sham male mice. Quantitative analysis revealed no significant differences between groups in (E) the total number of pubertally born cells in the adult male dentate gyrus. The small inlaid boxes show BrdU-labeled cells at a higher magnification. Low mag scale bar = 200 μm; high mag scale bar = 10 μm. Data are represented as mean ± SEM. * p < .001.

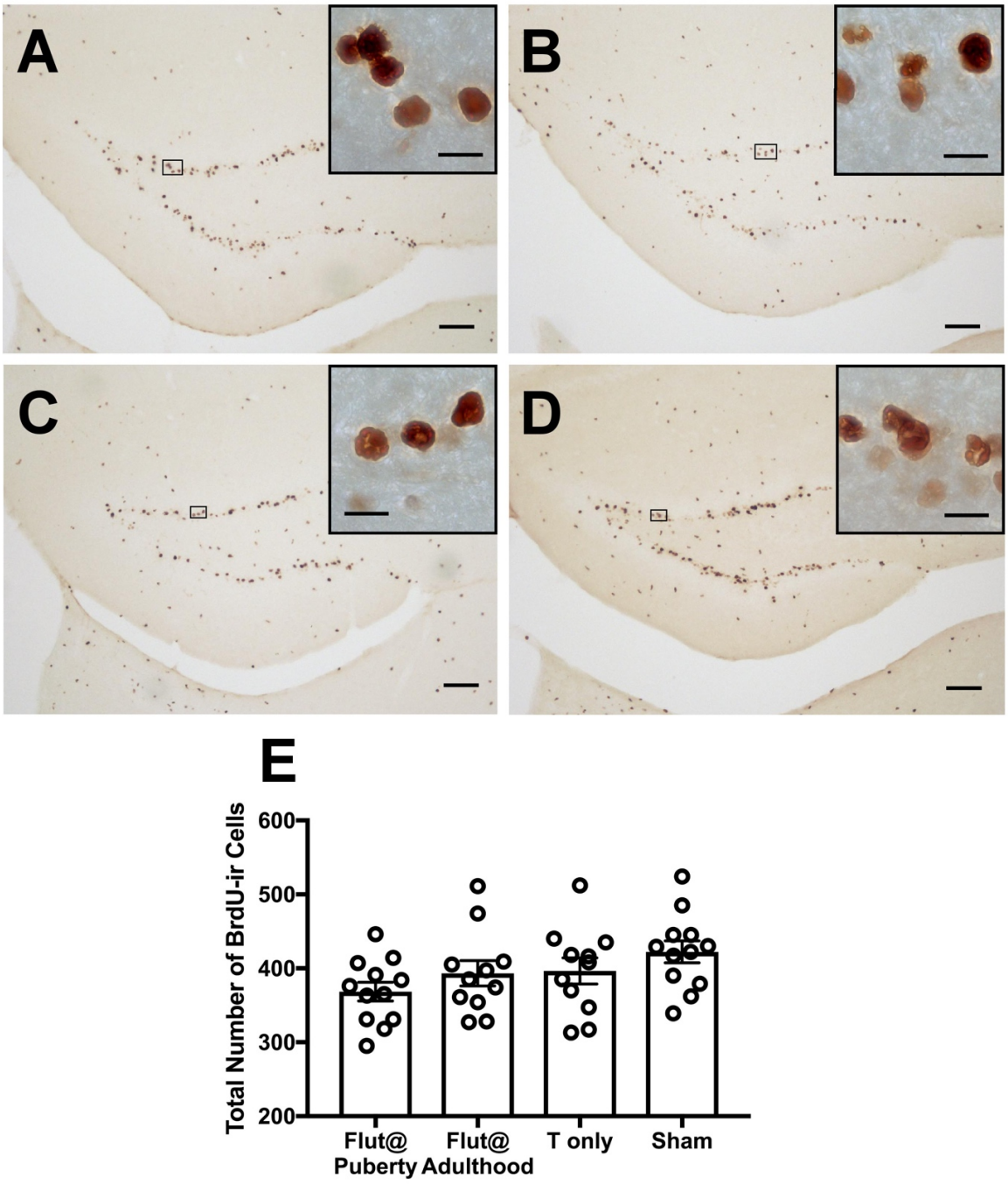


Figure 2.4. Blocking androgen receptors during puberty, but not adulthood, reduces the number of pubertally born cells that become neurons in the adult MePD. (A) A representative photomicrograph of a coronal section of the adult MePD in a sham male stained for BrdU (green), GFAP (red), and NeuN (blue). Quantitative analysis revealed that (B) Flut@Puberty male mice had significantly less pubertally born cells that colocalized with a mature neuron marker in the adult MePD than Flut@Adulthood, T Only, and Sham male mice. However, (C) no significant differences were observed between groups in the proportion of pubertally born cells that colocalized with an astrocytic marker in the adult MePD. Merged Z-stack images of BrdU-ir cells colocalized with (D) NeuN-ir and (E) GFAP-ir cells are shown at maximum intensity projections. Low mag scale bar = 200 μm; high mag scale bar = 10 μm. Data are represented as mean ± SEM.

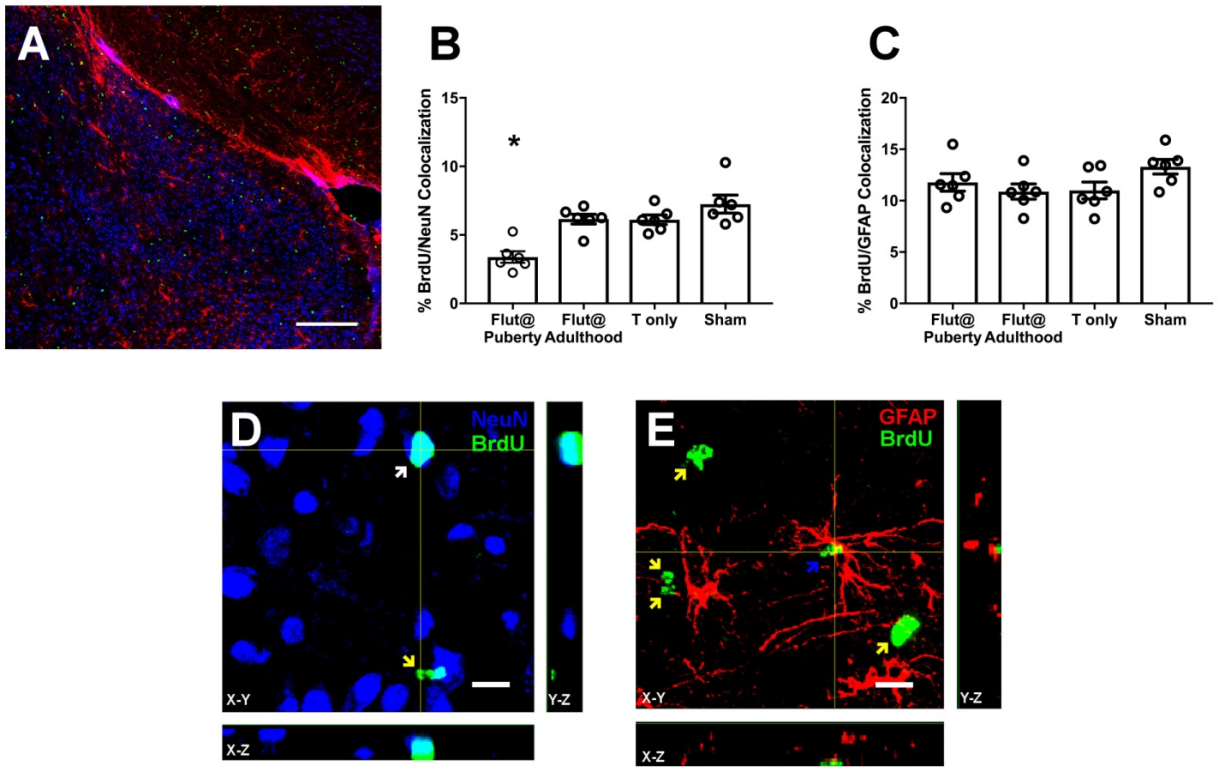


Figure 2.5. Androgen receptors are not required to enhance the number of pubertally born cells that colocalize with mature neurons or astrocytes in the adult VMH. (A) A representative photomicrograph of a coronal section of the adult VMH in a sham male stained for BrdU (green), GFAP (red), and NeuN (blue). Quantitative analysis revealed no significant differences between groups in the proportion of pubertally born cells that colocalized with (B, D, F) a mature neuronal marker or (C, E, G) astrocytic marker in the adult VMHvl, VMHdm, and VMHc. Scale bar = 200 μm. Data are represented as mean ± SEM.

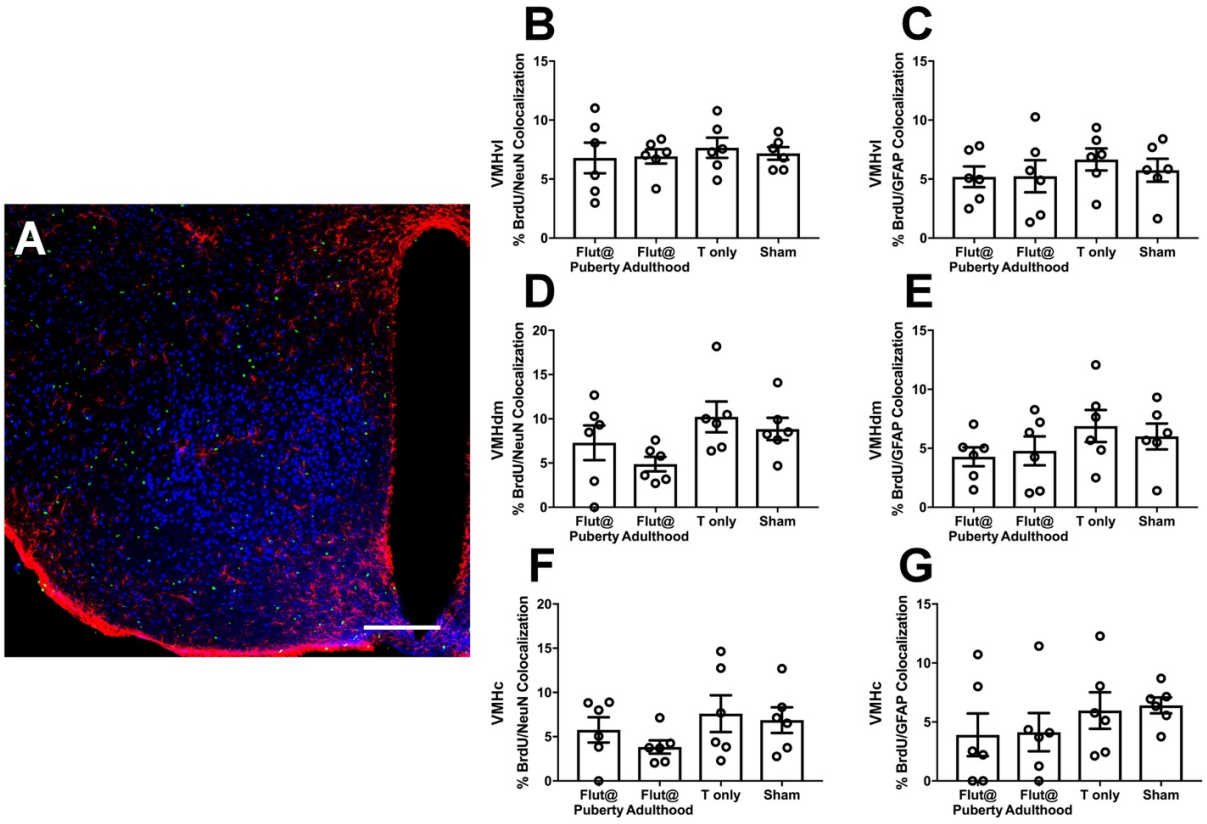
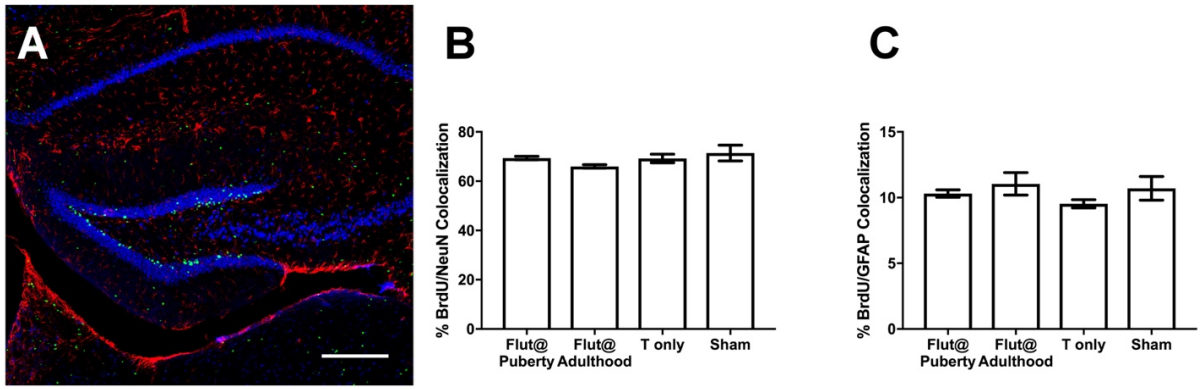


Figure 2.6. Androgen receptors are not required for pubertally born cells to differentiate into mature neurons or astrocytes in the adult dentate gyrus. (A) A representative photomicrograph of a coronal section of the adult DG in a sham male stained for BrdU (green), GFAP (red), and NeuN (blue). Quantitative analysis revealed no significant differences between groups in the proportion of pubertally born cells that colocalized with (B) a mature neuronal marker or (C) astrocytic marker in the adult DG. Scale bar = 200 μm. Data are represented as mean ± SEM.



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CHAPTER 3

Sociosexual interactions activate pubertally born cells in the posterodorsal medial amygdala and ventromedial hypothalamus of adult male mice

ABSTRACT

In adult mice, pubertally born cells are retained in three limbic regions: the posterodorsal medial amygdala (MePD), ventromedial hypothalamus (VMH), and dentate gyrus (DG). The MePD and VMH are both sexually dimorphic brain regions linked to sex-specific social behaviors, such as male sexual behavior and intermale aggression. A previous study in male hamsters showed that some pubertally born cells in the adult hamster brain develop into mature neurons and glia that are functionally incorporated into neural circuits involved in sex-specific social behaviors. In the current study, we examined whether pubertally born cells are activated by socially relevant stimuli in the MePD, VMH, and DG of adult male mice. We also determined the phenotype of pubertally born cells in each region of interest using NeuN, a mature neuron marker, and GFAP, an astrocytic marker. The subdivisions of the VMH were all analyzed as separate regions of interest: ventrolateral subdivision (VMHvl), dorsomedial subdivision (VMHdm), and central subdivision (VMHc). We administered 5-bromo-2'-deoxyuridine (BrdU) to male mice during the first week of puberty (P28-35) to label cells born during this period of time. On P63, each subject was exposed to one of three social interactions one hour prior to sacrifice: resident-intruder, receptive female, or no social interaction. Aggressive and sexual behaviors elicited about 0.43% and 0.56 of pubertally born cell activation in the MePD, respectively, while aggressive behaviors activated about 0.68% of pubertally born cells in the VMHvl and 0.66% of pubertally

born cells in the VMHdm. Furthermore, social interaction did not activate pubertally born cells in the VMHc and DG. In the MePD, about 6% and 12% of pubertally born cells differentiated into mature neurons and astrocytes, respectively. In the VMH, 7% of pubertally born cells were mature neurons and 7% were astrocytes in all subdivisions analyzed. In the DG, about 75% of pubertally born cells were neurons and 10% were astrocytes. These findings indicate that the pubertally born cells are incorporated into existing adult neural circuits known to regulate sociosexual behaviors.

INTRODUCTION

The pubertal brain undergoes extensive structural remodeling(Blakemore et al., 2010; Giedd et al., 1999; Sisk et al., 2004), which includes cytogenesis, or the addition of new cells (Ahmed et al., 2008; Mohr et al., 2016; Mohr et al., 2013; Saul et al., 2015; Staffend et al., 2014). Recent findings from our lab and the previous chapter in this dissertation have shown that new neurons and glial cells are added to the posterodorsal medial amygdala (MePD), ventromedial hypothalamus (VMH), and dentate gyrus (DG) during puberty and that a subset of these cells survives well into adulthood(Ahmed et al., 2008; Mohr et al., 2013). The MePD, VMH, and DG are involved in regulating sociosexual behaviors, such as male sexual behavior and intermale aggression(Falkner et al., 2014a; Kollack-Walker et al., 1992, 1995; Lee et al., 2014; Lin et al., 2011; Samuelsen et al., 2009a; Sano et al., 2016; Sano et al., 2013), which emerge during puberty to promote the survival and/or reproductive capabilities of adult rodents(Barkley et al., 1977; De Lorme et al., 2013; Schulz et al., 2006). However, it is currently unclear whether pubertally born cells are functionally integrated into brain regions known to regulate sociosexual behaviors such as the MePD, VMH, and DG.

The MePD is a subregion of the medial amygdala that receives strong afferent projections from the accessory olfactory bulb (AOB) (Cadiz-Moretti et al., 2016). The AOB sends chemosensory information to the MePD (Samuelsen et al., 2009b) and, in turn, the MePD categorizes socially relevant cues from the environment to help elicit context-appropriate behaviors (e.g. male sexual behavior in response to the presence of a sexually receptive female)(Samuelsen et al., 2009a). The MePD of male rodents is highly responsive to the scent of conspecifics. For instance, the adult male mouse MePD shows an increase in Fos expression, a marker of neural activation, in response to female urine and bedding soiled by a female (Samuelsen et al., 2009a; Taziaux et al., 2011). In addition, the male MePD is also markedly activated by physical social interactions as sexual interactions with a receptive female and aggressive encounters with an intruder male increase Fos expression(Hong et al., 2014; Kollack-Walker et al., 1995; Taziaux et al., 2011).

The VMH is part of the mediobasal hypothalamus and receives strong innervations from the MePD (Pardo-Bellver et al., 2012). The VMH is commonly associated with regulating female sexual behavior (Flanagan-Cato et al., 2001) and energy balance (King, 2006b), but recent studies have implicated the VMH in male sexual behavior, intermale aggression, and defensive behaviors as well (Choi et al., 2005; Falkner et al., 2014a; Falkner et al., 2014b; Lin et al., 2011; Wang et al., 2015). The ventrolateral subdivision of the VMH (VMHvl), in particular, is involved in gating male sexual behavior and intermale aggression in adult male mice(Choi et al., 2005; Lee et al., 2014; Lin et al., 2011). When neurons in the VMHvl are activated, males become aggressive, but inhibition of these neurons promotes mating behavior (Lin et

al., 2011). More specifically, the activation of neurons expressing estrogen receptor-α (ERα) in the VMHvl can elicit aggressive behavior in males (Lee et al., 2014). Furthermore, ablating ERα in the VMH or progesterone receptors in the VMHvl leads to significant reductions in male sexual behavior and intermale aggression (Sano et al., 2013; Yang et al., 2013). The dorsomedial and central subdivisions of the VMH (VMHdm and VMHc, respectively) mediate defensive behaviors in adult male mice(Kunwar et al., 2015; Mongeau et al., 2003; Wang et al., 2015). Stimulation of neurons in the VMHdm and VMHc elicits avoidance behaviors, such as fleeing and jumping, and immobility(Wang et al., 2015). In addition, a predator scent (i.e. cat collar) and exposure to a predatory rat induces Fos expression in the VMHdm of adult male rats (Dielenberg et al., 2001; Silva et al., 2013). Thus, subdivisions in the VMH serve different functions, with the VMHvl regulating male sexual behavior and intermale aggression, while the VMHdm and VMHc plays a role in controlling defensive behaviors.

The DG is a region in the hippocampus recognized for maintaining relatively high levels of postnatal neurogenesis well into adulthood. Throughout the years, the DG has been associated with controlling many different types of behaviors, such as spatial learning, mood regulation, object recognition, and more recently, social behaviors (Ibi et al., 2008; Jessberger et al., 2009; Shors et al., 2002; Wei et al., 2011). Social experiences tend to have a strong influence on neurogenesis in the DG in rodents. In adult male rats, sexual encounters with a receptive female enhances the number of new neurons in the DG (Leuner et al., 2010). Conversely, social isolation during adolescence leads to reduced levels of neurogenesis in the DG of adult male mice (Ibi et al., 2008). Moreover, it appears some pubertally born neurons in the DG are functionally distinct

from adult born neurons. Blocking pubertal neurogenesis in female mice reduces social exploration and increases social aversion during female-female social interactions, while blocking adult neurogenesis does not have an effect on these social behaviors (Wei et al., 2011). Thus, it seems that pubertally born neurons in the DG may facilitate the acquisition of adaptive behaviors (e.g. appropriate social behaviors for mating) required for adulthood.

While it is clear that the MePD, VMH, and DG are intimately involved in the regulation of adult sociosexual behaviors, the neurological changes that facilitate the development of these behaviors remains unknown. A previous study found that that a subset of pubertally born MePD cells in male hamsters is activated by a sexual encounter(Mohr et al., 2013). This was the first study to show that pubertally born cells had the capacity to mature into permanent fixtures in the brain to participate in the expression of adult social behaviors, like sexual behavior. Therefore, it is possible that pubertally born cells in the MePD, VMH, and DG also become functionally integrated into existing neural circuits and are activated by social interactions.

The current chapter aimed to expand on this breakthrough discovery and examine Fos expression in each region of interest (ROI) (i.e. MePD, VMHvl, VMHdm, VMHc, and DG) to detect region-specific activation in response to different social behaviors in adult males and determine whether pubertally born cells in the of adult male mice become functionally integrated into existing neural circuits that mediate adult sociosexual behaviors (i.e. male sexual behavior and intermale aggression). In addition, we determined what proportion of pubertally born cells in each ROI became mature neurons or astrocytes.

MATERIALS AND METHODS

Animal Subjects and Experimental Design

42 male mice from an in-house wildtype C57BL/6 mouse colony were used for experimental procedures described below. In addition, 14 female and 14 male mice from the same colony were used as stimulus animals for social interaction tests. All mice were weaned at P24 and group housed (4-5 mice/cage) in clear plexiglass cages (29 x 18 x 13 cm) in a 12:12 light:dark cycle (lights on: 7:00 AM, lights off: 7:00 PM). All subjects were provided ad libitum rodent chow (Harlan Teklad 8640 Rodent Diet, Madison, WI) and water. All female stimulus mice were ovariectomized on between P50-P60 and subcutaneously (s.c.) implanted with a Silastic capsule filled with 17β-estradiol (0.6 mm–inner diameter and 3.2 mm–outer diameter; 12 mm–effective release length, 1.8 cm–total capsule length, filled with 25 µg 17β-estradiol /ml sesame oil). All female stimulus mice participated in a social interaction test between P60-P70. Four hours prior to the social interaction test, all females received an injection of progesterone (0.5mg/0.1 ml in sesame oil, s.c.). All housing and experimental procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Michigan State University's Institutional Animal Care and Use Committee.

BrdU administration

During the first week of puberty (P28-P35), all subjects were administered BrdU (1.0 mg/ml, Sigma-Aldrich) dissolved in the drinking water. BrdU drinking water was prepared fresh every 3 days. During BrdU administration, all mice were housed in Innocage mouse cages (37 x 23 x 14 cm) with Aquavive amber water bottles (Innovive,

San Diego, CA). The use of the Aquavive amber water bottles allowed us to administer BrdU via drinking water in smaller volumes (about 100 ml of water/bottle) to avoid excess BrdU waste. The Aquavive amber water bottles also had minimal leakage and since BrdU is light sensitive, the amber bottles provided adequate protection from light. In addition, the use of disposable Innocage mouse cages avoided BrdU contamination of reusable animal cages.

Social Interaction Tasks

On P56, one week prior to social interactions and subsequent sacrifice, all male mice were housed in separate cages and pair-housed with a female mouse to provide a home cage territory and sexual experience. On P63, the female was removed from the home cage one hour prior to the social interaction tasks. The male mice were randomly assigned to one of three types of social interactions: resident-intruder paradigm (n=14), sexual behavior (n=14), or no social interaction (n=14). In the resident-intruder condition, a smaller, unfamiliar male intruder was introduced to the home cage of the resident at the start of the test. After fifteen minutes, the intruder was removed from the cage and the resident male was sacrificed one hour later. In the sexual behavior condition, an unfamiliar estrogen and progesterone primed female mouse was introduced to the male's home cage. After fifteen minutes, the female mouse was removed from the cage and the male subject was sacrificed one hour later. In the no social interaction condition, the male mice received no social stimulus and resided in their home cage until time of sacrifice. All social interactions were monitored to confirm that the appropriate social interaction (i.e. attack and chase behavior during resident-intruder paradigm and mounting and intromission during sex behavior) occurred as

intended. Henceforth, the following group names will be used: male-male interaction group (male mice that underwent the resident-intruder paradigm), male-female interaction group (male mice that experienced male sexual behavior), and no social interaction group (male mice that experienced no social interaction).

Tissue Collection and Processing

On P63, all mice were given an overdose of sodium pentobarbital (150 mg/kg, i.p.) and intracardially perfused with 0.9% saline followed by cold 4% paraformaldehyde in 0.1 M phosphate buffered saline (pH 7.4). Brains were removed and postfixed overnight in 4% paraformaldehyde, then switched to 30% sucrose solution at 4 °C until sectioning. All brains were sectioned within one week of perfusion. Brains were cut on a freezing microtome into 30 µm coronal sections (from about Bregma -0.82 to Bregma -2.74), and collected as 4 alternate series. The entire first series for each animal was Nissl stained with thionin, dehydrated, and coverslipped.

Double-Label BrdU/Fos Immunofluorescence

The entire second series of brain sections underwent immunofluorescence to label BrdU-immunoreactive (ir) and Fos-ir cells. Free-floating sections were rinsed in Tris-buffered saline (TBS; 0.05 M; pH 7.6) for 30 minutes and then incubated in 0.1% sodium borohydride for 10 minutes. After a 15-minute rinse in TBS, the sections were incubated in 2N HCl for 60 minutes at 37°C. Sections were then neutralized in 0.1M borate buffer (pH 8.5) for 10 minutes. Following a 30-minute rinse in TBS, the sections were blocked in TBS containing 0.01% Triton-X-100 and 3% donkey serum for 1 hour, and then incubated in a primary antibody cocktail at 4°C for 48 hours, which contained monoclonal rat anti-BrdU (catalog no. MCA2060; Serotec) at a working concentration of

1 µg/ml and polyclonal rabbit anti-c-Fos (catalog no. ABE457; Millipore) at a working concentration of 0.1 µg/ml. Sections were then rinsed in TBS for 30 minutes and incubated in a secondary antibody cocktail for 2 hours at room temperature, which contained Cy2 Affinipure donkey anti-rat secondary antibody (catalog no. 712-225-153; Jackson Immunoresearch) at a working concentration of 2.8 µg/ml and Cy3 Affinipure donkey anti-rabbit (catalog no. 711-165-152, Jackson Immunoresearch) at a working concentration of 2.8 µg/ml. Following a 30-minute rinse in TBS, all sections were mounted onto gelatin-subbed slides, dehydrated, and coverslipped.

Triple-Label BrdU/GFAP/NeuN Immunofluorescence

The third series of brain sections of 6 animals (2 mice/group) underwent immunofluorescence to label for BrdU, GFAP (a marker of astrocytes), and NeuN (a marker of mature neurons). Since differences in social interaction prior to sacrifice could not affect colocalization of pubertally born cells with NeuN or GFAP, a total of 6 mice were analyzed for the MePD and VMH, and a total of 3 mice were analyzed for the DG (1 mouse/group). Free-floating sections were rinsed in Tris-buffered saline (TBS; 0.05 M; pH 7.6) for 30 minutes and then incubated in 0.1% sodium borohydride for 10 minutes. After a 15-minute rinse in TBS, the sections were incubated in 2N HCl for 60 minutes at 37°C. Sections were then neutralized in 0.1M borate buffer (pH 8.5) for 10 minutes. Following a 30-minute rinse in TBS, the sections were blocked in TBS containing 0.01% Triton-X-100 and 3% donkey serum for 1 hour, and then incubated in a primary antibody cocktail at 4°C for 48 hours, which contained monoclonal rat anti-BrdU (catalog no. MCA2060; Serotec) at a working concentration of 1 µg/ml, polyclonal rabbit anti-GFAP (catalog no. Z0334; Dako) at a working concentration of 0.58 µg/ml,

and monoclonal mouse anti-NeuN (catalog no. MAB377, Chemicon) at a working concentration of 1 µg/ml. Sections were then rinsed in TBS for 30 minutes and incubated in a secondary antibody cocktail for 2 hours at room temperature, which contained Cy2 Affinipure donkey anti-rat secondary antibody (catalog no. 712-225-153; Jackson Immunoresearch) at a working concentration of 5.6 µg/ml, Cy3 Affinipure donkey anti-rabbit (catalog no. 711-165-152, Jackson Immunoresearch) at a working concentration of 6.0 µg/ml, and Cy5 Affinipure donkey anti-mouse (catalog no. 715-175-151, Jackson Immunoresearch) at a working concentration of 6.0 µg/ml. Following a 30-minute rinse in TBS, all sections were mounted onto gelatin-subbed slides, dehydrated, and coverslipped.

Microscopic Analysis

All microscopic analyses were performed by an observer blind to treatment group. The regions of interest for all microscopic analyses included the posterodorsal medial amygdala (MePD), the ventrolateral, dorsomedial, and central subdivisions of the ventromedial hypothalamus (VMHvl, VMHdm, and VMHc, respectively), and the dentate gyrus (DG). The boundaries of the MePD and VMH on the Nissl series were determined using a standard mouse brain atlas(Paxinos et al., 2004). To consistently sample anatomically-matched sections across animals, the following landmarks were used to trace the MePD: the shape of the optic tract, the size and shape of the lateral ventricles, the size and shape of the intercalated amygdalar nucleus, and the size and shape of the stria terminalis. For the VMH, the size, shape, and staining intensity of the nucleus itself, as well as the size and shape of the third ventricle were used to

consistently sample anatomically-matched sections across animals. For the DG, the two most rostral sections were analyzed.

Double-label BrdU/Fos Analysis

The double-label BrdU/Fos series was analyzed using an Olympus BX51 microscope under epi-illumination using Neurolucida version 10 (MBF Bioscience). The regions of interest were traced using the Nissl-stained series and contours were superimposed onto adjacent sections of the double-label BrdU series. The full rostral-caudal extent of the MePD, VMHvl, VMHdm, and VMHc was analyzed, which included a total of six MePD sections and five VMH sections. Animals that did not have all anatomically-matched sections were excluded from analysis. The final sample sizes were as follows: male-male interaction (n=13), male-female interaction (n=11), and no social interaction (n=14). The total number of Fos-ir cells was calculated by summing the number of Fos-ir cells across all sections analyzed. The total number of BrdU-ir cells was determined by summing the number of BrdU-ir cells across all sections analyzed. The % BrdU-ir cells that expressed Fos was calculated by dividing the total number of double-labeled BrdU-ir/Fos-ir cells by the total number of BrdU-ir cells multiplied by 100.

Triple-Label BrdU/GFAP/NeuN Analysis

The triple-label BrdU/GFAP/NeuN series was analyzed using an Olympus FluoView FV1000 confocal laser-scanning microscope. A z-stack orthogonal viewer was utilized to verify colocalization of BrdU/GFAP and BrdU/NeuN. All regions of interest were analyzed using the multi-line Argon gas laser (488 nm), green Helium Neon laser (543 nm), and red Helium Neon laser (633 nm) to detect BrdU-ir, GFAP-ir, and NeuN-ir cells, respectively. Again, the full rostral-caudal extent of the MePD, VMHvl, VMHdm,

and VMHc was analyzed. The % colocalization of BrdU with GFAP was calculated by dividing the total number of BrdU-ir/GFAP-ir cells by the total number of BrdU-ir cells multiplied by 100. The % colocalization of BrdU with NeuN was calculated by dividing the total number of BrdU-ir/NeuN-ir cells by the total number of BrdU-ir cells multiplied by 100.

Statistical Analysis

For the double-label BrdU/Fos analysis, group differences in total number of BrdU-ir cells, total number of Fos-ir cells, and % colocalization of BrdU-ir/Fos-ir cells were analyzed using a one-way ANOVA. For all analyses, a significance value of $p \leq 0.05$ was used and Cohen's d was calculated to determine effect sizes. For the triple-label BrdU/GFAP/NeuN analysis, no statistical analyses were performed because of small sample sizes (n=2/group). Only % colocalization of BrdU/GFAP and BrdU/NeuN were calculated, as described above, to determine the proportion of pubertally born cells that differentiated into astrocytes or mature neurons for each region of interest.

RESULTS

Social interaction activates pubertally born cells in the adult male MePD.

Pubertally born cells in the MePD were activated by social interactions in adult male mice as evidenced by Fos expression in BrdU-ir cells (Fig 3.1A). An ANOVA revealed a significant group difference in the proportion of pubertally born MePD cells that express Fos ($F(2,35)=11.731$, $p=0.000$) (Fig 3.1B). Post-hoc analyses showed that male-male social interaction ($p=0.006$, $d=1.35$) and male-female social interaction ($p<0.000$, $d=2.10$) significantly increased the proportion of BrdU-ir cells that colocalize with Fos-ir in the adult MePD when compared to no social interaction. No significant

differences were observed between the male-male and male-female social interaction groups (p=0.516). In addition, there were significant differences in the total number of Fos-ir cells in the adult MePD between social interaction groups (F(2,35)=33.409, p=.000) (Fig 3.1C). The expression of Fos was larger in response to male-male social interaction (p<0.000, d=2.48) (Fig 3.1E) and male-female social interaction (p<0.000, d=4.11) (Fig 3.1F) than no social interaction (Fig 3.1G), recapitulating previous studies showing increased activation of the adult MePD in response to social interactions with a male or female conspecific(Kollack-Walker et al., 1995). No significant differences were detected between the male-male social interaction and male-female social interaction groups (p=1.00). Furthermore, no group differences were observed in the total number of BrdU-ir cells (F(2,35)=1.029, p=0.368) (Fig 3.1D) showing that the number of pubertally born cells in the MePD was comparable across all groups.

Male-male social interactions activate pubertally born cells in the adult male VMHvl and VMHdm.

Double-labeled BrdU-ir and Fos-ir cells were identified in the adult male VMH (Fig 3.2A) with male-male social interactions enhancing the activation of pubertally born cells in the VMHvl and VMHdm. An ANOVA revealed a significant group difference in the proportion of pubertally born cells that express Fos in the VMHvl (F(2,35)=4.649, p=0.016) (Fig 3.2B) and VMHdm (F(2,35)=4.298, p=0.021) (Fig 3.2C). In the VMHvl, male-male interaction significantly increased the proportion of BrdU-ir cells that expressed Fos compared to no social interaction (p=0.013, d=1.23). The proportion of BrdU-ir cells that express Fos-ir after male-female interaction was not different from that of either the male-male (p=0.534) or no social interaction (p=0.417) groups, suggesting

that pubertally born cells in the VMHvl are only moderately activated in following social interaction with a female conspecific. Similar to the VMHvl, more pubertally born cells in the VMHdm were activated in response to male-male interactions than no social interaction (p=0.018, d=1.06). Moreover, the percent cell colocalization of BrdU-ir/Fos-ir in the male-female interaction group was not significantly different compared to the male-male interaction (p=0.306) or no social interaction (p=0.860) groups.

Quantitative analysis of Fos-ir cells revealed significant group differences in the total number of Fos-ir cells in the VMHvl (F(2,35)=14.707, p<.000) (Fig 3.2D), with male-male (p<0.000, d=1.80) (Fig 3.1H) and male-female social interactions (p=0.006, d=2.80) (Fig 3.1I) significantly increasing VMHvl activation compared with no social interaction (Fig 3.1J). Moreover, male-male and male-female social interactions increased Fos-ir expression in the VMHvl to comparable levels (p=0.264). Group differences in the number of Fos-ir cells were also detected in the VMHdm (F(2,35)=6.902, p=0.003) (Fig 3.2E). Post-hoc analyses revealed that male-male social interaction induced greater Fos-ir expression in the VMHdm than no social interaction (p=0.003, d=1.39). The number of Fos-ir cells in the male-female social interaction group did not significantly differ from the male-male interaction (p=0.808) or no social interaction groups (p=0.076). These results suggest that social interactions with either a male or female conspecific activates the adult male VMHvl, while only male-male social interaction elicits high levels of activation in the VMHdm. Interestingly, male-female social interaction also moderately increased VMHdm activation. Again, no group differences were found in the total number of BrdU-ir cells in the VMHvl (F(2,35)=0.886, p=0.421) (Fig 3.2F) and the VMHdm (F(2,35)=1.629, p=0.211) (Fig 3.2G).

Pubertally born cells in the adult male VMHc and DG are not activated by social interactions.

In the VMHc, double-labeled BrdU-ir and Fos-ir cells were observed in only 2 male mice, both of which were in the male-female social interaction group. ANOVA detected no significant group differences in the proportion of pubertally born cells that express Fos-ir ($F(2,35)=3.096$, $p=0.058$) (Fig 3.3A). However, ANOVA revealed group differences in the number of Fos-ir cells in the VMHv ($F(2,35)=6.902$, $p=0.003$) (Fig 3.3B). Male-male ($p<0.000$, $d=1.52$) and male-female ($p=0.041$, $d=1.56$) social interactions evoked greater VMHc activation compared to no social interactions. Male-male and male-female social interactions appeared to stimulate an equivalent Fos-ir response in the VMHc ($p=0.419$). Once more, no group differences were found in the total number of BrdU-ir cells in the VMHc ($F(2,35)=1.459$, $p=0.246$) (Fig 3.3C).

A small subset of pubertally born cells in the adult male DG expressed Fos-ir (Fig 3.4A). An ANOVA revealed no significant group differences in the percent colocalization of BrdU-ir/Fos-ir in the DG ($F(2,35)=1.154$, $p=0.327$) (Fig 3.4B) or the total number of BrdU-ir cells in the DG ($F(2,35)=3.103$, $p=0.060$) (Fig 3.4D). However, significant group differences were detected in the total number of Fos-ir cells in the adult DG ($F(2,35)=10.104$, $p=0.000$) (Fig 3.4C). Post-hoc analyses discovered male-male ($p=0.003$, $d=1.62$) (Fig 3.4F) and male-female ($p=0.001$, $d=1.38$) (Fig 3.4E) interactions significantly increased the number of Fos-ir cells in the adult male DG than no social interaction (Fig 3.4G) with no significant differences between the male-male and male-female interaction groups were detected in the total number of Fos-ir cells ($p=1.00$).

Pubertally born cells in the MePD, VMH, and DG differentiate into mature neurons and astrocytes.

Using triple-label immunofluorescence and confocal laser scanning microscopy, we discovered that a subset of pubertally born cells in the adult MePD, VMH, and DG differentiated into mature neurons (Fig 3.5A) and astrocytes (Fig 3.5B). These pubertally born cells are estimated to be about 4-5 weeks old, as BrdU was administered from P28-35 and tissue was collected at P63. The proportion of BrdU-ir cells that colocalized either NeuN-ir or GFAP-ir in the MePD, VMH, and DG is depicted in Fig 3.5C. Approximately 6% and 12% of pubertally born MePD cells colocalized with NeuN-ir and GFAP-ir, respectively. The proportion of BrdU-ir cells that differentiated into mature neurons or astrocytes was similar across the subdivisions of the VMH. About 6-7% of BrdU-ir cells colabeled with NeuN-ir in the VMHvl, VMHdm, and VMHc, while approximately 7% of BrdU-ir cells colabeled with GFAP-ir in all subdivisions of the VMH. In the adult male DG, most BrdU-ir cells colocalized with NeuN-ir (~75%) indicating that most pubertally born cells in the DG differentiate into neurons. A small subset of pubertally born cells in the DG also expressed GFAP (10%).

DISCUSSION

Here we discovered that a subset of pubertally born cells in the adult male MePD was activated in response to social interactions with a male or female conspecific, while a group of pubertally born cells in the VMHvl and VMHdm was activated in response to male-male social interaction. Social interactions did not activate pubertally born cells in the VMHc and DG. These findings demonstrate that cells generated during the first week of puberty can be activated in adulthood as a consequence of social interaction in

a brain region-specific manner. In addition, we found that overall neural activation, as indicated by the presence of Fos-ir cells, was increased in all ROIs (i.e. MePD, VMHvl, VMHdm, VMHc, and DG) in response to a male-male or male-female social interaction, replicating findings from previous work (Kollack-Walker et al., 1995). Therefore, in the MePD, the activation patterns of pubertally born cells mirrors that of overall neural activation in response to male-male and male-female social interactions. However, this is not the case for the subdivisions of the VMH or DG. Together these results indicate pubertally born cells in the adult MePD participate in both male sexual behavior and intermale aggression, while the activation of pubertally born cells in the VMHvl is only critical for intermale aggression. We also examined the phenotype of pubertally born cells in all ROIs, and that a portion of pubertally born cells in all regions differentiated into neurons or astrocytes. Thus, some pubertally born cells are capable of maturing into neurons or astrocytes in the adult male brain at ~4-5 weeks of age.

Social interactions activate pubertally born cells in the adult male MePD

Intermale aggression and sexual behavior activated about 0.4% and 0.5% of BrdU-ir cells in the adult male MePD, respectively. To our knowledge, this is the first study to demonstrate that pubertally born cells in the MePD respond to intermale aggression in adulthood. Intermale aggression emerges around the time of puberty (Barkley et al., 1977) when the pheromones of an unfamiliar conspecific male become aversive(Brown, 1979). Since the MePD is involved in the processing of chemosensory information, it is possible that new cells may be born during puberty to help mediate intermale aggression in adulthood. In a recent study, investigators found that pre-pubertal, but not adult, knockdown of ERα-expressing neurons in the medial amygdala

decreased aggressive behaviors in adult male mice (Sano et al., 2016; Sano et al., 2013). Therefore, pubertal estrogen has an organizational effect on the medial amygdala to facilitate the development of male aggressive behaviors. It is possible, in male mice, that estrogen, aromatized from testosterone, acts to promote the proliferation or survival of pubertally born cells that become incorporated into existing neural circuits to regulate intermale aggression in adulthood. Very little is known regarding the effects of estrogen on cytogenesis in the male MePD, but a study in adult male meadow voles revealed that testosterone or estrogen, but not DHT, increased cell proliferation in the medial amygdala (Fowler et al., 2003). However, we cannot rule out the possibility that pubertally born cells express ER, which by estrogen acts on these ER-expressing pubertally born cells to enhance survival. Furthermore, estrogen could stimulate the migration of cells into the MePD, which by then these cells mature and become incorporated into existing neural circuits.

In the current study, we identified about 0.5% active BrdU-ir cells in male mice in response to sexual behavior. While the proportion activated pubertally born cells may seem low, it should be noted that about 6% of pubertally born cells in the male mouse MePD become mature neurons. Since Fos is traditionally used to examine activation of neurons, it is not surprising to find a small percentage of BrdU-ir cells are activated in the adult male MePD. However, a previous finding in our lab showed roughly 2% of pubertally born cells in the adult MePD of male hamsters were activated by a sexual encounter (Mohr et al., 2013). These two studies used different methods of BrdU administration, which could also account for differences in pubertally born cells activation: in the hamster study, BrdU was injected daily (i.p.) for three weeks (P28-49),

while in the mouse study, BrdU was administered via drinking water for one week (P28-49). It is possible that cells born during mid to late puberty have a more prominent role in regulating male sexual behavior.

It is also important to consider the effects of environmental conditions on the functional incorporation of pubertally born cells. Environmental enrichment (i.e. larger cages, running wheel, cotton bedding, and cardboard igloo), after weaning, doubled the percent colocalization of BrdU-ir/Fos-ir cells in the adult male hamster MePD in response to sexual behavior (Mohr et al., 2013). It is now well established that environmental conditions can influence neurogenesis as introducing adult rodents to environmentally enriched conditions, exercise, and sexual experience enhances neurogenesis in the hippocampus (Fan et al., 2014; Kempermann et al., 2002; Leuner et al., 2010; Monteiro et al., 2014; Vivar et al., 2013), suggesting that these new neurons may play an important functional role in the behaviors that promote their survival. While the mice in the current study only received moderate environmental enrichment (i.e. cardboard igloo and cotton bedding), they were socially enriched (i.e. group housed). Social enrichment is also known to promote neurogenesis, particularly in the hippocampus (Gheusi et al., 2009; Lu et al., 2003; Westenbroek et al., 2004). Moreover, social isolation reduces hippocampal and amygdalar neurogenesis in female prairie voles (Fowler et al., 2002; Lieberwirth et al., 2012). Therefore, since the subjects in the current study received social enrichment and moderate environmental enrichment, we believe it is unlikely that the lack of additional enrichment (i.e. running wheel and larger cage) stunted the development of pubertally born cells in mice.

The MePD is most commonly known for regulating social interactions with conspecifics, however, it should be noted that the MePD has many additional functions including regulating pubertal timing, feeding, and reward motivated behaviors (Hu et al., 2016; King, 2006a; Li et al., 2015; Pardo-Bellver et al., 2012). Therefore, it is possible that these behaviors or other types of social interactions may also activate a subset of pubertally born cells in the MePD.

Male-male social interactions activate pubertally born cells in the VMHvl and VMHdm

A higher proportion of pubertally born cells in the VMHvl became activated in response to intermale aggression than sexual behavior or no social interaction in adult male mice. Past research has shown that Fos expression is increased in the male VMHvl by an aggressive encounter with a conspecific male(Kollack-Walker et al., 1995). In adult male mice, excitation of VMHvl neurons prompts males to attack all intruders, including conspecific males and females and even inanimate objects (e.g. gloves)(Lin et al., 2011). Due to these recent findings, the VMHvl is now recognized as a key regulator of intermale aggression; however, the VMHvl is also involved in male sexual behavior(Falkner et al., 2014b; Kollack-Walker et al., 1995; Yang et al., 2013), so it was unexpected to not see a significant increase in pubertally born cell activation by sexual behavior in adult male mice. These results could indicate the while the VMHvl are known to regulate male sexual behavior, it is possible that pubertally born cells do not respond to male sexual behavior, and they are more important for regulating intermale aggression.

In the VMHdm, the increase in BrdU-ir/Fos-ir cell colocalization in response to intermale aggression was quite surprising. Traditionally, the rodent VMHdm has been

implicated in initiating defensive behaviors (i.e. freezing or avoidance) in response to predators with minimal neural activation in response to social interactions with a conspecific (Dielenberg et al., 2001; Perez-Gomez et al., 2015). Although, it is possible that defensive behaviors initiated in response to interacting with an unfamiliar male mouse increased neural activation in the VMHdm. Group housing (4-5 mice/cage) may have inadvertently affected the development of aggressive and defensive behaviors in the male subjects. Group housed male rodents rapidly form stable dominance hierarchies usually comprised of one dominant male and several subordinate males(Poole et al., 1973; So et al., 2015). In dominance hierarchies, the dominant male mouse is typically the most aggressive, while the subordinate males display low levels of aggression (Poole et al., 1973). Therefore, when introduced to the resident-intruder paradigm, it is possible that the dominant male from each cage performed aggressively, while subordinate males displayed a mixture of aggressive and a greater amount of defensive behaviors in response to an unfamiliar intruder male. This was the case in male hamsters; a previous study showed subordinate males displayed more defensive behaviors than dominant males in response to an agonistic encounter, however, interestingly, the number of Fos-ir cells in the VMH did not differ between groups (Pan et al., 2010). The study did not examine the subdivisions of the VMH separately, thus, group differences in the VMHdm could have been overshadowed by increased activation in the VMHvl.

Similar to the MePD, the VMH, in general, is responsible for regulating many behaviors including energy homeostasis and feeding(Gavini et al., 2016; King, 2006b). Researchers have demonstrated that newly generated cells in the VMH of adult mice

become leptin-responsive 4 weeks post-BrdU treatment, indicating that new born adult cells in the VMH participate in energy balance regulation(Bless et al., 2016; Kokoeva et al., 2005). Thus, it is possible that pubertally born cells in the adult VMH are more sensitive to behaviors such as energy homeostasis.

Pubertally born cells in the VMHc and DG are not activated by social interactions

In the VMHc, there was very little, if any, activation of pubertally born cells in adult male mice. Out of all the subjects, pubertally born cells in the VMHc of only two male mice were activated by social interaction; therefore, statistically, there were no group differences in pubertally born cell activation. Unfortunately, not much is known regarding the function of the VMHc. In general, the VMHc is not included in neuroanatomical studies due to its sparse neuronal population, but on occasion, researchers will combine the VMHc with the VMHdm to create one entity (VMHdm/c). Recently, a study discovered that activation the VMHdm/c increased defensive behaviors in male mice (Wang et al., 2015), however, this behavioral effect could be due to the activation of the VMHdm alone. Thus, it is not surprising that pubertally born cells in the VMHc were not activated by social interactions.

Pubertally born cells in the DG were not activated in response to social interactions. The DG has been shown to be involved in social behaviors(Ahern et al., 2016; Ibi et al., 2008; Opendak et al., 2016), therefore, it was unexpected to discover there was no increase in pubertally born cell activation in the DG in response to social interactions. For instance, social isolation during adolescence significantly reduces neurogenesis in the DG and increases aggression in male mice; however, treating socially isolated mice with fluoxetine, an antidepressant, prevented these adverse

effects (Ibi et al., 2008). In addition, male rats that were group-housed after weaning show enhanced Fos expression in response to a social encounter in the DG than males raised in social isolation(Ahern et al., 2016). However, the DG of the hippocampus is a complex brain regions and participates in a myriad of different behaviors(Curlik et al., 2014; de Andrade et al., 2013; Duarte-Guterman et al., 2015), therefore, pubertally born cells in the adult DG may serve a different purpose than participating in social interactions with a male or female conspecific.

Although pubertally born cells in the VMHc and DG are not activated by social interactions, the overall neural activation pattern, as assessed by Fos expression, showed that both the VMHc and DG are activated by social interactions. This finding suggests that neurons in the VMHc and DG are activated by social interactions; however, they are born outside of the first week of puberty. It is possible that pubertally born cells during mid to late puberty in the VMHc and DG are more responsive to social interactions.

A subset of pubertally born cells in the MePD, VMH, and DG differentiate into mature neurons or astrocytes

In the current study, we confirmed that a portion of pubertally born cells in the adult mouse MePD, VMH, and DG differentiated into mature neurons or astrocytes, replicating the results of the previous chapter. These results also corroborate the previous findings that have shown some cells born in the postnatal born also differentiate into mature neurons and glial cells (i.e. astrocytes, microglia, and oligodendrocytes) (Bless et al., 2014; Fowler et al., 2003; Kokoeva et al., 2005; Mohr et al., 2013; Saul et al., 2015). However, the fact remains that most of the pubertally born

cells in the MePD and VMH did not colocalize with NeuN or GFAP. A study in male meadow voles found that approximately 35% of newly born cells in the amygdala of adult male meadow voles colabeled with NG2 (Fowler et al., 2003). In rats, 50% of pubertally born cells in the male rat amygdala colocalized with NG2 10 days after BrdU administration (Saul et al., 2015). NG2 is traditionally known as a marker for oligodendrocyte cell precursors and most NG2-expressing cells do mature into oligodendrocytes, however studies have shown that a subset of NG2-expressing cells can also go on to become astrocytes and neurons (Vigano et al., 2016). In male rats, about 15% pubertally born cells expressed doublecortin (DCX), an immature neuron marker, 4 and 8 weeks post-BrdU administration. Furthermore, a recent study in female rats showed about 23% of pubertally born cells in the female rat anteroventral periventricular nucleus expressed Iba1, a microglia marker (Mohr et al., 2016). Thus, while a significant portion of pubertally born cells did not express NeuN or GFAP in the mouse MePD and VMH, it is feasible that these pubertally born cells may be other cell phenotypes such as oligodendrocytes, microglia or neurons in an immature state. It should also be noted that not all astrocytes are known to express GFAP, so future studies could utilize other astrocytic markers, such as Aldh1L1(Cahoy et al., 2008).

Conclusions

The current study demonstrates that pubertally born cells are functionally integrated into three limbic structures in the adult mouse brain: the posterodorsal medial amygdala, the ventromedial hypothalamus, and the dentate gyrus of the hippocampus. Thus, a subset of cells born during the first week of puberty persist and survive into adulthood to contribute to the maturation of social behaviors and some cells differentiate

into mature neurons or astrocytes. In addition, we established that the MePD, VMH, and DG all have a role in sexual and aggressive behaviors. Documenting the functional incorporation of pubertally born cells into the behavioral circuitry in mice sets the stage to further explore and identify the neurochemical phenotype, connectivity, and function of these pubertally born cells in future studies.

Figure 3.1. Social interaction activates pubertally born cells in the adult male MePD. (A) Representative photomicrograph displaying a colocalized BrdU-ir (green) and Fos-ir (red) cell in a coronal section of an adult male MePD. (B) Quantitative analysis revealed that more pubertally born cells are activated in response to a male-male and male-female interactions than no social interaction. (C) Male mice exposed to a male or female mouse also have significantly more Fos-ir cells in the adult MePD than male mice that received no social interaction. (D) No significant differences were observed in the total number of BrdU-ir cells in the adult MePD between groups. Representative photomicrographs of the MePD display the BrdU-ir and Fos-ir cells of male mice in the (E) male-male, (F) male-female, and (G) no social interaction groups. Data are represented as mean ± SEM. Low mag scale bar = 100 μm, high mag scale bar = 10 μm.

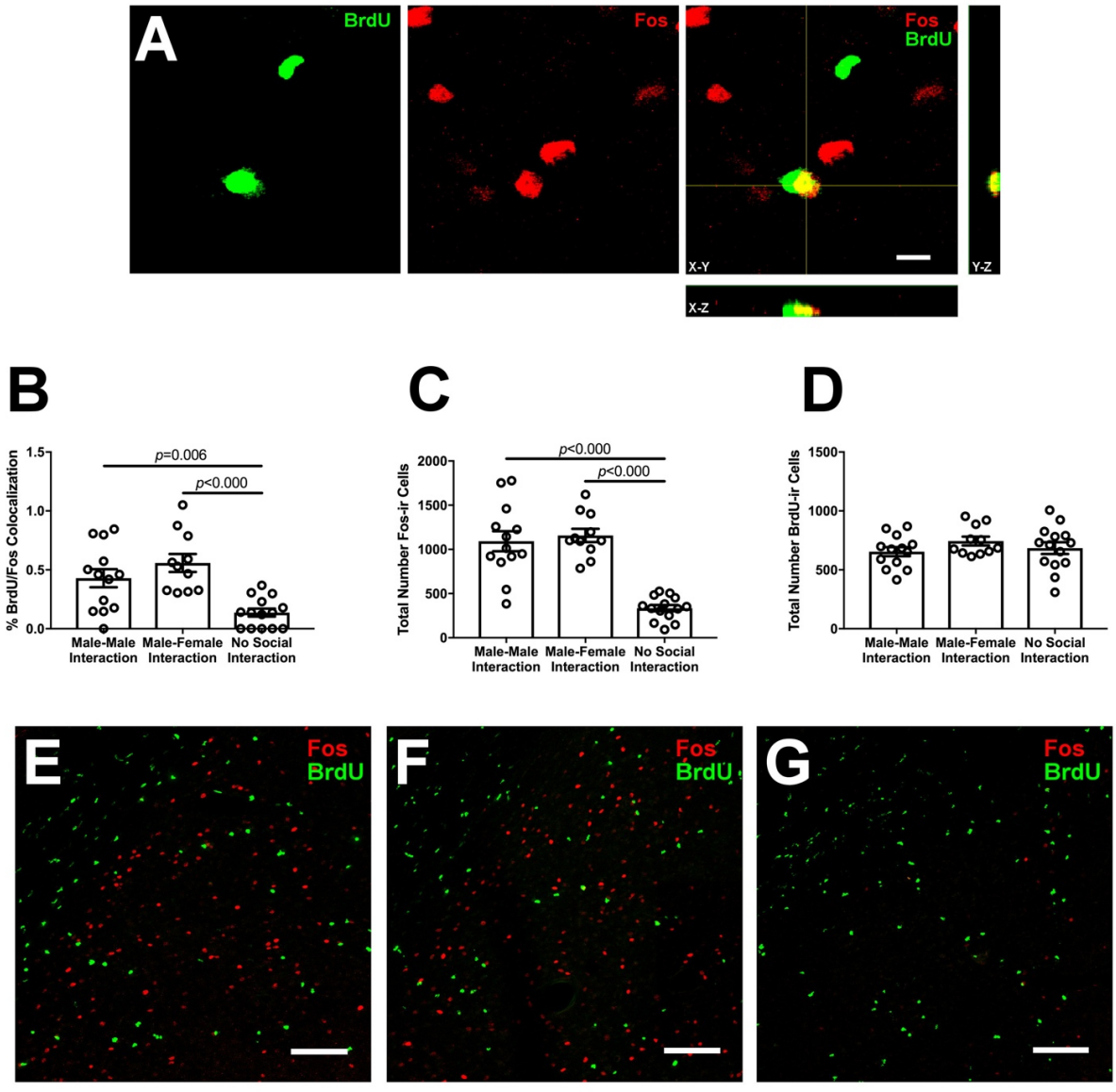


Figure 3.2. Male-male social interactions activate pubertally born cells in the adult VMHvl and VMHdm. (A) A representative image of a colocalized BrdU-ir and Fos-ir cell in the adult male VMHvl. Quantitative analysis revealed that more pubertally born cells were activated in the (B) VMHvl and (C)VMHdm in response to male-male interactions than no social interaction. (D) In the VMHvl of male mice had more Fos-ir cells in response to male-male and male-female interactions than no social interactions. (E) In the VMHdm, male mice had more Fos-ir cells during the male-male social interaction than no social interaction. No significant differences were found in the total number of BrdU-ir cells between groups in the (F)VMHvl and (G)VMHdm. Representative photomicrographs of the VMH show the BrdU-ir and Fos-ir cells of male mice in the (H) male-male, (I) male-female, and (J) no social interaction groups. Data are represented as mean ± SEM. Low mag scale bar = 100 μm, high mag scale bar = 10 μm.

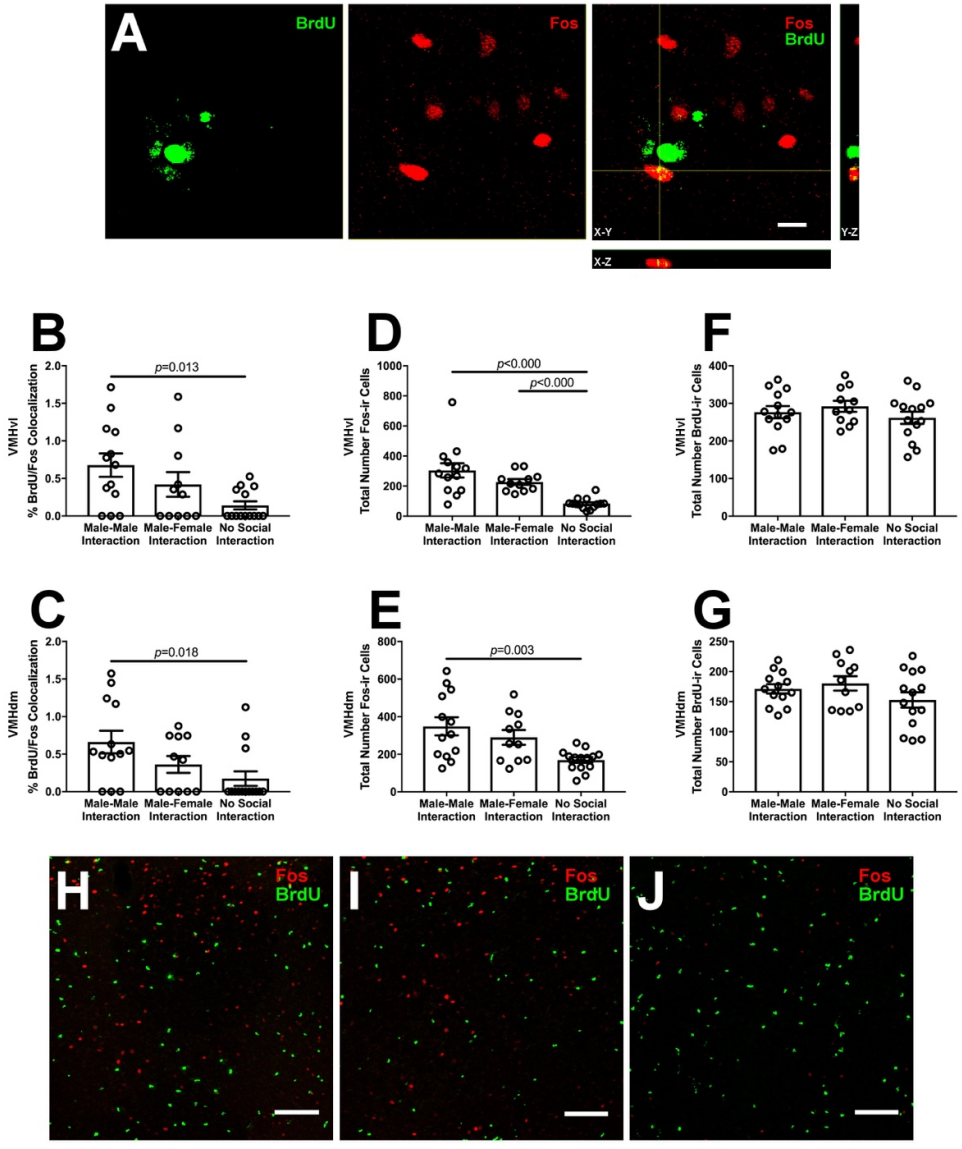


Figure 3.3. Pubertally born cells in the VMHc are not activated by social interactions. (A) An ANOVA revealed no significant group differences in the % colocalization of BrdU- and Fos-ir cells in the VMHc. (B) The VMHc also showed greater activation in response to a male-male and male-female social interaction compared to no social interaction. (C) No significant differences were found between groups in the total number of BrdU-ir cells. Data are represented as mean ± SEM.

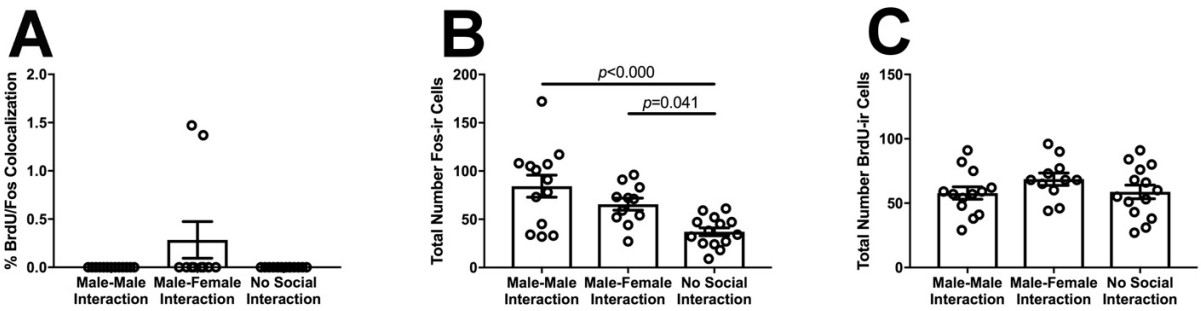


Figure 3.4. Pubertally born cells in the dentate gyrus are not activated by social interactions. (A) Representative image of a colocalized BrdU-ir and Fos-ir cells in the adult male dentate gyrus. (B) No significant differences were detected between groups in the % colocalization of BrdU-ir and Fos-ir. (C) Quantitative analysis revealed male-male and male-female social interactions increased the total number of Fos-ir cells in the adult DG compared to no social interaction. (D) No significant differences were found between groups in the total number of BrdU-ir cells. Representative photomicrographs show BrdU-ir and Fos-ir cells in the adult DG of male mice in the (H) male-male, (I) male-female, and (J) no social interaction groups. Data are represented as mean ± SEM. Low mag scale bar = 100 μm, high mag scale bar = 10 μm.

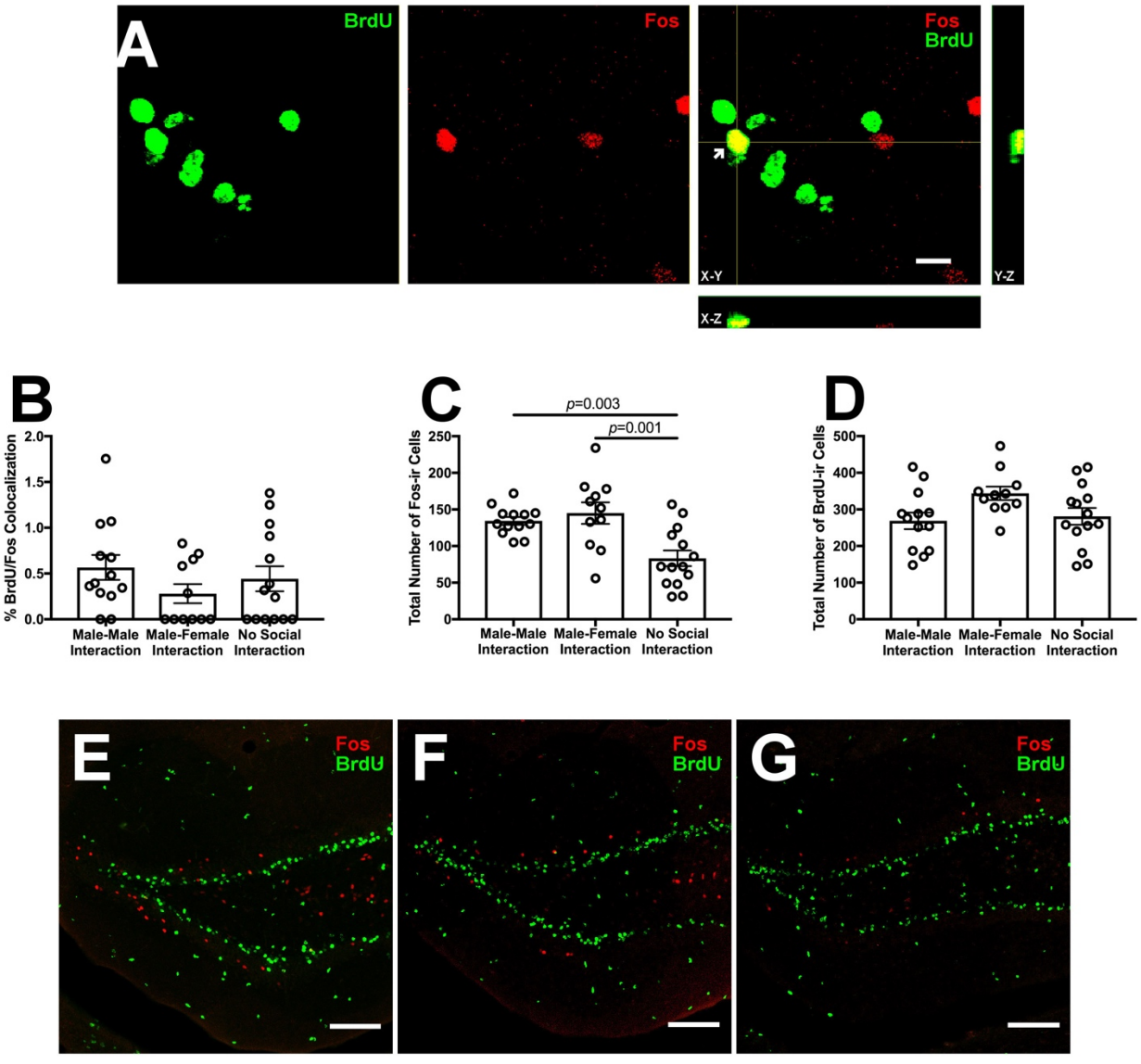
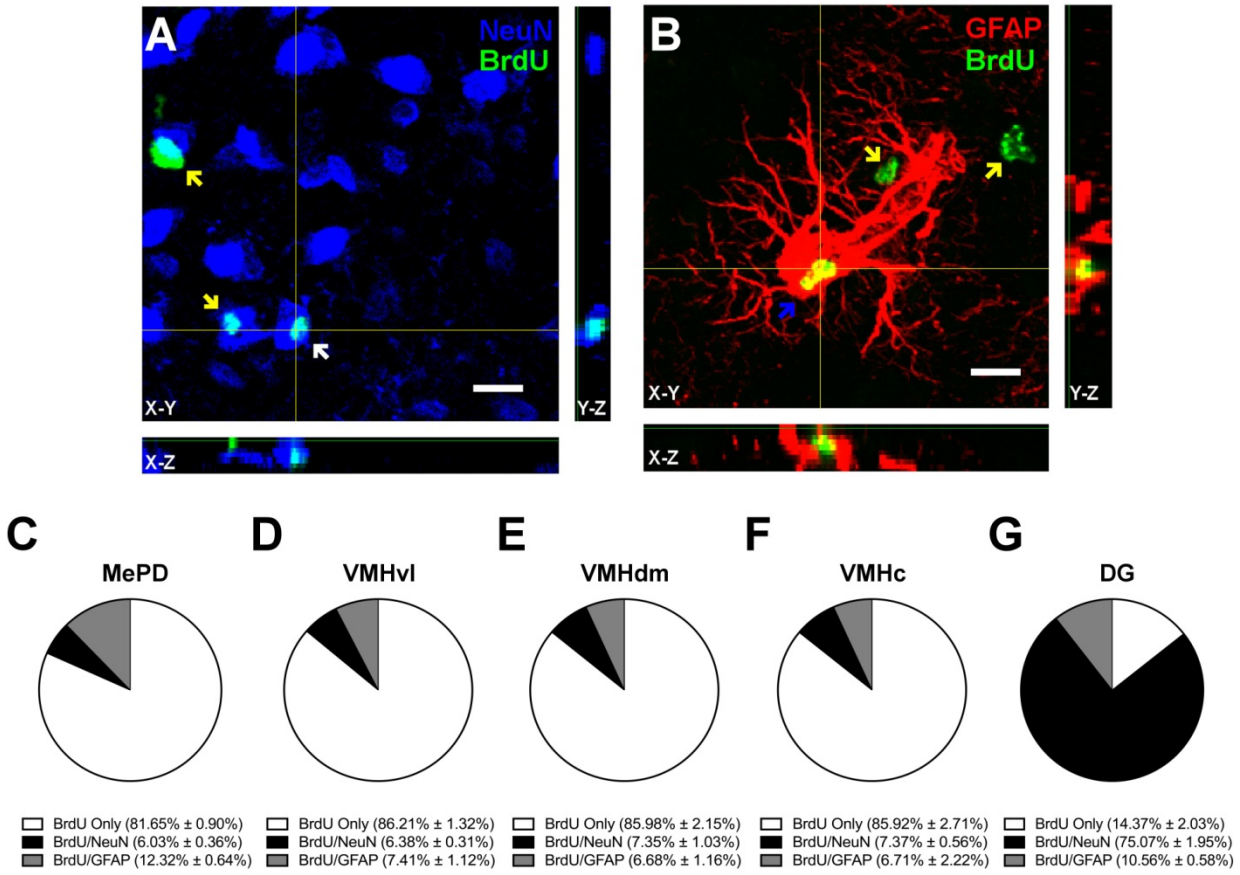


Figure 3.5. Pubertally born cells in the adult MePD, VMH, and DG differentiate into mature neurons and astrocytes. Representative photomicrographs of BrdU-ir cells in the adult male MePD colocalized with (A) NeuN, a mature neuron marker, and (B) GFAP, an astrocytic marker. Yellow arrows indicate cells that only express BrdU, white arrows indicate BrdU-ir cells that express NeuN, and blue arrows indicate BrdU-ir cells that express GFAP. Pie charts display the percent colocalization of all BrdU-ir cells with either NeuN-ir cells or GFAP-ir cells in the (C) MePD, (D) VMHvl, (E), VMHdm, (F) VMHc, and (G) DG. Data displayed show mean ± SEM.



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CONCLUSIONS

For several decades, the belief that the brain remained a static structure after the perinatal period dominated the field of neuroscience (Kaplan, 2001). Even though evidence of postnatal neurogenesis was first established in the 1960s (Altman et al., 1965), the controversial topic was fiercely debated until the 1980s, when Fernando Nottebohm made the groundbreaking discovery that new neurons were produced in the adult brain of canaries (Goldman et al., 1983). However, even with this enormous paradigm shift in our understanding of postnatal neurogenesis, skeptics touted this finding was unique to the avian species and not transferable to the mammalian brain (Rakic, 1985). This claim was, of course, debunked as several laboratories have demonstrated that new neurons are added to the adult brain in mice, rats, tree shrews, primates, and even humans (Cameron et al., 1993; Eriksson et al., 1998; Gould et al., 1997; Gould et al., 1999; Kuhn et al., 1996). Today, it is widely accepted that new neurons are added to the postnatal brain in the hippocampus and olfactory bulb; meanwhile, the idea that new neurons are added outside of these canonical neurogenic regions is still being disputed. The findings in this dissertation contribute to the body of work investigating postnatal neurogenesis and gliogenesis by 1) highlighting the potential mechanisms that regulate the addition of new neurons and glial cells in the postnatal amygdala and hypothalamus and 2) examining the functionality of these new cells.

Previous studies in adult rodents have found that several factors work together to influence the proliferation and survival of adult born neurons, which include environmental enrichment, stress and anxiety, physical exercise, and age(Kempermann

et al., 2002; Kempermann et al., 1997; Mirescu et al., 2006; Vivar et al., 2013; Westenbroek et al., 2004). It was discovered that gonadal hormones also participate in adult neurogenesis with androgens and estrogens influencing cell proliferation and cell survival in males and females(Fowler et al., 2003; Fowler et al., 2005; Hamson et al., 2013; Ormerod et al., 2003, 2004; Spritzer et al., 2007; Tanapat et al., 1999). More recently, a study demonstrated that gonadal hormones influence the addition of new cells to the pubertal brain as well (Ahmed et al., 2008). During puberty, a time when the brain is highly plastic (Ho et al., 2012; Saul et al., 2014; Staffend et al., 2014), individuals experience gonadal maturation leading to a rise in circulating gonadal hormones; this increase in gonadal hormone release (primarily testosterone in males and estrogen in females) contributes to structural and functional changes that help facilitate the emergence of adult sociosexual behaviors (Sisk et al., 2005). This is not surprising, as many brain regions known to regulate sociosexual behaviors, such as the posterodorsal medial amygdala (MePD) and ventromedial hypothalamus (VMH), are populated with androgen receptors (ARs) and estrogen receptors (ERs)(Simerly et al., 1990). A delicate balance between androgen and estrogen action in these brain regions is believed to control sex-specific behaviors in adulthood. Given this information, the overall aim of this dissertation was to examine the role of gonadal hormones on pubertal cytogenesis in the MePD and VMH, and determine whether these new born cells become functionally integrated into existing neural circuits that contribute to adult sociosexual behaviors.

In the initial study examining pubertal cytogenesis outside of the classic neurogenic zones, researchers discovered that there was a sex difference in the

addition of pubertally born cells to sexually dimorphic brain regions, such as the medial amygdala(Ahmed et al., 2008). The medial amygdala is a male-biased region (i.e., larger in males compared to females) which regulates social behaviors(Cooke et al., 2003; Morris et al., 2008a; Morris et al., 2008b). It was recently discovered that adult male rats had more pubertally born cells in the adult medial amygdala than females (Ahmed et al., 2008). The fact that this sex difference was hormone-dependent suggests that gonadal hormones may regulate the addition of new cells during puberty to establish or maintain sex differences seen in the adult medial amygdala. Thus, our first aim was to examine sex differences in pubertal cytogenesis in the MePD and ventrolateral subdivision of the VMH (VMHvl), both male-biased sexually dimorphic brain regions, using the mouse model. We found that adult male mice had significantly more pubertally born cells than females in the MePD and VMHvl, while no sex differences were observed in sexually monomorphic structures (i.e. dorsomedial and central subdivision of the VMH (VMHdm and VMHc, respectively) and dentate gyrus (DG)). Identifying sex differences in the MePD and VMHvl prompted us to further investigate whether testosterone, the primary male gonadal hormone, increase the number of pubertally born cells in these male-biased brain regions.

In the current dissertation, we concluded that testosterone acting via ARs increased the number of pubertally born cells in the MePD of adult male mice. In the adult MePD, adult testosterone alone did not eliminate sex differences in the number of pubertally born cells, but providing both pubertal and adult testosterone significantly increased the amount of pubertally born cells in males and females. In addition, we discovered that the presence of functional ARs in male mice was required to increase

the number of pubertally born cells. More in-depth analysis revealed that it was actually pubertal AR activity that promoted the increase in pubertally born cells in the adult male MePD. While we have determined pubertal testosterone acts via an AR-dependent mechanism to stimulate the proliferation and/or survival of pubertally born cells in the adult MePD, we are unable to exclude the contributions of ERs to increasing the number of pubertally born cells in the adult male MePD. Because blocking ARs in adulthood did not lead to a decrease in pubertally born cells, it is possible that adult testosterone is aromatized into estrogen and acts through ERs to promote the survival of pubertally born cells. A previous study in adult male rats revealed that ARs and ERs work together to masculinize the volume and neuronal soma size of the medial amygdala. Thus, testosterone may act on both ARs and ERs to promote the accumulation of pubertally born cells in the adult male MePD. A critical next step will be to block ER activity during puberty and/or adulthood in male mice, using an ER-antagonist, to examine the role of ERs on promoting the survival of pubertally born cells in the adult male MePD. As previously mentioned, pubertal and adult testosterone treatment also significantly increased the number of pubertally born cells in the adult female MePD. Yet, it remains unclear whether each metabolite of testosterone (i.e. estrogen and DHT, a potent androgen) individually contributes to the proliferation and/or survival of pubertally born cells in the MePD. Thus, future studies are needed to determine how gonadal hormones influence pubertal cytogenesis in the adult MePD of females by blocking ARs or ERs during puberty and/or adulthood. These results will further our understanding of how sex differences in the number of pubertally born cells are produced and maintained in the adult MePD.

In the VMHvl, adult testosterone increased the number of pubertally born cells in adult male and female mice, regardless of pubertal testosterone. Moreover, blocking pubertal and adult ARs did not reduce pubertally born cell density in the adult male VMHvl. Since adult testosterone treatment increased pubertally born cell density independent of AR activity, it is possible that testosterone acts via an ER-dependent mechanism to enhance the survival of pubertally born cells in the adult male VMHvl. However, since gonadally intact females are already exposed to high levels of estrogen at the onset of puberty, it seems unlikely that adult testosterone is acting through ERs to increase pubertally born cell density in the adult female VMHvl. On the other hand, it is possible that DHT, a metabolite of testosterone, acts via ARs in the adult VMHvl to promote the survival of pubertally born cells in females. This pattern of findings would suggest that gonadal hormones differentially effect the survival of pubertally born cells in the adult VMHvl between male and female mice. A previous study in adult female rats discovered that estrogen increased cell proliferation in the DG, however, in adult male meadow voles, treatment with estrogen did not enhance cell proliferation in the DG. Given these results, it is crucial to explore the differential effects of estrogen and DHT on pubertal cytogenesis in male and female mice. It should be noted that gonadal hormones prior to puberty may also organize the male and female brain leading to sex differences in the sensitivity to pubertal and/or adult hormones. Therefore, it will also be important to determine whether manipulating gonadal hormones during the perinatal period (e.g. gonadectomy vs. gonadally intact) affects hormone sensitivity in the brains of males and females during puberty and adulthood and leads to sex differences in pubertally born cells in the adult MePD and VMHvl.

In the current line of experiments, we established that gonadal hormones influence pubertal cytogenesis in the MePD and VMHvl. However, due to the experimental design used in these studies, we can only examine how gonadal hormones contribute to the survival of these pubertally born cells into adulthood. In this case, cell survival is defined as the total number of pubertally born cells present in the adult MePD and VMHvl at the time of sacrifice. Because all subjects were treated with BrdU during puberty and then sacrificed in adulthood, we are unable comment on how gonadal hormones affect other mechanisms that may contribute to generating sex differences in pubertally born cell number in adulthood such as cell proliferation, cell migration, cell death, and cell differentiation. Several studies have demonstrated that gonadal hormones can affect cell proliferation in brain regions like the DG and amygdala in adulthood. However, it is currently unknown how gonadal hormones influence cell proliferation in the pubertal brain. In order to parse out the differential role of gonadal hormones on cell proliferation and cell survival in the adult MePD, a time-course study must be conducted by administering BrdU during puberty and then examining the number of pubertally born cells after varying time points (e.g. 24 hours, 2 days, 7 days, 14 days, 21 days, and 56 days). An examination of pubertally born cell number more proximally following BrdU administration (30 minutes, 1 hour, 2 hours, 6 hours, 10 hours) may provide insight into how gonadal hormones influence cell migration. Although, the migratory pathway of pubertally born cells to brain regions such as the MePD and VMH are not known, recent studies have identified the third ventricle and circumventricular organs as possible neurogenic niches where progenitor cells may

proliferate and reside. Therefore, it is possible that pubertally born cells originate in these neurogenic niches rather than the subventricular zone or subgranular zone.

Gonadal hormones are also known to shape sex differences in the adult brain by mediating cell death. Within the anteroventral periventricular nucleus (AVPV), a female-biased brain region in which adult female rodents have a larger volume and more neurons than males, the sex difference is largely regulated by cell death through apoptotic testosterone signaling among males. Interestingly, by deleting genes that promote cell death (Bax gene), the sex difference in AVPV neuron number is eliminated in adult mice. It is therefore possible that sex differences in pubertally born cell number in the adult MePD and VMHvl are a result of increased cell death in females. Using transgenic mice to disable the Bax gene, future studies can administer BrdU during puberty and examine the number of pubertally born cells in the MePD and VMHvl to determine whether Bax-mediated cell death is required to establish sex differences in pubertally born cell number in adulthood. Understanding the mechanism by which sex differences in the number of pubertally born cells are established will help to provide more insight on how gonadal hormones work to shape the adolescent brain to promote sex differences in structure and function in adulthood.

Cell differentiation may also act to promote sex differences within the brain. We discovered that pubertal ARs enhance the number of pubertally born neurons in the adult male MePD, but not the VMHvl, while pubertal and adult ARs did not influence the number of pubertally born astrocytes in the MePD or VMHvl. Thus, in the adult male MePD, testosterone may act via ARs to promote the differentiation of pubertally born cells into a neuronal phenotype. However, it is also possible that blocking ARs during

puberty stimulates apoptosis of pubertally born neuron. A time-course study would be necessary to determine whether fewer pubertally born cells differentiate into neurons or more pubertally born neurons die as a result of blocking pubertal ARs. Currently, it is unclear how the presence of androgens works to increase the number of pubertally born neurons in the MePD. It is possible that androgens may act indirectly to enhance the genesis and/or survival of pubertally born neurons. For example, astrocytes in the MePD have been shown to express ARs (Johnson et al., 2012); therefore, androgen could act on ARs expressed in astrocytes to stimulate the release of neurotrophic factors that can contribute to the survival of pubertally born neurons in the MePD. However, if ARs are present on pubertally born cells themselves, it is possible that androgens may act directly on pubertally born cells to drive either their genesis and/or survival of pubertally born neurons that may go on to influence adult social behaviors. Thus, another critical next step will be to investigate if and when pubertally born cells in the MePD begin to express ARs. Furthermore, it was rather surprising to find that blocking ARs during puberty or adulthood did not affect the number of pubertally born astrocytes in the adult MePD, as a previous study found the addition of new astrocytes during puberty in male rats is dependent on the presence of functional ARs (Johnson et al., 2013). However, since we only evaluated astrocytes born during the first week of puberty, it is possible that the survival of astrocytes born during mid or later puberty depend on the functional androgen receptors, contributing to sex differences in astrocyte number in the adult MePD.

Lastly, in this dissertation, we discovered that a subset of pubertally born cells in the mouse MePD, VMHvl, and VMHdm are functionally incorporated into existing neural

circuits and participate in adult sociosexual behaviors. This is the second study to demonstrate that pubertally born cells outside of the canonical neurogenic zones become functionally integrated into the adult brain. A previous study in male hamsters found that a group of pubertally born cells in the amygdala and hypothalamus are activated in response to a sexual encounter (Mohr et al., 2013). In male mice, we discovered that social interaction with a male or female conspecific activated pubertally born cells in the MePD, while social interactions with a male activated pubertally born cells in the VMHvl and VMHdm. Although most pubertally born cells were not activated by a male or female social encounter in adult male mice, it is possible that these pubertally born cells may be activated by other behaviors other than male and female social interactions. Thus, future studies could examine the response of pubertally born cells to other stimuli, such as administering glucose or leptin to enhance the Fos in the VMH, or perhaps examining the Fos response in the MePD to anxiety-inducing tasks or a predator scent.

Another important next step will be to examine whether gonadal hormones actually play an indirect role in regulating pubertal cytogenesis. It is possible that pubertal testosterone elicits behaviors that, in turn, influence pubertal cytogenesis. For instance, studies have shown that male mice that are socially isolated have reduced neurogenesis in the hippocampus compared to males that are group housed despite the males being gonadally intact (Lieberwirth et al., 2012; Spritzer et al., 2011). Thus, social interaction may facilitate the preservation of these newly generated cells. A study also found that testosterone treatment increased self-grooming in male mice (Achiraman et al., 2014). In early development, high levels of maternal licking and grooming resulted in

increased neuronal survival in the hippocampus of offspring compared to those that received low levels of maternal care (Bredy et al., 2003). Therefore, it is possible that an increase in self-grooming during adulthood, as a result of testosterone treatment, may have a similar effect which leads to an increase in cell number in the brain. Follow-up investigations should examine how gonadal hormones control behaviors that may facilitate the changes in proliferation and/or survival of pubertally born cells in the brain.

In many mental health disorders, such as depression, disruption in social behaviors is a core symptom (Segrin, 2000). Interestingly, the prevalence of mental health disorders increases during puberty with females being twice more likely to develop these disorders compared to males (Paus et al., 2008). Therefore, another important future consideration will be to examine how cytogenesis in brain regions involved in regulating social behaviors differs between males and females in animal models of mental illness. In adulthood, decreased hippocampal neurogenesis has been observed in animal models of depression and anxiety (Hill et al., 2015; Snyder et al., 2011). Antidepressant drugs, such as fluoxetine, increase neurogenesis in the hippocampus, which may work to alleviate depressive symptoms (Klomp et al., 2014). Interestingly, a study found that fluoxetine affected adult male and female rodents differently, increasing cell proliferation in males, but not females (Hodes et al., 2009). However, these effects were not observed during puberty, as males and females treated with saline or fluoxetine both had high levels of cell proliferation (Hodes et al., 2009). These results suggest that antidepressant drugs have an age-specific and sex-specific effect. It is clear that antidepressant medications, one of the most popular treatment options utilized by clinicians today, may not be appropriate for all age groups

or sexes. Therefore, it is essential that we further our understanding of the aberrant neurological changes that can occur in both sexes during adolescence as well as adulthood in order to develop more effective therapeutic options to treat mental health disorders.

In conclusion, the current dissertation has provided evidence that pubertal testosterone acts via ARs to increase the number of pubertally born cells in the male MePD, but not VMHvl. Moreover, pubertal ARs are required to increase the number of pubertally born neurons, but not astrocytes in the adult male MePD. We have also confirmed that a subset of pubertally born cells in the MePD and VMHvl are activated in response to social interactions. While the current results contribute important insights on the role of gonadal hormones on pubertally born cells in the MePD and VMHvl, there are still many unanswered questions. It will be important for future projects to assess the unique contributions of new neurons and glia depending on a distinct pubertal period (i.e. early, mid, or late puberty). The development of the pubertal brain is a gradual process, and it is possible that new cells born during discrete pubertal time periods may have specific functions in the adult brain. In addition, recent studies have shown that sexual differentiation of the central nervous system involves both peripheral and central ARs (Swift-Gallant et al., 2012). Therefore, it will be important to distinguish the contributions of peripheral ARs from central ARs on promoting pubertal cytogenesis; and follow-up studies may aim to control the temporal and spatial specificity of AR expression in male mice by reducing AR activity starting at puberty and only in the nervous system.

This dissertation contributes evidence that the adolescent brain gains new neurons and astrocytes, and furthermore, cells born during puberty are activated by sociosexual behaviors in adulthood. In male mice, pubertal androgens play an integral role in enhancing the number of pubertally born neurons in the adult MePD. Based on the understanding that pubertal neurogenesis and gliogenesis in the amygdala and hypothalamus occur across at least three different species (i.e. rats, hamsters, and mice)(Ahmed et al., 2008; Mohr et al., 2013; Saul et al., 2015; Staffend et al., 2014), it is likely that this phenomenon exists in other species as well, including humans. Therefore, it is necessary to work toward a better understanding of the mechanisms regulating the proliferation and survival of pubertally born cells as we also begin to grasp the functional role of these cells in adolescent development. During puberty and adolescence, the prevalence of mental health disorders substantially increases (Paus et al., 2008), and research has shown that stress, anxiety, and depression are associated with decreased neurogenesis and gliogenesis (de Andrade et al., 2013; Hill et al., 2015; Kreisel et al., 2014; Rajkowska et al., 2007; Saaltink et al., 2014). A full understanding of atypical development is currently hampered by a dearth of knowledge about the neurological changes in the adolescent brain associated with healthy or typical social maturation. Thus, continued research on pubertal neurogenesis and gliogenesis will have profound ramifications for understanding healthy social development as well as identifying new targets for intervention when adolescent development goes awry.

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