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Regulation of male sexual and territorial behaviors by the androgen receptor

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**Regulation of male sexual and territorial behaviors by the androgen receptor**

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## Abstract

All vertebrates require testosterone for male sexual and territorial behaviors. Testosterone acts on numerous tissues throughout the body, including the brain and reproductive tract. Additionally, testosterone acts at multiple developmental stages. Early in development, testosterone masculinizes the brain; in adulthood testosterone permits these masculinized neural circuits to generate male-typical sexual and territorial behaviors. However, how testosterone acts in the brain at a cellular and molecular level to regulate the development and activation of circuits that control behavior remains unclear.

Surprisingly, estrogen is also necessary and sufficient for male behaviors in the mouse. All estrogen is derived from androgens such as testosterone, via the enzyme aromatase. Aromatase and estrogen receptors are expressed in brain regions known to control sexually dimorphic behavior, which suggests that the behavioral effects of testosterone may result from the conversion of testosterone to estrogen within the brain, and the subsequent activation of estrogen receptors. Indeed, treatment of female neonatal mice with estrogen is sufficient to masculinize mating and territorial behavior patterns. Estrogen signaling is also necessary for male-typical behaviors: male mice with deletions of the estrogen receptors or aromatase exhibit dramatic reductions in male mating and aggression. These results raise the possibility that testosterone may act simply as a

prohormone for estrogen in the brain, and that testosterone's cognate receptor, androgen receptor (AR), may be dispensable in the brain for male behaviors.

In my thesis, I present data demonstrating that AR is required for males to exhibit wildtype levels of mating and territorial behaviors, indicating that AR regulates activation of these behaviors. However, when the behaviors do occur, they appear qualitatively male, suggesting that the development of the neural circuits for male behaviors proceeds independent of AR. This finding is corroborated by a dearth of AR-expressing neurons in the developing brain during the time window critical for masculinization. Together with previous findings, these results suggest a model in which estrogen signaling controls the development of neural circuits for male sexual and territorial behaviors, while AR regulates the extent of these behaviors in adulthood.

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# CHAPTER 1

## Introduction

All sexually reproducing species display behaviors typical for each sex. These behaviors can be elicited in naïve animals, suggesting that the neural circuits mediating them are genetically hardwired. However, how the brain integrates information from its environment and its internal state to produce an appropriate behavior remains unclear. The innate behaviors of courtship, territory defense, and parental care are sexually dimorphic, controlled by processes during development or adulthood that are themselves sexually dimorphic. Those signaling pathways that control the sexual differentiation of the animal provide genetic entrées to dissect how the brain generates these complex social behaviors.

Sex determination utilizes diverse mechanisms across species. In some species, sex is determined by a chromosomal mechanism, and is invariant throughout the life of the animal (Manolakou et al., 2006). Other systems include environmental determination of sex, while others change sex in response to outside cues. In most eutherian mammals, including mice and humans, sex is determined by the Y chromosome. The early embryo contains bipotential gonads which may differentiate into either testes or ovaries. The *Sry* gene carried on the Y chromosome of males instructs the bipotential gonads to differentiate into testes (Sekido and Lovell-Badge, 2009). The testes of the developing animal produce testosterone, which in turn masculinizes target tissues, including the reproductive tract and the brain. Testosterone production is also high during post-pubertal life, which permits stereotyped mating behavior and intermale aggression in

adult males. In the absence of *Sry*, the bipotential gonad develops into an ovary, and there is little to no circulating testosterone. The resulting female produces estrogen and progesterone in adulthood, and displays sexual receptivity and maternal behaviors.

The effects of testosterone can be grouped into two main categories: those effects during development that control the differentiation of the brain ("organizational" effects), and acute effects in adulthood that controls the production of male-typical behaviors ("activational" effects) (Phoenix et al., 1959). In mice, the testes produce a surge of testosterone on the first postnatal day of life (Motelica-Heino et al., 1988). Testosterone levels rise rapidly after birth, and then return to a low baseline level within hours, and remain low until puberty. This perinatal testosterone drives sexual differentiation of the brain. Adult testosterone is required for the generation of male mating and aggression by the perinatally masculinized brain (Beeman, 1947; Champlin et al., 1963). An effect of testosterone during these two time windows has been been well established, however the cellular and molecular events underlying these hormonal effects, and how they contribute to behavior, remain unclear.

**Control of male behavior by testosterone**

Testosterone is required in the adult brain to elicit mating and aggression in male mice. After castration, adult male mice cease to mate or fight, but these behaviors can be rescued by treatment with testosterone (Beeman, 1947; Champlin et al., 1963). Control of male behavior by testosterone is not limited to adult life, however. The perinatal testosterone surge in mice corresponds to a time when this hormone is important for the

subsequent display of the full complement of male behavior in adulthood. Mice deprived of testosterone in the first postnatal hours of life by castration do not display male-typical levels of these behaviors, even when supplemented with testosterone at later time points (Motelica-Heino et al., 1993; Peters et al., 1972; Quadagno et al., 1975). In contrast, castration of mice after the testosterone surge has subsided does not prevent the adult display of these behaviors, provided testosterone is supplemented in adulthood. Treatment with testosterone during the perinatal period is sufficient to masculinize the brain. A study by Phoenix et al (1959) showed that female guinea pigs treated during development with testosterone exhibit male-typical mating in adulthood. This perinatal masculinization was later shown to be common to many vertebrates. Treatment of female mice with testosterone on the day of birth is sufficient to produce adult mice that fight at levels comparable to males, provided they receive testosterone in adulthood (Edwards, 1968; Edwards, 1969; Edwards and Burge, 1971a). These experiments show that there is a critical time window early in development during which testosterone masculinizes the brain and behavior, and that testosterone is required in adulthood to activate those masculinized neural circuits.

**Regulation of male behavior by the androgen receptor**

Testosterone mediates its effects through its cognate receptor, the androgen receptor (AR). Male mice bearing a nonfunctional allele of AR exhibit behavioral phenotypes reminiscent of castrates. The *testicular feminization* (*Tfm*) allele contains a spontaneous mutation in the coding sequence of AR which results in a premature stop codon (Charest et al., 1991), and an inability to bind testosterone (Goldstein and Wilson, 1972). Due to a

requirement for AR in the development of male genitalia, *Tfm* mice appear anatomically feminized. Additionally, these mice have atrophied testes and lack male accessory sex organs such as seminal vesicles and prostate gland (Goldstein and Wilson, 1972; Lyon and Hawkes, 1970). These findings have been replicated in mouse lines harboring engineered null mutations of AR (De Gendt et al., 2004; Holdcraft and Braun, 2004; Notini et al., 2005; Sato et al., 2004; Yeh et al., 2002).

Behavioral studies of mice with a null mutation in AR reveal a dramatic loss of male-typical behavior. AR mutant males do not mate with females, nor do they attack male intruders (Ohno et al., 1974; Olsen, 1992; Sato et al., 2004). These experiments demonstrate that AR is necessary for the masculinization of male-typical behaviors. However, AR mutant male mice do not exhibit female sexual behavior (Ohno et al., 1974; Sato et al., 2004). This indicates that sexual differentiation of male behavior involves two dissociable processes, masculinization and defeminization, and that AR regulates masculinization but not defeminization.

*Mechanism of AR gene regulation*

How does testosterone masculinize its target tissues on a cellular level? AR is a ligand-activated zinc-finger transcription factor that regulates the transcription of target genes (Chang et al., 1988; Lubahn et al., 1988). Testosterone binding to AR results in a conformational change, causing it to dissociate from a complex containing chaperone proteins. AR can then dimerize and bind to DNA sequences called androgen response elements. AR also recruits co-activators or repressors, which may enhance or inhibit the

transcription of nearby genes. Although the binding sites of AR have been mapped in a prostate cancer cell line (Wang et al., 2009), the identities of genes directly regulated by AR in the brain remain unknown.

In addition to its canonical role in controlling gene expression, AR has been suggested to activate intracellular signaling cascades in a transcription-independent manner. *In vitro* studies, primarily in non-neural cell lines, have shown AR-mediated signaling via PI-3K and MAPK, and regulation of ion channel permeability (Michels and Hoppe, 2008). The rapid effects of testosterone treatment on male-typical behavior suggests that a non-transcriptional mechanism of AR may play a role, however these effects have yet to be conclusively shown.

**Estrogen signaling controls male-typical behaviors**

In keeping with the requirement of AR for the function of the male reproductive tract, AR mutant males have very low levels of circulating testosterone (Goldstein and Wilson, 1972; Sato et al., 2004). As metabolites of testosterone such as estrogen are known to bind receptors in the brain, the reduction in male behaviors in AR mutant mice could be secondary to the reduced levels of testosterone in the brain. Indeed, in addition to its role in female behavior, estrogen signaling plays a critical role in male behaviors. Testosterone is converted to estrogen by the enzyme aromatase, which is expressed in the male brain (Lauber and Lichtensteiger, 1994; Naftolin et al., 1971; Wagner and Morrell, 1996; Wu et al., 2009). Estrogen has two cognate receptors, estrogen receptors alpha (ERα) and beta (ERβ), both of which are expressed in the brain (Shughrue et al., 1997;

Simerly et al., 1990). Estrogen signaling is necessary for the differentiation of male behavior, as male mice lacking both ERα and β do not exhibit mating or intermale aggression (Ogawa et al., 2000). A third potential estrogen receptor, Gpr30, has been proposed, but its function is unknown (Revankar et al., 2005). In complementary studies, male mice mutant for aromatase and unable to synthesize estrogen (Fisher et al., 1998) exhibit a severe reduction in mating and aggression (Honda et al., 1998; Toda et al., 2001a; Toda et al., 2001b). Estrogen is also sufficient to masculinize the male brain: treatment of female mice on the day of birth with estrogen results in male-typical aggression as adults (Baum et al., 1974; Simon et al., 1984; Wu et al., 2009). Similarly, males castrated as adults, then treated with estrogen continue to exhibit mating and aggression (Dalterio et al., 1979; Edwards and Burge, 1971b; Simon and Gandelman, 1978). Thus, estrogen is necessary and sufficient for the display of male behaviors.

These findings bolster the idea that testosterone may simply be a prohormone for estrogen in the brain (Naftolin and Ryan, 1975). Could all of testosterone's effects be mediated subsequent to aromatization into estrogen? Experiments using AR null mutant mice do not adequately address this question, as these mutants do not produce male-typical levels of testosterone, and consequently lack the substrate for estrogen production. In fact, treatment of AR null mutant mice with estrogen is sufficient to activate male mating and aggression (Olsen, 1992; Sato et al., 2004). However, estrogen treatment of either AR null males or adult castrates does not restore male-typical behavior to full wildtype levels (Finney and Erpino, 1976; Sato et al., 2004; Wallis and Luttge, 1975). This may result from various non-mutually-exclusive scenarios. First, AR may be

required solely in peripheral tissues such as the male reproductive tract for the full display of male behavior. The diminished behavior may result from the lack of male genitalia in AR null mutants. Second, AR may be required in the brain to drive full male levels of behavior, or AR may control specific aspects of male behavior. This may result from a requirement for AR in a subset of brain regions or at only specific developmental stages. Third, as estrogen rescue experiments provide this hormone at supraphysiological levels, the resulting male behavior may not be indicative of its effects under normal conditions. At physiological concentrations, estrogen and testosterone may each be required to masculinize the brain. These possibilities will be addressed using a genetic strategy in Chapter 3.

**Neural control of male mating and aggression**

Where do testosterone and estrogen act to control the generation of male-typical behavior? These hormones control how male and female brains process the same environmental cues differently, resulting in behavioral patterns that are divergent between sexes. The most parsimonious mechanism by which this can occur is that gonadal hormones bind their cognate receptors in the brain, resulting in gene transcription that affects the neuroanatomy or electrophysiological properties of these circuits. AR, ERα, and ERβ are expressed in discrete cell populations in the brain, as well as in peripheral tissues including the reproductive system. Within the brain, the highest concentrations of these receptors are found in brain regions implicated in sexually dimorphic behaviors by several lines of evidence. These limbic centers include the preoptic hypothalamus (POA), medial amygdala (MeA), bed nucleus of the stria

terminalis (BNST), ventromedial hypothalamus (VMH), lateral septum, and the premammillary hypothalamus. These brain regions are extensively interconnected with one another, and they are activated during mating and aggression (Newman, 1999). Many of these regions exhibit sexual dimorphism in anatomy or gene expression (Guillamon and Segovia, 1996), and lesions of any of these brain regions results in a deficit in one or more sexually dimorphic behaviors (Meisel and Sachs, 1994). The sensory stimuli that can elicit sexually dimorphic behaviors in any given species also drive neural activity these brain regions. The same brain regions control the output of sexually dimorphic behaviors across many vertebrate species, suggesting that the functions of these neural circuits are evolutionarily conserved. These brain regions are activated by social stimuli through various sensory modalities in a species-specific manner. For example, male birdsong activates neurons in the avian analogs of the VMH, MeA, and BNST of the female white-throated sparrow (Maney et al., 2008), while in terrestrial mammals such as mice, the bedding of female rodents activates regions including the POA, BNST, and MeA of males (Pfaus and Heeb, 1997). For mice, pheromonal cues are often critical releasers of social behaviors (Dulac and Torello, 2003). These findings in diverse vertebrate species suggest that a core network of steroid-sensitive brain regions controls the generation of sexually dimorphic behaviors. Although the sites of AR, ERα, and ERβ expression have been mapped, which of these brain regions control the dimorphic processing of information from sensory systems to generate sex-specific outputs remains unclear. Additionally, how steroid receptor signaling affects those brain regions to change how information is processed is beginning

to be elucidated. Two classes of sex differences could establish the sexual dimorphisms in behavior: in anatomy, in firing properties, or both, to be discussed below.

**Neuroanatomical correlates of sexually dimorphic behaviors**

*Sexual dimorphism in cell number*

Sexual dimorphisms are observed in various anatomical parameters in the brain. One of the most easily quantified sex differences is in the number of cells in a given brain region. The first such dimorphism described in the mammalian brain was in a subnucleus of the preoptic hypothalamus (Gorski et al., 1978). This region is larger in male than female rats, resulting from an increase in the total number of cells. The POA is critical for mating and aggression in numerous vertebrate species, suggesting that this sexual dimorphism may be functionally relevant. Sexual dimorphisms in cell number have been subsequently observed in various brain regions, usually in regions known to participate in sexually dimorphic behaviors such as the POA, BNST, and the anteroventral periventricular hypothalamus (AVPV) (Guillamon and Segovia, 1996; Simerly, 2002). In many of these cases, testosterone or estrogen has been shown to control the dimorphism. This suggests that during development or adulthood, gonadal hormones regulate genes that affect the rate of cell birth, apoptosis, or cell migration. Neurogenesis is largely complete by the time of the perinatal testosterone surge (al-Shamma and De Vries, 1996; Bayer, 1980; Bayer and Altman, 1987), thus differential cell birth is unlikely to contribute to the observed dimorphisms. A role for sex differences in cell migration is also not thought to contribute to the difference in cell numbers (Simerly, 2002), although a sex difference in motility of neurons in the POA prior to the testosterone surge has been

documented (Knoll et al., 2007). Regulation of apoptosis seems to be the most common method by which testosterone or estrogen effect sexual dimorphisms in cell number. Treatment of female mice with testosterone or estrogen is sufficient to masculinize the rate of apoptosis in various dimorphic brain regions (Arai et al., 1996; Davis et al., 1996; Holmes et al., 2009; Wu et al., 2009). To test whether apoptosis is necessary for the sexual dimorphisms, one can take advantage of a mouse mutant for the proapoptotic gene Bax. When programmed cell death is thus blocked, many of the observed dimorphisms in cell number are prevented (Forger, 2006). These studies suggest that sexual dimorphism in apoptosis, likely mediated by steroid hormones, is responsible for most sex differences in cell number in the nervous system. How a change in the number of neurons in a given brain region affects the output of neural circuits, however, remains a matter of conjecture (see Chapter 4).

*Sexual dimorphism in connectivity*

Information may be processed differentially by male and female brains due to differences in connectivity within or between brain regions. This could result from a number of cellular dimorphisms, including alterations in axonal targeting, dendritic arborization, or synaptogenesis. Alteration of each of these processes by gonadal hormone signaling has been observed. A classical sexual dimorphism is the organization of synapses onto the dendrites of the POA. Raisman and Field counted the number of synapses formed on the spine or the shaft of dendrites in the POA, from axons originating in the amygdala or elsewhere in the brain. They found in females, axons of non-amygdaloid origin synapse upon POA dendritic spines at a higher rate than in males (Raisman and Field, 1971). The

pattern of input to neurons may also be sexually dimorphic due to changes in the dendritic field. The size of the dendritic arbor in brain regions critical for sexually dimorphic behavior has been shown to differ by sex (Chen et al., 1990; Griffin and Flanagan-Cato, 2009; Hammer, 1984), and may be sensitive to testosterone (Rand and Breedlove, 1995). On a finer scale, individual dendrites may be sexually dimorphic as well. The number of dendritic spines is sexually dimorphic in the POA (Schwarz et al., 2008; Todd et al., 2007). Such dimorphisms in inputs to specific brain regions may contribute to sex differences in neural activity leading to sexual dimorphisms in behavior.

Axonal outgrowth may also be differentially regulated between sexes. Males extend more neurons from the BNST to the AVPV, and this dimorphism is regulated by testosterone (Ibanez et al., 2001). Thus, the outputs from a given neuronal pool may vary by sex, potentially altering information flow within the nervous system. These structural dimorphisms ultimately result from differences in gene expression patterns, which are likely to be direct or indirect targets of steroid receptor signaling.

**Control of neuronal activity by gonadal hormones**

*Sexual dimorphism in gene expression*

In addition to the structural dimorphisms described above, changes in gene expression may control the activity of neurons in the circuits regulating sexually dimorphic behavior. Numerous sexual dimorphisms have been observed in the expression pattern of specific genes. These genes are often necessary for the synthesis of ligands implicated in social behavior (Clarkson and Herbison, 2006; Simerly et al., 1985; van Leeuwen et al., 1985;

Wu et al., 2009), or receptors for signaling pathways that mediate such behaviors (Ikeda et al., 2003; Orikasa et al., 2002; Shah et al., 2004; Simerly et al., 1990). These sexual dimorphisms may regulate the development or activity of the neural circuits that control male-typical behaviors. In particular, the sexual dimorphism in AR and ER expression is consistent with the key role played by these hormones in male-typical behaviors. Although it remains unclear whether the dimorphism *per se* is important for these behaviors, these receptors are nevertheless critical components of this neural circuit.

*Sexual dimorphism in neuronal physiology*

Differential processing of information between male and female brains results in different behavioral output. This may result from dimorphisms in the physical structure of the circuits mediating sexually dimorphic behavior as described above, in the neural activity of these circuits, or both. Relatively little is known about sex differences in electrophysiological properties of the neurons. Kendrick and colleagues found that neurons that project via the stria terminalis, connecting the amygdala with the BNST and hypothalamus, exhibit a sexual dimorphism in the refractory period after stimulation (Kendrick and Drewett, 1979). Neurons in the male stria terminalis have a shorter refractory period after stimulation than do those in the female stria terminalis, a characteristic controlled by estrogen in adulthood (Kendrick and Drewett, 1980). This property may result in the increased firing of these neurons in males relative to females, perhaps regulating the display of male-typical behavior. This may be an emergent property of the circuit, resulting from one of the dimorphisms discussed above, such as differential synaptogenesis. Alternatively, it may result from cell-intrinsic differences in

the electrophysiological properties of the individual neurons in the stria terminalis. Genes that control the sexual dimorphisms in electrophysiological properties remain thus far elusive, although calbindin, nitric oxide synthase, and T-type calcium channels have been suggested as candidates. These genes are expressed in a sexually dimorphic pattern, under the control of testosterone, and presumably AR or estrogen signaling (Lephart, 1996; Martini et al., 2008; Watson et al., 1998; Zhang et al., 2009). Direct tests of the contribution to behavior of these putative steroid hormone target genes have yet to be performed.

**Experimental approach**

In my thesis work, I have sought to understand how male mating and territorial behaviors are generated. To approach this question, I have asked what role, if any, AR in the brain plays in the control of these behaviors. To disentangle the functions of AR and estrogen signaling, we require an approach that will delete AR in the neural circuits controlling behavior, while maintaining high circulating testosterone levels. To avoid the caveat that AR is required in the testes for production of testosterone (and hence estrogen), I generated mice with a nonfunctional AR in the brain, but normal masculinization of peripheral tissues. This approach has allowed us to develop a model by which testosterone masculinizes male pattern behavior via estrogen signaling, but testosterone signaling through AR in adulthood controls the levels of male mating and territorial behaviors.

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# CHAPTER 2

## A genetic approach to dissect sexually dimorphic behaviors

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**Abstract**

It has been known since antiquity that gender-specific behaviors are regulated by the gonads. We now know that testosterone is required for the appropriate display of male patterns of behavior. Estrogen and progesterone, on the other hand, are essential for female typical responses. Research from several groups also indicates that estrogen signaling is required for male typical behaviors. This finding raises the issue of the relative contribution of these two hormonal systems in the control of male typical behavioral displays. In this review we discuss the findings that led to these conclusions and suggest various genetic strategies that may be required to understand the relative roles of testosterone and estrogen signaling in the control of gender specific behavior.

## Introduction

All animals exhibit sex differences in behavior that are characteristic of the species. Such gender typical behaviors are often used to court mates, to defend territory and other resources, and to procure food for mates and offspring. These sexual dimorphisms in behavior are often innate and can be displayed without prior social experience or training, suggesting that the neural circuits that mediate these behaviors are developmentally hardwired in the brain. Nevertheless, the display of these behaviors is tightly regulated by external sensory cues as well as internal physiological regulators such as hormones. Such dual control ensures that animals engage in these behaviors only at the appropriate time and circumstance. While we have learned much about the sensory and hormonal control of sexually dimorphic behaviors (Morris et al, 2004; Arnold, 2004; Simerly, 2002; Scordalakes et al, 2002; Dulac and Torello, 2003; Axel, 1994), several major issues remain to be resolved. Recent advances in genetic engineering in mice should permit a sophisticated dissection of many of these issues. In this review we focus on how such advances will eventually permit a mechanistic understanding of the spatial and temporal requirements of androgen receptor signaling in male typical behaviors in mice.

Rodents exhibit sexual dimorphisms in many social behaviors. These include qualitative and quantitative differences in patterns of aggression, the scent marking of territory, courtship vocalizations, mating, sexual partner preference, and nursing (Goy and McEwan, 1980; Meisel and Sachs, 1994). Note that throughout this review we use the terms “sexually dimorphic behaviors,” “sex specific behaviors” and “sex typical behaviors” interchangeably. Testosterone appears to be necessary and sufficient for the

display of most, if not all, male typical behaviors in rodents (Goy and McEwan, 1980; Meisel and Sachs, 1994; Burns-Cusato et al, 2004; Gandelman, 1980; Beeman, 1947). The sensory and hormonal regulation of sexually dimorphic behaviors in various vertebrate species has been exhaustively reviewed elsewhere (Perkins and Roselli, 2007; Pfaus and Heeb, 1997; Kollack-Walker and Newman, 1995; Heeb and Yahr, 1996; Wade and Arnold, 2004), and we limit our discussion to the hormonal control of mating and aggression in male mice. Testosterone is required in a reversible manner in adult rodents for the display of male typical behaviors. Mice and other rodents castrated as adults rapidly lose male typical mating and aggressive patterns of behavior. These deficits, however, can be reversed by supplementation with testosterone (Table 2.1) (Wallis and Luttge, 1975; Luttge et al, 1974; Edwards and Burge, 1971a; Edwards, 1969; Beeman, 1947). This adult requirement is also referred to as the "activational role" of testosterone in male specific behavior (Phoenix et al, 1959). In addition to this activational role, testosterone also appears to be required perinatally in rodents for the subsequent expression of the full complement of male specific behaviors. For example, male mice castrated within a few hours of birth show significant deficits in mating and intermale aggression as adults (Quadagno et al, 1975; Motelica-Heino et al, 1993; Peters et al, 1972). Importantly, these deficits cannot be corrected in adult life with exogenous testosterone (but see also vom Saal et al, 1976). This irreversible effect of testosterone during early life is also referred to as the "organizational role" for testosterone in male behaviors (Phoenix et al, 1959). Testosterone provided perinatally and in adult life also elicits male typical patterns of mating and aggression in female rodents, suggesting that it

is sufficient to drive these behaviors (Edwards and Burge, 1971a; Edwards, 1968; Edwards, 1969).

Testosterone initiates organizational and activational changes in the brain by binding its cognate receptor, the androgen receptor (AR) (Chang et al, 1988; Lubahn et al, 1988). Additionally, testosterone is a prohormone, and it can be metabolized into dihydrotestosterone (DHT) or estrogen (Lephart, 1996; Imperato-McGinley and Zhu, 2002). DHT also binds and activates AR whereas estrogen binds distinct cognate receptors. Estrogen binds to estrogen receptor α (ERα), estrogen receptor β (ERβ), and the G-protein coupled receptor (GPCR), GPR30 (Walter et al, 1985; Kuiper et al, 1996; Revankar et al, 2005). AR and ERα and β are ligand-activated nuclear hormone receptors that modulate the transcription of target genes. By contrast GPR30 is a transmembrane receptor that binds estrogen and initiates heterotrimeric G-protein mediated signaling. While androgens can rapidly activate intracellular signaling cascades independent of direct transcriptional modulation by liganded AR, the functional relevance of these activities for male mating and aggression remains to be elucidated (Michels and Hoppe, 2007; Sun et al, 2006).

Each of the receptors for testosterone and estrogen is expressed in many brain regions, including those that have been implicated in the control of male typical mating and aggression (Shah et al, 2004; Merchenthaler et al, 2004; Simerly et al, 1990; Brailoiu et al, 2007; Lein et al, 2007; Meisel and Sachs, 1994). Such brain regions include the medial amygdala (MeA), the bed nucleus of the stria terminalis (BNST), and the medial

preoptic area of the hypothalamus (POA). The enzymes that catalyze the conversion of testosterone into DHT and estrogen are also found in the rodent brain, including in the regions mentioned above (Lauber and Lichtensteiger, 1994; Wagner and Morrell, 1996; Melcangi et al, 1998). This suggests a scenario in which testosterone may also act as a prohormone in the rodent brain (Naftolin et al, 1972). Indeed, estrogen signaling is also required for most male typical behaviors in mice. Adult castrated mice supplemented with estrogen show recovery of most components of male typical mating and fighting (Dalterio et al, 1979; Edwards and Burge, 1971b; Simon and Gandelman, 1978). However, such studies suggest that a complete recovery of male specific patterns of behavior is observed when the castrates are supplemented with both estrogen and DHT (Wallis and Luttge, 1975; Finney and Erpino, 1976). The genetic background is an important factor—in some strains DHT alone can rescue mating and aggression in castrate mice (Luttge et al, 1974; Luttge and Hall, 1973a; Luttge and Hall, 1973b; Burns-Cusato et al, 2004; Maxson et al, 1983). These classic hormone supplementation and deprivation paradigms have provided a strong demonstration of the critical role of androgen and estrogen signaling in male behaviors.

The genetic strategies we discuss in this review should shed additional light on the temporal and spatial requirements of hormone signaling in the control of rodent male behaviors. These two approaches, hormonal manipulations and gene targeting, often provide complementary information. For example, the loss of male mating after adult castration suggests that testosterone is required for this particular behavior. The resumption of male mating following testosterone replacement provides evidence that

testosterone is sufficient to initiate these displays. Such studies do not reveal whether testosterone signaling through AR is required for male sexual behaviors nor do they suggest which particular brain regions respond to testosterone to mediate male mating. As genetic deletion of AR also abolishes male mating routines, this approach strongly implicates testosterone signaling through AR in regulating sexual behavior. One limitation of this interpretation is that AR may have a ligand-independent role in modulating male mating. However, as both AR and its cognate ligand, testosterone, are required for male mating, the parsimonious explanation is that testosterone acts via AR to mediate male mating. In other words, a combination of hormonal manipulations and gene targeting often offers a more nuanced insight into the neuroendocrine control of behavior and other physiological processes. We discuss several examples of such a combined approach in the following sections. Note that as the genetic deletion of AR is constitutive this experiment does not reveal when and where AR function is required for male sexual behavior (see Nelson, 1997, for an extended discussion). In later sections, we discuss various genetic approaches that have been devised to generate deletions in a regionally or temporally restricted fashion in order to bypass this limitation.

**The role of estrogen signaling in male mating and aggression in mice**

Targeted deletion of aromatase, the enzyme that converts testosterone into estrogen, abrogates all estrogen production in the body (Fisher et al, 1998). Male mice null for aromatase have profound deficits in mating and aggression (Table 2.2) (Honda et al, 1998; Matsumoto et al, 2003; Toda et al, 2001a; Toda et al, 2001b). In a standard mating assay, these mutant males mount a female less frequently than wildtype males, exhibit

reductions in intromissions, and rarely ejaculate. Aromatase mutant males also exhibit a severe reduction in aggression towards wildtype males in standard resident-intruder assays. These observations directly implicate a role for estrogen synthesis in the control of male typical behaviors.

Adult males null for ERβ appear to exhibit wildtype levels of mating and aggression (Ogawa et al, 1999; Temple et al, 2003). Male mice homozygous null for ERα on the other hand do exhibit partial deficits in mounting, intromission, and ejaculation in standard tests of sexual behaviors (Ogawa et al, 1997; Wersinger et al, 1997; Ogawa et al 1998). Note however that these deficits stand in stark contrast to the abrogation of mating observed in adult castrates, suggesting that multiple hormonal mechanisms control male mating. Consistent with this notion, there is a complete loss of all male typical mating behavior in mice homozygous null for both ERα and β (Ogawa et al, 2000). This more profound mating deficit in the ERα and β double mutants suggests a functional redundancy between ERα and ERβ in the control of male sexual behavior. In contrast to this functional redundancy in the control of male mating behavior, intermale aggression appears to require only a functional ERα as ERα mutant males display minimal levels of intermale aggression, a phenotype that strongly resembles the deficits observed in castrates. Taken together, these observations suggest that estrogen signaling underlies the appropriate expression of many components of male mating and aggression.

Note that the mating deficits observed in males doubly mutant for ERα and β are

more severe than those in males lacking aromatase. What might account for the difference in phenotypes between males unable to synthesize estrogen and those unable to bind estrogen using nuclear hormone receptors? One possible explanation for this discrepancy is that the ERs have estrogen-independent activities that also control various components of male mating behavior. Alternately, the estrogen deficiency in aromatase null animals may be partially rescued by dietary estrogen. In any event, these genetic studies demonstrate that estrogen signaling via the nuclear hormone receptor type ERs is required for male specific patterns of mating and fighting.

**The role of androgen signaling in male mating and aggression in mice**

A functional AR is essential for masculinization of the external somatic phenotype and of sex typical behavioral displays in many species. Naturally occurring mutations in *AR* have been described in rats, mice, cattle, and humans (Bardin et al, 1970; Lyon and Hawkes, 1970; Short, 1967; Morris and Mahesh, 1963). In each instance, males bearing a null allele of *AR* have feminized external genitalia and other secondary sexual characteristics. Such mutants fail to exhibit male typical behaviors characteristic of the species, and in some cases even display feminized behaviors. Studies in human populations reveal a large spectrum of clinical presentations of men with mutations in *AR*, ranging from a mild feminization of the external phenotype to completely feminized patients who are karyotypically male (XY). This syndrome, referred to as the Androgen Insensitivity Syndrome (AIS) in the clinical literature, provides dramatic evidence of the influence exerted by gonadal steroid hormones on the development of gender typical physical and socio-sexual traits (McPhaul, 2002).

Mice also require an intact AR for the display of male typical patterns of mating and aggression (Ohno et al, 1974; Olsen, 1992). The *tfm* (*testicular feminization*) allele is a naturally occurring mutation which leads to a frameshift in the first exon of the mouse *AR* locus (Charest et al, 1991). This frameshift leads to a prematurely truncated protein, which lacks the DNA- and ligand-binding domains and is therefore likely to be non-functional. As in other species, *tfm* males have feminized external genitalia (Lyon and Hawkes, 1970). When presented with females in estrus, *tfm* males exhibit virtually no mounting or other consummatory aspects of male typical mating behavior (Ohno et al, 1974). However, this demasculinization is not accompanied by the feminization of sexual behavior. *Tfm* males do not display female typical sexual receptivity towards wildtype males (Ohno et al, 1974). This absence of sexual receptivity persists even when AR null mutants are castrated and primed with estrogen and progesterone, a hormonal regimen that induces sexual receptivity in ovariectomized female mice (Sato et al, 2004). This defeminization of sexual behavior is likely mediated by estrogen signaling through ERβ. Castrated ERβ null males primed with estrogen and progesterone show enhanced female typical receptive behavior compared with WT males, indicating that ERβ signaling is necessary for the defeminization of male behavior (Scordalakes et al, 2002). Therefore, the low-to-normal titers of testosterone observed in *tfm* males may provide sufficient substrate for the neural synthesis of estrogen, which likely activates ERβ to defeminize sexual behavior.

Unlike wildtype male residents, *tfm* male residents are not aggressive toward wildtype male intruders in the standard resident-intruder assay (Ohno et al, 1974). This deficit in male resident typical behavior also manifests in altered urine marking. Wildtype males appear to mark their home cage by depositing small quantities of urine all over the cage floor (Desjardins et al, 1973). By contrast, wildtype females and *tfm* males deposit their urine in large pools in a corner of the cage (N. Shah, unpublished observations). The loss of aggression observed in *tfm* males resembles the deficits in males null for ERα or ERα and β. ER mutant males have not been tested in urine-marking assays. Nevertheless these findings suggest a dual requirement for AR and ER signaling in the control of male typical territorial marking and defense.

So far we have described the behavioral deficits in mice null for ER or AR mediated signaling. Interestingly, AR also appears to be essential for generating cues that permit other conspecifics to recognize the animal as being male. *Tfm* intruder males do not elicit aggression from resident wildtype males (Ohno et al, 1974). By contrast, ERα null intruder males are recognized as males and are attacked in resident-intruder tests (Scordalakes and Rissman, 2004). Male mice castrated as adults also do not elicit aggression in such testing, consistent with a role for AR in generating male specific cues (Mugford and Nowell, 1970). Adult castrate males are attacked when their backs are swabbed with urine obtained from wildtype male mice, suggesting that AR may regulate the production of male typical pheromonal signatures (Maruniak et al, 1986).

**The relative contributions of AR and ER signaling to male mating and aggression**

Male mice homozygous null for ERα and β do not engage in male specific patterns of mating and fighting. Similarly, *tfm* males also do not mate or fight. This dual genetic requirement suggests that these two hormonal systems operate independently to regulate these male specific behaviors (Figure 2.1). Alternately, the estrogen and androgen signaling systems may act sequentially within the same pathway to regulate male mating and fighting. Note that the models shown in Figure 2.1 simply provide a genetic framework to understand the relative contributions of AR and ER in the control of male mating and fighting. These models do not reveal the cellular basis for the requirement for AR or ER in these behaviors.

The behavioral deficits in mice mutant for AR or ER do not demonstrate that these receptors directly activate mating or fighting by signaling within the neural circuits that mediate these behaviors. It is possible that the phenotype observed in these mutants arises from secondary effects of the deletion. For example, it is formally possible that a group of neurons that participates in the neural circuit that mediates male mating co-expresses both AR and ERα. In this hypothetical scenario, AR may regulate neuronal survival whereas ERα may modulate transcription of a set of genes whose products are required for the neurons to regulate male mating. In *tfm* males therefore, the abrogation of male sexual behavior could result from the loss of this particular group of neurons. In this instance, crossing the *tfm* allele into a mouse strain over-expressing an anti-apoptotic gene such as *Bcl-2* in these neurons should prevent their cell death, thereby "rescuing" the mating deficit (Zup et al, 2003; Forger et al, 2004). However, males null for ERα

will exhibit a mating deficit that cannot be rescued even in the presence of the transgenic Bcl-2. In this example therefore, AR would play a permissive role in mediating male mating as its function does not directly control the activity of the neural circuit for male sexual behavior (cf. differentiation of the spinal nucleus of the bulbocavernosus, Morris et al, 2004). By contrast, ERα signaling would be a pre-requisite for neuronal function during male mating, and consequently this receptor would play an instructive role in this process. In an alternate scenario, it is possible that both AR and ER play instructive roles in regulating mating behavior. For example, if ER signaling regulates AR signaling, and in turn AR regulates genes required for the neurons to participate in the neural circuit, then both AR and ER could be said to mediate mating in an instructive manner. In fact, estrogen signaling has been shown to regulate AR expression in the rat brain during development, consistent with the notion that disruption of ER signaling may mediate some male sex-typical behaviors by modulating AR (McAbee et al, 1999). Testosterone or AR have also been shown to regulate the expression of aromatase in several species (Balthazart and Foidart, 1993; Roselli et al, 1987; Veney et al, 2000). While such regulation remains to be demonstrated in the mouse brain, these studies suggest a sequential, instructive role for AR and ER signaling in the control of male behavior (Figure 2.1b).

The preceding discussion highlights the fact that much needs to be done to sort out the relative contributions of AR and ER signaling in male mating and aggression. Inducible genetic manipulations of AR and ERα and β should resolve many of these outstanding issues. As discussed below, gonadectomy followed by appropriate hormonal

supplementation also offers the possibility of revealing potential interactions between the two hormone systems. Such hormonal supplementation studies, which are analogous to inducible transgenic rescue experiments, provide additional insight into the role of gonadal hormones in organizing and activating sex specific behaviors.

Mating, as well as fighting, can be rescued in adult castrated AR null males by supplementation with estrogen (Olsen, 1992; Sato et al, 2004; Scordalakes and Rissman, 2004). It is striking that in males constitutively null for AR, adult administration of estrogen restores inter-male aggression to essentially wildtype levels. Does this mean that hormonal signaling is dispensable in neonatal life for adult displays of aggression? This remains to be tested, as males with a constitutive deletion of AR have normal levels of testosterone neonatally (Sato et al, 2004). In such males, testosterone levels subsequently decline due to atrophy of the testes later in life. In other words, neonatal aromatization of testosterone to estrogen may be required for permitting aggressive displays in the adult mutants. In contrast to the essentially complete rescue of intermale aggression, estrogen treatment of AR mutant males only partially rescues various consummatory components of male typical mating. This suggests that either the hormonal supplementation is inadequate or that there is a neonatal requirement for intact AR signaling for male mating behaviors.

Testosterone or DHT supplementation of adult castrated ERα null males partially rescues mating and aggression (Ogawa et al, 1998; Scordalakes and Rissman, 2003; Sato et al, 2004). This rescue by DHT suggests that the low levels of mating and aggression

observed in intact ERα null males are dependent on AR-mediated signaling. Given that mice mutant for both ERα and β exhibit significantly more profound behavioral deficits than those of the single mutants alone, it is also possible that the low levels of mating and aggression present in intact ERα null males are independent of AR signaling. Note that such androgen supplementation experiments have not been performed to date in adult males doubly homozygous null for ERα and β.

The observation that hormonal supplementation can rescue many, and in some cases most, deficits in mating and aggression in male mice bearing mutations in AR or ER provides insight into the mechanisms that underlie these behaviors. For example, the finding that estrogen administration to adult *tfm* males can rescue intermale aggression in these mutants immediately suggests that the neural pathways that mediate male fighting can differentiate in the absence of functional AR. It is also possible that the estrogen provided to these males activates an alternate neural pathway to regulate aggression, and that this pathway develops normally in the absence of AR function (Figure 2.1a). In both instances, the supplementation with estrogen may simply be required because of the testicular atrophy observed in *tfm* males, which would presumably lead to a decline in local, neural synthesis of estrogen. In an alternate scenario, *tfm* males may have a homeostatic, compensatory upregulation of ER expression or function in regions that can modulate intermale aggression. Such compensation could permit a functional rescue of fighting when the mutant male is supplemented with estrogen. Compensatory mechanisms have been observed in many other processes during development as well as in adult life in many tissues, including the brain (Davis, 2006; Nelson, 1997). For

example, skeletal muscle differentiation is under the control of two basic helix-loop-helix transcription factors, MyoD and Myf-5. Mice doubly null for MyoD and Myf-5 fail to form any skeletal muscle (Rudnicki et al, 1993). However, mice mutant only for MyoD exhibit essentially normal muscle differentiation (Rudnicki et al, 1992). Myf-5 is expressed at higher than wildtype levels in MyoD mutants, indicating that the loss of function of MyoD has been compensated for by the up-regulation of Myf-5 (Weintraub, 1993). The rescue of aggression in *tfm* males with estrogen administration could also reflect true redundancy in the hormonal mechanisms that regulate this behavior. In other words, both AR and ER could function redundantly to mediate male fighting. We wish to operationally define such redundancy as the existence *in vivo* of two or more mechanisms that subserve the same process in the wildtype state (Thomas, 1993). If these mechanisms are truly redundant, one should observe normal biological function without alteration of activity or expression of the individual components of one pathway when the other is rendered non-functional genetically. An example of such redundancy is demonstrated in glial scarring subsequent to neural injury. Astrocytes and other cells in the nervous system migrate towards an injury, proliferate, and clear debris to aid wound healing. Vimentin and glial fibrillary acidic protein (GFAP), the two major intermediate filament proteins of the astrocyte cytoskeleton, appear to be redundant for glial scar formation. Mice singly null for vimentin or GFAP appear to undergo normal wound healing after injury to neural tissue (Pekny et al, 1999). Importantly, neither GFAP nor vimentin appear to be upregulated in these single mutants, suggesting true redundancy rather than homeostatic compensation (Eliasson et al, 1999). By contrast, mice doubly mutant for vimentin and GFAP have defective glial scar formation, accompanied by

increased mortality subsequent to the injury (Pekny et al, 1999). Taken together, these observations suggest that vimentin and GFAP participate in glial scarring in a redundant manner. We should point out that mice singly mutant for vimentin or GFAP do have deficits in processes unrelated to glial scar formation (Shibuki et al, 1996; Terzi et al, 1997), providing a good example of redundant function for some but not all biological processes in which these two proteins participate. It is often difficult to distinguish true redundancy from homeostatic compensation, and the term "functional redundancy" could be used to describe the phenotype until the underlying mechanism is understood.

To summarize, the rescue of deficits in mating or fighting in AR and ER mutants with estrogen or testosterone, respectively, may result from one or a combination of several mechanisms. Identifying the mechanism which operates in vivo will require a molecular understanding of the target genes regulated by sex steroid receptors as well as the identification of specific neural circuits that regulate various routines in male typical mating and fighting. In the section that follows we suggest genetic strategies to understand the role of AR in restricted neuronal populations in regulating mating and aggression in male mice. These genetic strategies are also applicable to the estrogen receptors, and provide a powerful complementary approach to the hormonal manipulations discussed above.

**Dissecting the temporal and spatial roles of AR in male behavior**

As the findings discussed above illustrate, there are several outstanding issues that need to be resolved about the role of AR in regulating mating and aggression in the mouse. It

remains to be genetically demonstrated whether AR is required in the brain to mediate male typical behaviors. Alternately, AR may only function in non-neural, peripheral tissues such as the gonads, indirectly regulating behavior through its effects on the neuroendocrine axis. Thus it is possible that the behavioral deficits observed in *tfm* males result from a disruption of neuroendocrine regulation, rather than from a loss of AR function within the neural circuits that mediate mating and fighting. If AR is indeed required within the brain to control male specific behaviors, is the requirement purely developmental, adult, or both? And finally, which particular subclasses of neurons require AR to control male typical aggression and mating?

It has so far been difficult to distinguish homeostatic compensation for AR deficiency from redundancy in the requirement for either AR or ER mediated signaling in the regulation of male typical behaviors. Engineering an inducible deletion of AR in the adult brain would effectively bypass any developmental compensatory mechanisms. However, even this approach may not distinguish between redundancy and acute homeostatic mechanisms that may be activated in the face of adult AR deficiency. Additional insight into this issue may be afforded by experiments that determine whether AR or ERs function within the same sets of neurons to regulate fighting or mating. Clearly there is a pressing need for experimental manipulation of AR and ER function that can be performed with spatial and temporal precision. The Cre-loxP system (see Box 1) offers a genetically tractable approach to achieve such precise control of AR function. Indeed, over the past five years, several groups have generated different alleles of *AR* containing loxP-flanked ("floxed") exons (Figure 2.2) (De Gendt et al, 2005;

Holdcraft and Braun, 2004; Notini et al, 2005; Sato et al, 2004; Yeh et al, 2002). The exons that have been flanked encode the N-terminal activation domain or the DNA binding domain. Deletion of these exons, which occurs when Cre recombinase is provided in *trans*, leads to a loss-of-function allele of *AR*. Indeed, when mice bearing such a floxed *AR* allele are bred with mice expressing Cre recombinase under the control of an ubiquitous promoter, the male progeny bearing both the floxed *AR* allele and the transgenic Cre recombinase appear to recapitulate the *tfm* phenotype (De Gendt et al, 2005; Holdcraft and Braun, 2004; Notini et al, 2005; Sato et al, 2004; Yeh et al, 2002). In the section that follows, we present several genetic strategies utilizing the Cre/lox system that will permit the deletion of AR in a spatially and temporally controlled manner.

*Spatially restricted manipulation of AR function*

The *tfm* mouse lacks AR signaling in all tissues. However, AR is likely to have important roles in diverse tissues, including the gonads, the pituitary, and the nervous system. The behavioral deficits of AR null mice could in principle result from a disruption of AR signaling in the pituitary or from a lack of AR signaling in the brain. Using the Cre-lox system, brain-specific deletions of AR can be generated with the available Cre and floxed AR lines (Figure 2.3a) (Cinato et al, 2001; Goebbels et al, 2006; Korets-Smith et al, 2004; Tronche et al, 1999; Tsien et al, 1996; Zhu et al, 2001). Deficits in either mating or aggression in such mice would provide a convincing demonstration of a neural requirement of AR in these behavioral routines. If neural AR is indeed necessary for these behaviors, is it required in the neural circuits that regulate

the hypothalamic-pituitary-gonadal (HPG) axis, or in circuits that directly control male behaviors? These two (non-mutually exclusive) possibilities can be distinguished by profiling circulating hormone titers. Any dysfunction of the HPG axis could be bypassed by castration and supplementation with testosterone in order to test for additional deficits in the neural circuits that control aggression and mating.

*Temporally controlled manipulation of AR function*

Classical experiments have shown that hormonal signaling is required both developmentally and in adulthood for male typical behaviors. However, the relative contribution of AR versus ER signaling at various time points has been difficult to establish using traditional hormone supplementation experiments. The advent of temporally controlled Cre systems now provides a novel way to test the necessity of AR signaling at defined time points. In order to distinguish between the developmental and adult roles of AR in controlling male mating and aggression, one has to compare the behavioral consequences of constitutive deletion of AR in the brain to those resulting from the deletion of AR exclusively in the adult. The generation of ligand-activated Cre recombinase offers an elegant, general solution to this problem (Metzger et al, 1995). In the most widely used version, Cre recombinase is fused to the ligand-binding domain of a mutated version of human ERα ($Cre^{ERT2}$), which does not recognize endogenous estrogens, but binds tamoxifen with high affinity (Feil et al, 1997). The $Cre^{ERT2}$ fusion protein will only enter the nucleus upon administration of tamoxifen, allowing Cre to access loxP sites in the genome (Figure 2.3b). Cre needs only to be expressed transiently to recombine floxed targets, so tamoxifen can be administered for a short, defined period.

Alternative systems for the temporally restricted manipulation of Cre function are also available (Kellendonk et al, 1996; Gossen and Bujard, 2002). At least a few transgenic lines bearing a $Cre^{ERT2}$ allele under the control of a brain restricted promoter have been generated (Erdmann et al, 2007; Kuo et al, 2006), and the use of such strains should permit the inducible deletion of neural AR at defined time points.

If AR is required in the adult brain, it is likely that only a subset of the neurons that express AR control mating and aggression. AR is expressed in pools of neurons in diverse brain regions (Shah et al, 2004; Simerly et al, 1990). In order to define the behavioral role of AR in these subsets, it is necessary to delete AR function selectively within such candidate populations. One approach to achieve such regionally and temporally restricted deletion of AR is to stereotactically deliver Cre recombinase using a viral vector. Among the most promising viral vectors are those generated using either lentiviral or adeno-associated virus (AAV) backbones (Miyoshi et al, 1998; Burger et al, 2005). Both viruses are amenable to routine molecular biological manipulations and can be generated in high titers in the laboratory. These viruses can infect post-mitotic cell types, including neurons, and appear to be relatively non-toxic, allowing for long term survival and behavioral analysis of virally transduced animals. Several groups have demonstrated the feasibility of delivering Cre recombinase stereotactically to the adult mouse brain using either a lentiviral or an AAV preparation (Ahmed et al, 2004; Heldt et al, 2001; Thevenot et al, 2003; Kaspar et al, 2002; Pfeifer, 2001; Rajji et al, 2003; Scammell, 2003). As viral vectors are modular, they can be injected into different regions of the brain in adult mice bearing the floxed *AR* allele, bypassing the need for the

generation of multiple region-specific Cre transgenic lines. The use of virally delivered Cre recombinase will permit a facile analysis of how AR in specific brain regions may mediate male typical behaviors.

In this review we have attempted to synthesize the numerous observations on the roles of androgen and estrogen signaling in the control of male mating and fighting in mice. While we have discussed male typical behaviors in terms of mating and aggression, in fact each of these two behaviors consists of distinct subroutines. Additionally these behaviors have motivational and consummatory components (Wersinger and Rissman, 2000; Bodo and Rissman, 2007; Bakker et al, 2002), and for the most part we have limited our discussion to the consummatory aspects. Nevertheless, the Cre/lox reagents we describe will also be useful in understanding the control of each of these individual subroutines by androgen and estrogen signaling.

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**Box 1: Cre Recombinase**

The P1 bacteriophage gene *Cre* encodes Cre recombinase, which recognizes sequence-specific target sites in DNA referred to as loxP sites (Sauer, 1998). Each loxP element is a pseudopalindromic 34 base pair sequence. This asymmetry in the loxP site has practical consequences. The DNA target flanked by loxP sites ("floxed") in head to tail orientation will be deleted in the presence of Cre. By contrast, a DNA target flanked by two loxP sites in head to head orientation will be inverted. In the former instance, the deletion of the floxed target is accompanied by the loss of a loxP element, leaving behind a single loxP element as a residue of the recombination event. As the loxP element is 34 base pairs long, it occurs only infrequently in long stretches of genomic DNA. In fact, the sequenced mouse genome appears to contain no endogenous loxP sites. This means that one can safely flox DNA target sites in the mouse genome, as the provision of Cre in *trans* should lead to recombination only at that locus. Note that appropriate placement of loxP sites in the target locus should not interfere with normal gene function. To confirm that gene function has not been altered, mice carrying the floxed allele, but no Cre, must be analyzed as well.

Various versions of Cre are available, including one fused with a fluorescent reporter protein to enable visualization of the Cre fusion protein (Gagneten et al, 1997). In another variant, the activity of Cre recombinase is ligand inducible (see text). Finally, Cre belongs to a class of recombinase proteins which share the property of mediating recombination events at DNA targets flanked by unique recognition sequences. At least one of these, FLPe, is now widely used in place of Cre in the mouse (Branda and Dymecki, 2004). FLPe recognizes frt sites, which are distinct from the loxP sites

recognized by Cre recombinase. Consequently, it is now possible to design experiments using both Cre and FLPe to mediate deletions of distinct targets in the genome of the same animal.

**Figure Legends**

Figure 2.1: Models for the control of male typical behaviors by androgen and estrogen signaling

AR and ER may directly regulate male behavior through parallel (a) or sequential (b) pathways in the brain. (a) Androgen and estrogen signaling operate independently of one another to masculinize the brain and behavior. (b) AR and ER may function within the same circuits to masculinize behavior. In this scenario, AR and ER are postulated to interact epistatically, such that either one could function upstream of the other receptor.

**Figure 2.1**

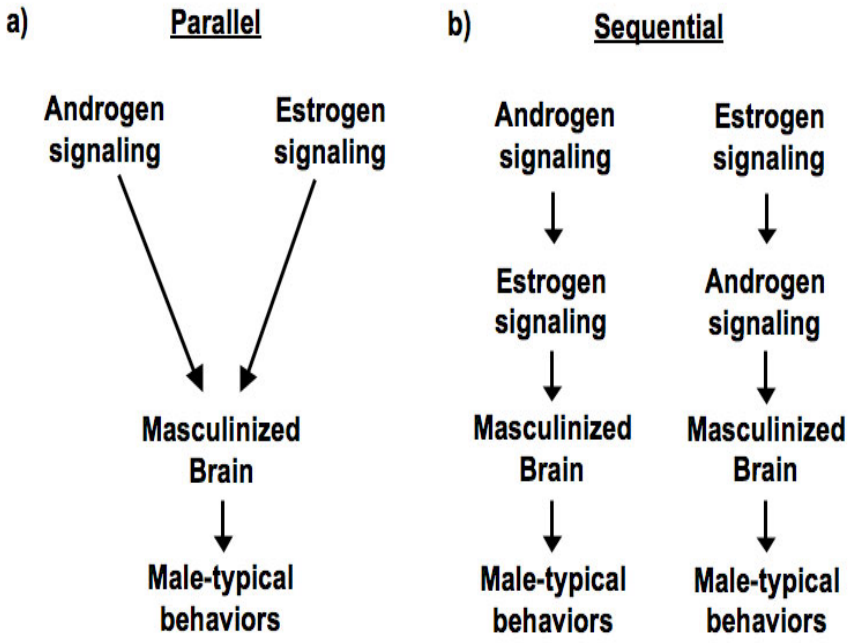
a)
Parallel
Androgen signaling
Estrogen signaling
Masculinized Brain
Male-typical behaviors
b)
Sequential
Androgen signaling
Estrogen signaling
Masculinized Brain
Male-typical behaviors
Estrogen signaling
Androgen signaling
Masculinized Brain
Male-typical behaviors

Figure 2.2: Summary of published loxP flanked *AR* alleles

a) This schematic depicts the various functional domains of AR, including the N-terminal transactivation domain (dark gray), the DNA binding domain (white), the hinge region (hatched), and the ligand binding domain (light gray). The various exons encoding each of the functional domains are shown using the same schema (b-e). Several groups have generated loxP flanked ("floxed") AR alleles. The exons that have been floxed include either those that encode the N-terminal transactivation domain (b: Sato et al, 2004; c: Holdcraft and Braun, 2004) or the DNA binding domain (d: Yeh et al, 2002 and DeGendt et al, 2005; e: Notini et al, 2005). Note that the configuration of loxP sites in (c) will result in the inversion of exon 1 rather than a deletion. The loxP sites are denoted as solid arrowheads.

**Figure 2.2**

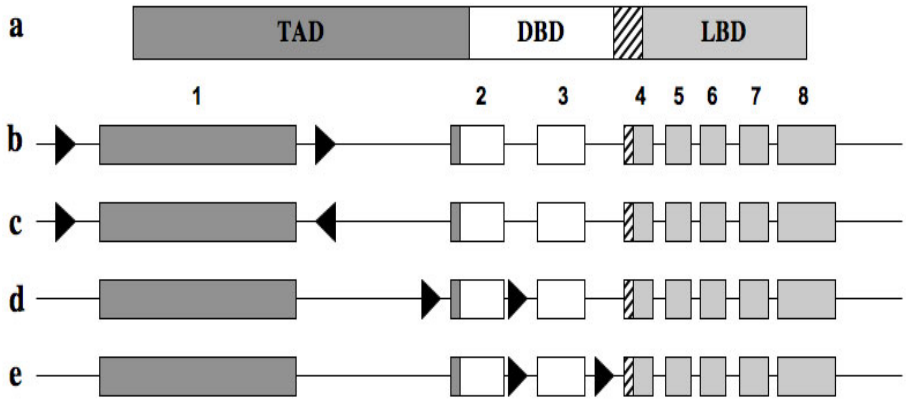

Figure 2.3: Spatial and temporal control of *AR* function with Cre recombinase

(a) To achieve tissue-specific recombination, the Cre transgene is placed under the control of tissue specific regulatory elements. As Cre expression is spatially restricted, excision of the floxed *AR* allele occurs only in defined regions, leaving behind a single loxP site. (polyA: polyadenylation sequence.)

(b) To obtain spatial and temporal control of recombination, the $CreER^{T2}$ fusion protein is expressed under the control of tissue specific regulatory elements. In the absence of tamoxifen, $CreER^{T2}$ is sequestered in the cytoplasm and cannot act on floxed *AR* alleles in the nucleus. Binding of tamoxifen induces $CreER^{T2}$ translocation to the nucleus where $CreER^{T2}$ is now free to recombine the floxed *AR* allele.

**Figure 2.3**

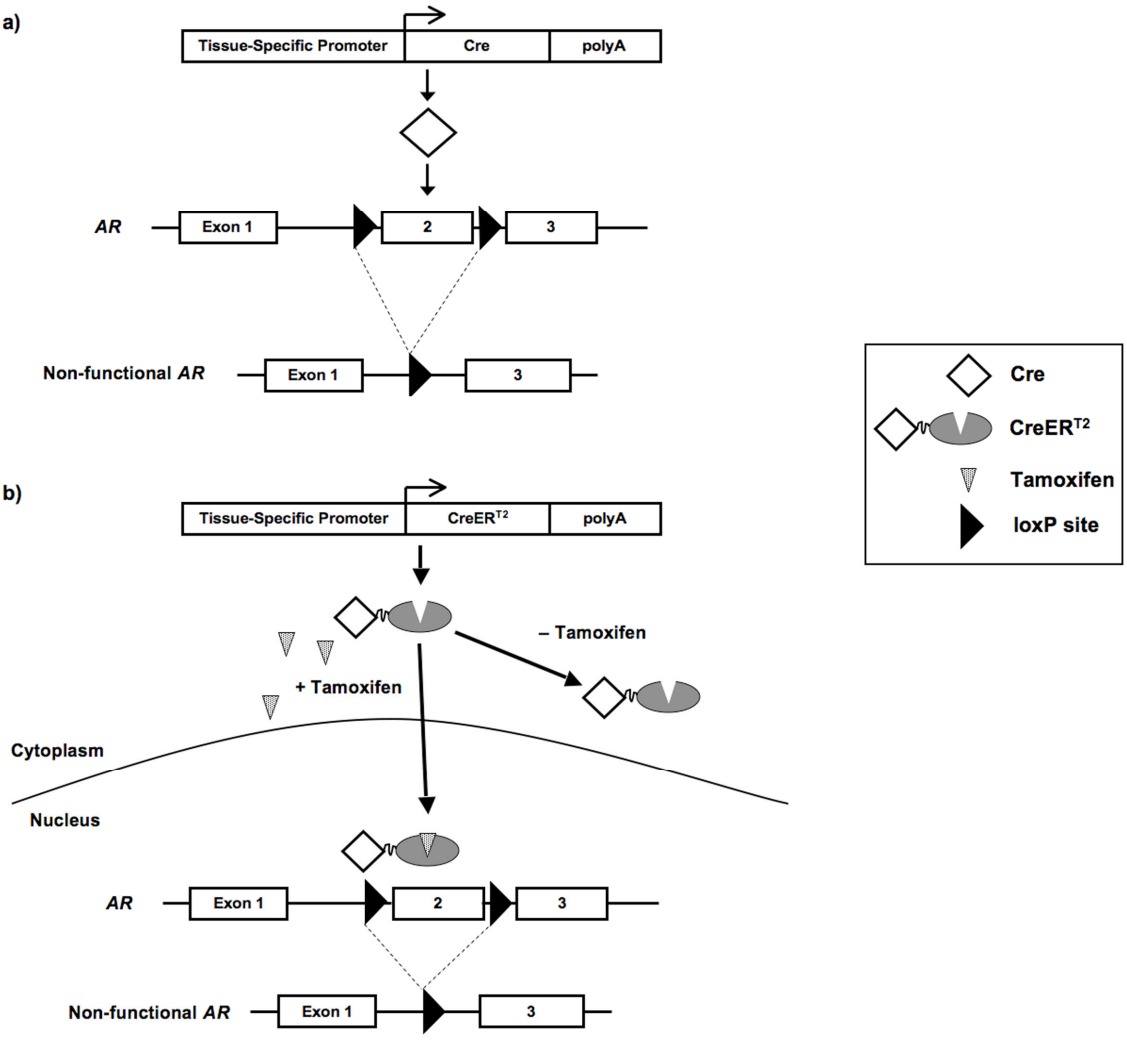
a)
Tissue-Specific Promoter
Cre
polyA
AR
Exon 1
2
3
Non-functional AR
Exon 1
3
b)
Tissue-Specific Promoter
CreER$^{T2}$
polyA
– Tamoxifen
+ Tamoxifen
Cytoplasm
Nucleus
AR
Exon 1
2
3
Non-functional AR
Exon 1
3
Cre
CreER$^{T2}$
Tamoxifen
loxP site

Table 2.1: Hormonal rescue of male mating and aggression deficits

* A different study reports no recovery of aggression with testosterone supplementation.

**Table 2.1**

| | Adult Castrate | | $AR^{-/Y}$ | | $ER\alpha^{-/-}$ | |
|---|---|---|---|---|---|---|
| | **Mating** | **Aggression** | **Mating** | **Aggression** | **Mating** | **Aggression** |
| **Testosterone** | full[a] | full[d] | partial[f] | not tested | partial[i,j] | none[i] |
| **DHT** | partial[b] | partial[e] | no[e,f] | partial[e] | partial[e,i] | partial[e] |
| **Estradiol** | partial[c] | full[e] | partial[e,f,g] | full[e,h] | none[e] | not tested |

a) Luttge et al, 1974
b) Wallis and Luttge, 1974
c) Edwards and Burge, 1971b
d) Edwards, 1968
e) Sato et al, 2004
f) Olsen, 1992
g) Bodo and Rissman, 2007
h) Scordlakes and Rissman, 2004
i) Ogawa et al, 1998
j) Scordlakes and Rissman, 2003

Table 2.2: Deficits in mating and aggression in male mice mutant for sex steroid receptors or aromatase

**Table 2.2**

| | AR$^{-/Y}$ | ERα$^{-/-}$ | ERβ$^{-/-}$ | ERαβ$^{-/-}$ | Aromatase$^{-/-}$ |
|---|---|---|---|---|---|
| **Mount Frequency** | none[a,b] | decreased[e,f,g] | unchanged[h] | none[i] | decreased[j,k] |
| **Intromission Frequency** | none[a,b] | decreased[e,f,g] | unchanged[h] | none[i] | decreased[j,k] |
| **Ejaculation Frequency** | none[a,b] | none[e,f,g] | unchanged[h] | none[i] | decreased[j,k] |
| **Aggression Towards Males** | none[a,b] | decreased[e,f,g] | unchanged[h] | decreased[i] | decreased[j,k] |
| **Attacked by Resident Males** | no[c,d] | yes[c] | not tested | not tested | not tested |

a) Olsen, 1992
b) Sato et al, 2004
c) Scordalakes and Rissman, 2004
d) Ohno and Geller, 1974
e) Ogawa et al ,1997
f) Ogawa et al, 1998
g) Wersinger et al, 1997
h) Ogawa et al ,1999
i) Ogawa et al, 2000
j) Honda et al, 1998
k) Matsumoto et al, 2003

# CHAPTER 3

## The androgen receptor governs the execution, but not programming, of male sexual and territorial behaviors

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**SUMMARY**

Testosterone and estrogen are essential for male behaviors in vertebrates. How these two signaling pathways interact to control masculinization of the brain and behavior remains to be established. Circulating testosterone activates the androgen receptor (AR) and also serves as the source of estrogen in the brain. We have used a genetic strategy to delete AR specifically in the mouse nervous system. This approach permits us to determine the function of AR in sexually dimorphic behaviors in males while maintaining circulating testosterone levels within the normal range. We find that AR mutant males exhibit masculine sexual and territorial displays, but they have striking deficits in specific components of these behaviors. Taken together with the surprisingly limited expression of AR in the developing brain, our findings indicate that testosterone acts as a precursor to estrogen to masculinize the brain and behavior, and signals via AR to control the levels of male behavioral displays.

## INTRODUCTION

All sexually reproducing animals exhibit gender dimorphisms in behaviors that are characteristic of the species. Such sex differences in behaviors can be observed in many displays, including in mating, territorial defense, and parental care. Gonadal steroid hormones play a critical role in the neural circuits that mediate sexually dimorphic behaviors: they organize the differentiation of these circuits in the developing animal, and activate these neural pathways to influence sex specific behaviors in the mature organism. Such an "organizational" effect is thought to lead to irreversible modifications in subsequent behavior, whereas the "activational" function of the hormones results in acute changes in the behavioral repertoire (Arnold et al., 2003; Goy and McEwen, 1980; Morris et al., 2004; Phoenix et al., 1959). Male typical patterns of behavior are controlled by both testosterone and estrogen in many vertebrates, including mammals. However, the relative contribution of these two hormone signaling pathways to the masculine differentiation of brain and behavior remains to be determined.

The requirement of estrogen for male behaviors appears counter-intuitive as this circulating ovarian hormone is essentially undetectable in the males of most species. Testosterone, or a related androgen, is an obligate precursor of estrogen, and circulating testosterone in males can be metabolized into estrogen in the brain by the enzyme aromatase (Balthazart and Ball, 1998; MacLusky and Naftolin, 1981; Naftolin and Ryan, 1975). It is this target derived estrogen that controls male behaviors, and male mice null for aromatase display profound deficits in male typical mating and aggression (Honda et al., 1998; Toda et al., 2001a; Toda et al., 2001b). The precursor-product relation between

testosterone and estrogen raises the possibility that the sole function of testosterone in the neural control of male behaviors is to serve as a circulating prohormone for estrogen (Figure 3.1A). Alternatively, testosterone may act not only as a precursor for estrogen, but it may also signal via AR in neurons to drive male behaviors (Figure 3.1B). Consistent with the latter scenario, male mice constitutively mutant for AR do not mate or fight, and pharmacological studies also indicate a role for this receptor in controlling these behaviors (Finney and Erpino, 1976; Ohno et al., 1974; Sato et al., 2004; Wallis and Luttge, 1975). Importantly, these studies do not necessarily distinguish between a peripheral versus a neural-specific function of AR in regulating male behaviors. Moreover, such pharmacological studies often utilized dihydrotestosterone (DHT) to examine AR function, as this steroid is a non-aromatizable androgen. Recent evidence indicates, however, that 3βAdiol, a DHT metabolite found in vivo, is an estrogenic steroid capable of signaling via nuclear estrogen receptors (Ishikawa et al., 2006; Pak et al., 2005; Sikora et al., 2009; Wahlgren et al., 2008; Weihua et al., 2001). This makes it difficult to unambiguously define a role for AR in controlling male behaviors using DHT. The use of constitutive AR mutants is also not definitive in defining a role for AR in male behaviors as these animals have low, often undetectable, levels of circulating testosterone (Sato et al., 2004) (SAJ, unpublished observations) resulting from postnatal testicular atrophy. Consequently, the behavioral deficits in constitutive AR mutant males could result solely from inadequate estrogen synthesis and signaling in the brain due to the low levels of circulating testosterone.

In contrast to the uncertainty regarding the role of AR in the neural circuits that control male mating and territoriality, the contribution of estrogen signaling to these behaviors is firmly established. Male mice doubly mutant for the nuclear estrogen receptors ERα, β display a complete abrogation of these masculine behaviors despite normal levels of circulating testosterone, indicating that this hormone cannot elicit male typical sexual and aggressive behaviors solely by signaling via AR (Dupont et al., 2000; Ogawa et al., 2000; Ogawa et al., 1997; Ohno et al., 1974; Sato et al., 2004; Wersinger et al., 1997) (MVW, unpublished observations). Both testosterone and estrogen, however, appear critical during development and adult life in males for the display of dimorphic behaviors such as inter-male aggression (Bakker et al., 2006; Finney and Erpino, 1976; Motelica-Heino et al., 1993; Wu et al., 2009). Consistent with the apparent non-redundant requirement for these hormones in male mating and fighting, their cognate receptors are expressed in overlapping, but not identical, sexually dimorphic patterns in neuronal populations critical for these behaviors (Perez et al., 2003; Shah et al., 2004; Simerly et al., 1990). Thus, AR and ERα, β are widely expressed in inter-connected limbic regions such as the medial amygdala, the bed nucleus of the stria terminalis, and the preoptic hypothalamus. Despite numerous studies documenting a role for testosterone in male specific patterning of gene expression and behavior, the extent to which this hormone signals through AR for masculinizing the brain and behavior remains unclear (Juntti et al., 2008).

Our findings indicate that AR is unlikely to play a major role in the differentiation of the neural circuits that control male typical behaviors. We find a

surprisingly sparse expression of AR in the developing brain in areas such as the bed nucleus of the stria terminalis and preoptic hypothalamus that are thought to be important for dimorphic behaviors. By comparison, the estrogen receptors and aromatase are expressed by many neurons in these regions at birth, and we show that estrogen signaling is necessary and sufficient for the sexual differentiation of AR within these populations. In addition, we find that male mice bearing a nervous system restricted deletion of AR exhibit a masculine repertoire of sexual and territorial behaviors with diminutions in specific components of these displays. Adult male mice mutant for AR in the brain have normal levels of circulating testosterone, and these behavioral deficits therefore reflect a requirement for AR in male behaviors rather than inadequate circulating testosterone for estrogen synthesis in the brain. Taken together, our results indicate that testosterone signaling via AR does not control masculine differentiation of the brain and behavior. Rather, AR signaling regulates the extent of male typical behavioral displays.

## RESULTS

### Sparse expression of AR in the developing brain

There is a male specific transient spike in serum testosterone at birth (postnatal day 1, P1) followed by a sharp drop in circulating titer within 36 hours, and these low baseline levels of testosterone persist until puberty. By contrast, the ovaries are quiescent during the neonatal period, and there is little circulating estrogen (or testosterone) in female pups (McCarthy, 2008; Motelica-Heino et al., 1988). The male specific surge of testosterone and its subsequent conversion to estrogen is thought to be critical for the sexual differentiation of the neural circuits that control many dimorphic behaviors (Motelica-

Heino et al., 1993; Peters et al., 1972; Phoenix et al., 1959; Whalen and Nadler, 1963; Wu et al., 2009). We wished to identify the neurons that respond to this critical testosterone surge via AR at birth. We analyzed AR expression in mice bearing a previously described knock-in AR allele (AR-IPIN allele) that permits faithful co-expression of the sensitive, genetically encoded reporter, nuclear β-galactosidase (βgal), in all cells that express AR (Shah et al., 2004). Previous work has implicated AR expressing regions such as the medial amygdala (MeA), posterior medial component of the medial subdivision of the bed nucleus of the stria terminalis (BNST), and the preoptic hypothalamus (POA) as being critical for the display of male typical mating, aggression, and territorial marking (Commins and Yahr, 1984; Kondo et al., 1998; Liu et al., 1997; Meisel and Sachs, 1994). Surprisingly, we only detected occasional, faintly AR positive cells in these regions at P1 (Figures 3.2A-C and 3.8D). There were >15-90 fold fewer AR expressing cells in these regions at birth (BNST 12.1 ± 5.7; MeA 16.4 ± 10.3; POA 59.8 ± 20.3; n = 6) than in older animals (Figure 3.3) (Shah et al., 2004; Wu et al., 2009). Similar results were obtained by directly immunolabeling for AR (data not shown). We could detect more AR positive cells in the MeA, BNST, and POA at P4, a timepoint by which the testosterone surge has already subsided (Figures 3.2D-F and 3.8E), but even at this age there appeared to be significantly fewer AR expressing cells than observed in adults. In contrast to this sparse and faint AR labeling in the BNST, POA, and MeA, AR expression could be reliably detected in a small pool of neurons in the vicinity of the arcuate (ArcN) and ventromedial (VMH) nuclei of the hypothalamus at P1 and P4 (Figure 3.8A, B). The expression pattern of AR in the BNST, POA, and MeA resolved

into widespread, intense labeling in these areas at P7, resembling the pattern observed in the adult brain (Figures 3.2G-L, 3.8, and data not shown).

The fetal testis produces testosterone from E13 (Crocoll et al., 1998) and we wondered if this hormone signaled via AR in the prenatal brain to masculinize neural pathways. We did not observe AR in the brain at E13.5, using βgal expression to visualize AR positive cells in mice bearing the AR-IPIN allele (Figure 3.9A-D). By contrast, at E15.5 and E17.5, we could visualize AR expression in the neurons near the ArcN and VMH but not in the BNST, POA, or MeA (Figure 3.9E-L). The ontogeny of AR expression in the BNST, POA, and MeA makes it unlikely that this hormone receptor plays a major, cell-autonomous role in masculinizing these neural pathways for male typical behaviors prenatally or at the time of the neonatal testosterone surge.

**Estrogen is necessary and sufficient for sexual differentiation of AR expression**

Previous work demonstrates that adult AR expression is sexually dimorphic such that there are more AR positive neurons in the BNST, POA, and the basal forebrain in males compared to females (Shah et al., 2004). AR expression in the BNST and POA in the P7 male resembles that observed in the adult male (Figure 3.2G-L) whereas we did not observe AR positive cells in the basal forebrain at P7 (data not shown). We asked whether the adult pattern of sexual dimorphism in AR expression was also apparent at P7 in the BNST and POA. Immunolabeling for βgal at P7 revealed significantly more AR positive cells in the male BNST and POA than in these regions in the female (Figure 3.3),

consistent with previous reports of sexual dimorphism in these regions (McAbee and DonCarlos, 1998; Shah et al., 2004).

The sexual dimorphism in AR expression is unlikely to arise from testosterone signaling via AR as this receptor is expressed in few cells in these regions at the time of the testosterone surge. Rather it is likely to result from the autonomous action of forebrain patterning genes or from estrogen signaling (Arnold et al., 2003; Hoch et al., 2009; Wu et al., 2009). Previous work indicates that both nuclear estrogen receptors (ERα, β) as well as aromatase are expressed in the early neonatal brain (Harada and Yamada, 1992; Wolfe et al., 2005; Wu et al., 2009). Indeed, we observed abundant expression of ERα, β and aromatase in the BNST and POA at P1 (Figure 3.4A-F), consistent with the notion that testosterone may masculinize AR expression after its conversion into estrogen.

If testosterone does masculinize AR expression in the BNST and POA subsequent to aromatization to estrogen, then either of these two hormones should be sufficient to drive male pattern differentiation of AR in these regions. Indeed, we find that administering testosterone or estrogen to P1 females masculinizes the number of AR positive cells in the P7 BNST and POA (Figure 3.4G-M), consistent with previous pharmacological studies in other vertebrates (Kim et al., 2004; McAbee and DonCarlos, 1999a, b). To determine whether the conversion of testosterone to estrogen is essential for the development of these sex differences in AR expression, we examined the BNST and POA of P7 aromatase$^{-/-}$ males (Honda et al., 1998). In these animals, the number of

AR positive cells is indistinguishable from that observed in control females, and significantly lower than in control males (Figure 3.4G, N, O). Taken together, these results show that estrogen controls the sexual differentiation of AR expression in the BNST and POA in males. We note that the few AR positive cells in the P1 BNST and POA may also respond to the testosterone surge at birth by inducing sexual differentiation of AR in neighboring cells that do not express this receptor; in such a scenario, both estrogen and testosterone signal via their cognate receptors to regulate the sex difference in AR expression in a redundant manner. Nevertheless, our findings demonstrate that estrogen is necessary and sufficient to drive masculinization of AR expression in these brain regions.

**Genetic deletion of AR in the nervous system**

Our results suggest that testosterone serves primarily as a precursor for estrogen during the neonatal period of sexual differentiation of the brain. Consequently, testosterone signaling via AR is unlikely to be essential for the differentiation of the male typical repertoire of dimorphic behaviors. Constitutive deletion of AR in all tissues results in feminization of the external genitalia, and eventual testicular atrophy, leading to a loss of circulating testosterone in adults (Lyon and Hawkes, 1970; Sato et al., 2004). To bypass the requirement for AR in the testes, we used a Cre-loxP strategy to engineer a deletion of AR specifically in the nervous system. We crossed mice bearing a previously described loxP-flanked allele of AR ($AR^{loxP}$) (De Gendt et al., 2004) to animals harboring the Nestin-Cre transgene (Nes-Cre) (Tronche et al., 1999) that drives Cre recombinase specifically in neural stem cells and glia (Figure 3.5A). This strategy should yield an

early, nervous system restricted deletion of AR in males carrying the X-linked $AR^{loxP}$ and the Nes-Cre transgenic alleles. Indeed, in contrast to the external phenotypes observed in constitutive AR mutant males, an examination of adult $AR^{loxP/Y}$; Nes-Cre ($AR^{NsDel}$) mice suggests normal AR function in non-neural tissues (Figure 3.5B-D, F): the genitalia of these mice are masculinized, and the testes and seminal vesicles, sensitive peripheral tissues responsive to AR signaling, are similar in weight to those of their control littermates (wildtype (WT), $AR^{loxP/Y}$, and Nes-Cre males). While there appears to be an elevation in circulating testosterone in $AR^{NsDel}$ mice, this is not statistically different when compared to the titers of this hormone in males of the control genotypes ($p = 0.157$, Kruskal-Wallis test for multiple group comparisons) (Figure 3.5E). The titers of circulating estrogen in these mutants are also comparable to those of the control males (Figure 3.5E). These findings suggest that testicular function is likely unimpaired in the absence of AR in the nervous system. Indeed, we find that $AR^{NsDel}$ mutants can sire litters when co-housed with wildtype females (data not shown), and that AR transcript levels in the testis as well as the pituitary are comparable between $AR^{NsDel}$ mice and their controls (Figure 3.5I, J).

Inadvertent deletion of AR in non-neural tissues such as muscle could result in a failure to thrive, a generalized motor deficit or muscular weakness. However, $AR^{NsDel}$ mice appear indistinguishable from their controls in body length and weight (Figure 3.5G). We also did not observe abnormal gait or gross motor deficits in our behavioral assays with these mutants (data not shown). In addition, $AR^{NsDel}$ males were similar to their control littermates in general motor activity and social interactions such as grooming

(Figure 3.10A-C). When assayed for motor performance on the rotarod, $AR^{NsDel}$ mice performed equivalently to control males (Figure 3.5H). In accord with these findings, quantitative RT-PCR (qPCR) also reveals comparable levels of AR mRNA in skeletal muscle obtained from $AR^{NsDel}$ and control males (Figure 3.5J).

In contrast to these findings in non-neural tissues, we observe a profound reduction in AR expression in the brain of adult $AR^{NsDel}$ males. Using qPCR, we find a large diminution in the levels of AR mRNA in various brain regions known to express this receptor (Shah et al., 2004; Simerly et al., 1990), including in the MeA, BNST, POA and other parts of the hypothalamus, olfactory bulbs, cingulate cortex, lateral septum, and the hippocampus (Figure 3.5J). Nes-Cre drives recombination in neural stem cells, and we therefore asked if the postnatal expression of AR was abolished in $AR^{NsDel}$ pups. We find a dramatic decrease in AR expression at P1 as well as P7 in these mutants compared to their control male littermates (Figure 3.5I). In agreement with these results, immunolabeling reveals very few AR positive cells in the forebrain of adult $AR^{NsDel}$ males compared to controls (Figure 3.5K-V). Taken together, our genetic strategy yields male mice that have intact peripheral masculinization and circulating testosterone and a deletion of AR that appears restricted to the nervous system. These mutants therefore afford the opportunity to assess the contribution of testosterone signaling via AR in the neural circuits that control male typical behaviors.

**AR increases the frequency of male sexual behavior**

In order to determine whether AR mediated signaling in the nervous system is essential for male sexual behavior, we examined the behavior of $AR^{NsDel}$ mice in mating assays. In mice, mating consists of a series of stereotyped routines that include mounting, intromission or penetration (as visualized by pelvic thrusting), and ejaculation. These behaviors can be reliably elicited in a 30 minute assay in which a WT estrous female is introduced into the cage of a singly housed WT male (Mandiyan et al., 2005; McGill, 1962). A statistically similar percent of $AR^{NsDel}$ residents (42%; n = 24 mice) mounted at least once when tested in 2-3 assays for sexual behavior with different estrous females when compared to control residents (Figure 3.11A). When we analyzed the percent of all assays with mounts, we observed mating in fewer assays with $AR^{NsDel}$ residents compared to males in the control cohort (Figure 3.6B). Notably, we find that some of the $AR^{NsDel}$ mice never mate, whereas when they do exhibit sexual behavior, these mutants mate in most assays (85% ± 6), similar to control residents (Figure 3.11G). The lowered probability of initiating mating across all assays was also reflected in the diminution of the number of mounts and intromissions as well as in the duration of intromissions exhibited by $AR^{NsDel}$ animals (Figure 3.6C, D). Male mice do not always achieve ejaculation within a 30 minute assay (McGill, 1962), and we observed ejaculation at a similar, low frequency in males of all genotypes (Figure 3.6B and 3.11A). The deficits in various parameters of sexual behavior in $AR^{NsDel}$ mice reflected an analysis of all assays, including those in which the resident did not initiate mating. We also examined these behavioral parameters by restricting our analysis to include only the assays in which

males mated. Strikingly, this analysis revealed that once $AR^{NsDel}$ mice initiate sexual behavior, they mate in a manner similar to their controls (Figure 3.6E, F and 3.11C-F).

Thus, while $AR^{NsDel}$ mutants are less likely to mate, once sexual behavior is initiated, its display appears similar to that of wildtype males. This suggests that AR controls the probability of triggering male mating but not the pattern of this complex behavioral routine. Alternatively, the lowered likelihood of initiating mating behavior could simply reflect a large variability in the extent of AR deletion in the brain. In this scenario, $AR^{NsDel}$ mice who do not mate may have little residual AR in the brain compared to the mutants who do initiate sexual behavior. Several lines of evidence favor the notion that AR regulates the probability of triggering male mating. First, qPCR analysis of AR deletion in individual $AR^{NsDel}$ males reveals a consistent, strong diminution of AR message in all mutants, regardless of their performance in mating assays (Figure 3.10D). Second, when $AR^{NsDel}$ mutants who did not mate in any of the three 30 minute mating assays were co-housed with females, they successfully sired litters (SAJ, preliminary observations). Finally, only a subset of constitutive AR mutant males attempted to mate when supplemented with testosterone (or estrogen) at doses that recapitulate wildtype circulating levels of this steroid hormone (Olsen, 1992; Sato et al., 2004) (MVW, unpublished observations). Taken together, these findings are consistent with the notion that AR functions in the brain to regulate the likelihood of initiating male sexual behavior.

Chemosensory cues emanating from females are essential for triggering male sexual behavior, and WT males engage in extensive anogenital chemoinvestigation of females prior to initiating sexual behavior (Keverne, 2004; Mandiyan et al., 2005; Yoon et al., 2005). The reduced frequency of male sexual behavior we observe with $AR^{NsDel}$ mice may be a consequence of deficits in such chemoinvestigation. However, we find that $AR^{NsDel}$ animals chemoinvestigate females in a manner comparable to their control male littermates (Figure 3.11B). Chemosensory processing of cues emanating from the two sexes leads to gender discrimination, one consequence of which is female-directed ultrasonic vocalizations by the resident male (Nyby et al., 1977; Pankevich et al., 2004; Stowers et al., 2002). We observe that $AR^{NsDel}$ mice vocalize to female, but not to male, intruders in their cage in a manner similar to their control counterparts, suggesting that sex discrimination is intact in these animals (Figure 3.6A). Taken together, these results suggest that the reduced frequency of male sexual behavior of $AR^{NsDel}$ mice is not a consequence of reduced chemoinvestigation of females or an inability to distinguish the sexes.

**AR controls the degree of male territorial behaviors**

We next tested $AR^{NsDel}$ animals in assays of male territorial behaviors. Singly housed WT male but not female resident mice attack male intruders in their homecage (resident-intruder aggression test) (Miczek et al., 2001). $AR^{NsDel}$ males were less aggressive than control residents by several measures. Although there was no statistical difference in the number of attacks or in the percent of tests with aggression (Figures 3.7A, 3.12A, B), the mutants spent significantly less time fighting compared to control residents, and this

deficit persisted even when we restricted our analysis to the assays in which we observed aggressive interactions (Figure 3.7B, C). The reduction in total duration of attacks cannot be explained by alterations in chemoinvestigation, the latency to first attack, or by the total attack number, as these parameters were statistically similar in all resident males (Figures 3.12B-E). Rather, our analysis revealed a deficit in the pattern of aggression following the first fight initiated by $AR^{NsDel}$ males (Figures 3.7D-F and 3.12F). Compared to the control residents, $AR^{NsDel}$ mice spent less time fighting with the intruder and exhibited a longer interval between successive attacks (Figure 3.7D, F). While $AR^{NsDel}$ residents and their controls attacked a similar number of times in an assay (Figure 3.12E), the mutants exhibited a lower attack rate (Figure 3.7E), initiating fewer attacks per unit time following the first fight. Unlike the attack number metric, a measurement of attack rate eliminates from analysis the variable latency to the first attack in any particular assay, and as such represents a corrected, perhaps more sensitive, measure of the frequency of fighting. As part of territorial behavior, resident WT male mice mark their territory by depositing many urine spots across the cage floor, whereas females pool their urine in one or a few large spots in a corner of the cage (Desjardins et al., 1973; Kimura and Hagiwara, 1985). Thus, there is a dimorphism in the number as well as the pattern of urine marks deposited by male and female mice. The male pattern of urine marking appears independent of AR function in the nervous system as $AR^{NsDel}$ residents also distribute their urine marks across the cage floor, similar to WT males (Figure 3.7H). By contrast, we find that $AR^{NsDel}$ residents deposit fewer urine marks compared to control resident males (Figure 3.7G). Importantly, $AR^{NsDel}$ males deposit more urine marks (10.2 ± 2.1 spots) than WT females who pool urine (1.7 ± 0.5 spots)

(Wu et al., 2009), suggesting that AR is not required to masculinize this parameter of urine marking, but rather AR enhances the display of this behavior. Taken together, these findings show that AR functions in the nervous system to control specific parameters of male typical urine marking and fighting.

## DISCUSSION

We find that male mice lacking AR in the nervous system can initiate masculine sexual and territorial displays. However, these mutants exhibit striking deficits in the pattern or the extent of these behaviors. Taken together, our findings demonstrate that AR is not essential for the masculinization of mating, aggression, and urine marking. Rather, AR signaling serves to amplify the display of this behavioral repertoire in males.

Our genetic strategy permits us to define a functional contribution of AR signaling in the neural circuits that mediate male behaviors. Using an approach identical to ours, a recent study also showed deficits in mating and fighting in males lacking AR in the nervous system (Raskin et al., 2009). Quantitative differences in the deficits reported in that study compared to our findings likely arise from differences in the experimental design. Here, we have significantly refined the analysis of $AR^{NsDel}$ mutants to provide mechanistic insight into the role of AR in masculinizing the brain and behavior. We have compared $AR^{NsDel}$ males to each of the control genotypes (WT, Nes-Cre, $AR^{loxP/Y}$) to assess the contributions of these distinct genetic backgrounds to the phenotype of the mutants. We have also developed analytical tools to examine in an extensive manner the behavioral deficits in sexual and territorial behaviors of $AR^{NsDel}$ males. We find that even

in the absence of AR function in the nervous system, males discriminate between the two sexes and initiate appropriate behavioral responses, mating with females and fighting with males. Our analysis of male mating reveals that AR regulates the probability of triggering sexual behavior but not the pattern of various components of male mating. Our analysis of territorial behaviors reveals a previously unreported role of AR in controlling the duration and pattern of inter-male aggression. We find that $AR^{NsDel}$ mutants mark their territory in a male pattern, but they deposit far fewer urine marks, indicating a deficit in this component of male territorial display. Our studies also indicate that it is unlikely that the masculine behaviors observed in AR mutants result from a failure to delete AR prior to the early neonatal sexual differentiation of the brain. Indeed, we find that there is minimal AR expression in regions known to be critical for dimorphic behaviors during this period, when gonadal hormones orchestrate sexual differentiation of the brain (Meisel and Sachs, 1994; Morris et al., 2004; Motelica-Heino et al., 1993), and this sparse neonatal AR expression is largely lost in $AR^{NsDel}$ males. Importantly, we demonstrate that the sexual differentiation of AR expression itself is controlled by estrogen signaling. The sparse perinatal expression of AR in the brain suggests that the behavioral phenotype of $AR^{NsDel}$ mice results from activational rather than organizational effects of testosterone acting on AR. We cannot exclude the possibility that AR also functions during the later postnatal period, including puberty, to influence the maturation of the neural circuits that drive male behaviors (Schulz et al., 2009). Regardless of the exact timepoint at which AR functions to control behaviors, our findings indicate that AR is not a master regulator for male behaviors, but rather, it serves as a gain control mechanism to regulate the extent of male sexual and territorial displays.

We find minimal AR expression in various brain regions of $AR^{NsDel}$ males, demonstrating that most neurons do not need to signal via this receptor to drive male behaviors. As we have deleted AR in the developing and adult animal, we cannot exclude the possibility that the masculine differentiation of behaviors in these mutants reflects compensatory mechanisms that are activated in the absence of AR signaling. Mice also exhibit a large array of behavioral dimorphisms, and it will be important in future studies to determine whether AR function in the nervous system is essential for the appropriate display of other male typical behaviors (Zuloaga et al., 2008a; Zuloaga et al., 2008b). Nevertheless, our study indicates that AR functions in the nervous system to control various parameters of male sexual and territorial behaviors, but it is not essential for the masculinization of this behavioral repertoire in mice.

**A model for hormonal control of male sexual and territorial behaviors**

Testosterone is essential for male behaviors. We set out to distinguish two competing models of testosterone's function in male behaviors: in one scenario, testosterone simply serves as a prohormone for estrogen in the brain, and it is estrogen signaling via its cognate receptors that masculinizes the brain and behavior (Figure 3.1A). Alternatively, testosterone may serve not only as a precursor for estrogen, but it may also signal via AR to control male behaviors (Figure 3.1B). We find that male mice mutant for AR in the nervous system do not exhibit male typical levels of mating and territorial behaviors. AR can regulate the activity and expression of aromatase (Roselli et al., 2009), and therefore might serve to amplify male behaviors by regulating the levels of local estrogen

synthesis. However, supplementation of constitutive AR mutants with estrogen does not restore mating and territorial displays to wildtype levels (Olsen, 1992; Sato et al., 2004; Scordalakes and Rissman, 2004). Such studies therefore suggest that AR in the brain may also control the expression of other genes that modulate the levels of male behavioral displays. Irrespective of the exact molecular mechanisms, our data demonstrate that testosterone signaling via AR is essential for wildtype male behavior.

The dual requirement for estrogen and testosterone in masculinizing the brain for sexual and territorial behaviors immediately poses the question of whether these two signaling pathways operate independently or via epistatic interactions. Mice of both sexes exhibit male mating behavior, whose display can be modulated by sensory as well as hormonal cues (Edwards and Burge, 1971; Jyotika et al., 2007; Kimchi et al., 2007; Martel and Baum, 2009). These findings suggest that the neural circuit for male mating is present in both sexes. Nevertheless, the neural control of some components of sexually dimorphic behaviors is thought to differentiate under the control of the perinatal testosterone surge (Arnold et al., 2003; Morris et al., 2004). The sparse expression of AR in the perinatal period, however, suggests that the masculinization of neural pathways in response to the testosterone surge at birth proceeds primarily under the control of estrogen.

We have recently demonstrated that the sexual differentiation of aromatase expressing neurons in the BNST and the MeA is independent of AR and is controlled by estrogen (Wu et al., 2009). Similarly, estrogen has been shown to regulate the dimorphic

expression of other genes in these regions as well as in the POA (Amateau and McCarthy, 2004; Scordalakes and Rissman, 2004; Simerly et al., 1997). It is difficult to completely exclude a function of AR in sexual differentiation of these limbic regions (Bodo and Rissman, 2008; Han and De Vries, 2003). However, our data constrains such a requirement to operate via a cell non-autonomous mechanism as AR is not expressed in the vast majority of cells in these areas at the time of the testosterone surge. By contrast, the perinatal expression of AR we observe in the vicinity of the VMH could potentially direct the previously described sexual differentiation of this nucleus (Dugger et al., 2007) in a cell autonomous manner. We show here that the masculinization of AR expression in the BNST and POA, two limbic regions previously implicated in sexual and territorial behaviors, is controlled by estrogen signaling. This postnatal sexual differentiation of AR is unlikely to result from estrogen regulated neurogenesis as neurons that populate these regions are born prenatally (al-Shamma and De Vries, 1996; Bayer, 1980; Bayer and Altman, 1987). Previous work has implicated dimorphic apoptosis as playing a critical role in the sexual differentiation of the BNST, POA, and other brain regions (Arai et al., 1996; Davis et al., 1996; Forger, 2009; Holmes et al., 2009; Waters and Simerly, 2009; Wu et al., 2009). It is therefore possible that the dimorphism in AR expression is a consequence of estrogen regulated cell survival. Estrogen may also control the dimorphism in AR expression by directly regulating the transcription of this gene via its nuclear hormone receptors. Regardless of the exact mechanism, our findings indicate that estrogen signaling drives the sexual differentiation of AR expression, and that it is also likely to control much of the perinatal masculinization of the brain.

The behavioral deficits of $AR^{NsDel}$ males are strikingly reminiscent of the behavioral phenotype of females treated with neonatal estrogen. As adults, such neonatally estrogen treated females respond to endogenous estrogen by exhibiting male patterns of mating and territorial displays at reduced levels compared to WT males (Wu et al., 2009) but similar to those observed in the $AR^{NsDel}$ males. Unlike $AR^{NsDel}$ males, however, these females do not have masculine levels of circulating testosterone, and depend on ovarian hormones to demonstrate male typical behaviors. Upon provision of exogenous testosterone in adult life, such neonatally estrogen treated females appear to mate, fight, and mark territory in a manner comparable to WT males. Taken together, these complementary findings suggest that testosterone signals via AR in the adult male to augment the male pattern behaviors that have differentiated under the control of estrogen signaling. Such a model is also consistent with the observation that testosterone signaling via AR is insufficient to elicit masculinized sexual or territorial behaviors in male mice doubly mutant for ERα, β. These diverse findings suggest a model for the control of male pattern behaviors in which estrogen masculinizes the neural circuits for mating, fighting and territory marking, and testosterone and estrogen signaling generates the male typical levels of these behaviors. It will be interesting in future studies to identify the molecular and circuit level mechanisms that are controlled by these hormones.

## EXPERIMENTAL PROCEDURES

### Animals

Mice were housed in a rodent barrier facility at UCSF with a 12 hour: 12 hour light-dark cycle. All experiments involving animals were performed in accordance with IACUC protocols at UCSF. The AR-IRES-PLAP-IRES-nlsLacZ (AR-IPIN) and aromatase knockout mice have been previously described (Honda et al., 1998; Shah et al., 2004). Animals bearing the $AR^{loxP}$ allele (De Gendt et al., 2004) or the Nes-Cre transgene (Tronche et al., 1999) were maintained on a mixed background (C57Bl/6J and 129/Sv). We mated females heterozygous for $AR^{loxP}$ to males hemizygous for the Nes-Cre transgene to generate males bearing both alleles ($AR^{loxP/Y}$; Nes-Cre) as well as the control males (WT, $AR^{loxP/Y}$, and Nes-Cre). Animals were group-housed by sex after weaning at 3 weeks of age.

### Behavioral assays

We used adult, singly housed male mice in behavioral assays, which were performed in the dark cycle. The behavioral testing was done as described previously (Wu et al., 2009). In brief, the male was first tested for territorial urine marking for 1 hour in a fresh cage, and then returned to the homecage. The males were subsequently tested for male sexual behavior for 30 min with an intruder female primed to be in estrus. Following the mating tests, the mice were tested for aggression for 15 min in the resident-intruder paradigm, using an adult male intruder who was group-housed with other intruders between testing sessions. The males were subsequently tested for ultrasonic vocalizations for 3 min in response to an intruder in their cage. Each resident was tested

for vocalization separately with a male and a female intruder. All animals were tested 2-3 times each in assays of sexual behavior and aggression, and once each for urine marking and ultrasonic vocalization. The experimental animals were always exposed to intruder mice they had not encountered previously, and each assay was separated by ≥ 2 days. All tests were scored by an experimenter blind to the genotype of the resident with a software package we have developed in Matlab.

**Histology**

We used age matched mice for all histological experiments. We visualized βgal activity at all ages in 20 μm thick sections using brightfield optics. Fluorescent immunolabeled sections (20 μm thickness, P7 and 65 μm thickness, adult) were imaged using a Nikon confocal microscope. The primary antisera used in this study are monoclonal rabbit anti-AR (1:750, Epitomics) and mouse anti-βgal (1:2500, Promega). The anti-AR antibody is specific to AR as we did not observe AR+ cells in various brain regions in the constitutive Tfm mutant males (Figure 3.10E-X). Staining for βgal activity and fluorescent immunolabeling were performed as described previously (Shah et al., 2004; Wu et al., 2009). Quantitation of cell numbers was performed using stereology and other experimental approaches (Supplementary Text).

Brain regions were identified based on landmarks as defined in standard atlases of the adult and developing mouse brain (Paxinos and Franklin, 2001; Paxinos et al., 2007). At P1 and P7, βgal expressing cells in the anterior hypothalamus and the BNST were found within the POA and the posterior medial component of the medial subdivision of

the BNST (also referred to as the principal nucleus of the BNST). Thus, the differences in cell number we observe between males and females and other experimental animals cannot simply be accounted for by changes in local distribution or cell density. All quantitation was performed by an investigator blind to sex, genotype, and hormone treatment. Similar results were obtained when the quantitation was done by a second investigator, who was also blinded to the same parameters.

**Statistical analysis**

To analyze categorical data, we used Fisher's exact test followed by a post-hoc Bonferroni's correction for multiple group comparisons. For all other comparisons, we first analyzed the distribution of the data with the Lillefors' goodness-of-fit test of normality. Datasets not violating this test were analyzed with parametric tests (Student's t-test for pairwise comparisons, and the one-way ANOVA for multiple group comparison), otherwise we used non-parametric analyses (Kolmogorov-Smirnov (KS) test for two group comparisons, and the Kruskal-Wallis test for multiple group comparison). We employed the Tukey's post hoc test subsequent to the one-way ANOVA and the Kruskal-Wallis tests to determine which groups differed significantly. We deemed an effect of the $AR^{loxP/Y}$; Nes-Cre genotype to be statistically significant only if this genotype differed from each of the 3 control cohorts (WT, $AR^{loxP/Y}$, and Nes-Cre).

**qPCR**

We collected testes, pituitary, the gastrocnemius muscle, and brain from $AR^{NsDel}$ mice and each of the control groups to quantitate the levels of AR mRNA. Adult and P7 brains

were dissected to obtain tissue from the olfactory bulb, septum, rostral hypothalamus (including the bed nucleus of the stria terminalis), caudal hypothalamus, cingulate cortex, hippocampus, and medial amygdala, using anatomical landmarks and stereo-coordinates of the mouse brain (Paxinos and Franklin, 2001; Paxinos et al., 2007). We defined the demarcation between the rostral and caudal hypothalamus as the coronal plane 0.6 mm caudal to bregma (adult), or 4.5 mm caudal to the anterior tip of the olfactory bulb (P7). P1 brains were dissected to obtain tissue from the hypothalamus. Each tissue and brain region for individual animals (P1, P7 or adult) was processed separately for RNA extraction (Qiagen RNeasy kit), oligo-dT primed cDNA synthesis (SuperScript III, Invitrogen), and qPCR. We used separate qPCR reactions to detect AR (5' primer in exon 1: GTGAAATGGGACCTTGGATG; 3' primer in exon 2: AGGTCTTCTGGGGTGGAAAG) as well as the ubiquitous ribosomal protein Rpl32 (5' primer: CGGTTATGGGAGCAACAAGAAAAC; 3' primer: GGACACATTGTGAGCAATCTCAGC) that was used for normalization of AR expression. As AR expression was similar across the three control genotypes but was significantly different from $AR^{NsDel}$ mice in all brain regions, the normalized AR mRNA levels from these animals were combined and compared to those of $AR^{NsDel}$ males. For visualization purposes, this data is presented in Figures 3.5(I, J) and 3.10D as the percent of AR mRNA in $AR^{NsDel}$ males in various tissues compared to the control cohort.

**Hormones**

Serum testosterone and estradiol titers were determined with kits from DRG International and Cayman Chemicals, respectively. We induced estrus in adult, ovariectomized 8-24

week old mice with sequential daily injections of 10 μg and 5 μg of 17β-estradiol benzoate (Sigma), and 50 μg of progesterone (Sigma) dissolved in 50-100 μL sesame oil (Sigma) (Beach, 1976). The females were used for sexual behavior 4-6 hours after progesterone injection. Females were allowed to recover for ≥ 1 week between assays. For hormonal manipulation of neonates, females were treated on the day of birth (P1) with a single 50 μL subcutaneous injection of hormone or vehicle. We injected either 100 μg testosterone propionate (Sigma) or 5 μg of estradiol benzoate dissolved in sesame oil.

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## FIGURE LEGENDS

**Figure 3.1: Models for the role of testosterone in masculinizing the brain and behavior**

Schematics illustrating possible mechanisms whereby testosterone controls male typical behaviors.

(A) Testosterone simply acts as a circulating prohormone for estrogen synthesis via the action of aromatase in the brain. In such a scenario, it is this locally derived estrogen that regulates sexual differentiation of the brain and behavior in males.

(B) Alternately, testosterone not only acts as a prohormone for estrogen, but it also activates its cognate receptor, the androgen receptor (AR), to influence directly the neural circuits that control male behaviors.

**Figure 3.1**

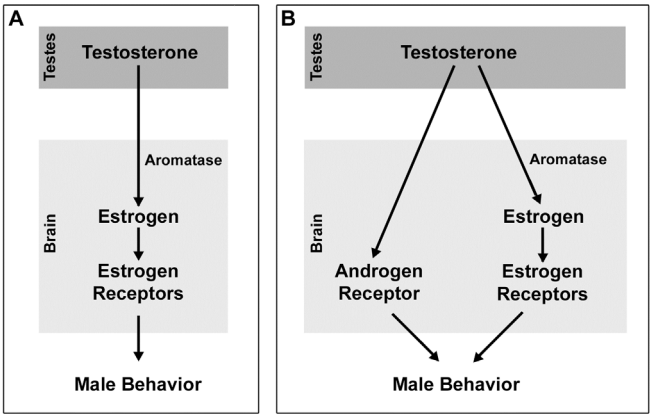

A
Testes
Testosterone
Aromatase
Brain
Estrogen
Estrogen
Receptors
Male Behavior
B
Testes
Testosterone
Aromatase
Brain
Estrogen
Androgen
Receptor
Estrogen
Receptors
Male Behavior

**Figure 3.2: Limited expression of AR in the newborn brain**

(A-L) Representative coronal sections through the brain of male P1 (postnatal day 1), P4, P7 and adult mice bearing the AR-IPIN allele stained for βgal activity. There are few AR positive cells in the BNST or POA at P1 (arrowhead, C). There are more AR expressing cells at P4 in these areas, and by P7 the number of AR positive cells in the BNST and POA approximates that observed in the adult brain. Scale bar equals 500 μm (top row), and 100 μm (bottom two rows).

(M-O) Nissl stained sections depicting the location of the BNST (M, N) and the POA (M, O).

$n \geq 3$ at P1, P4, P7.

**Figure 3.2**

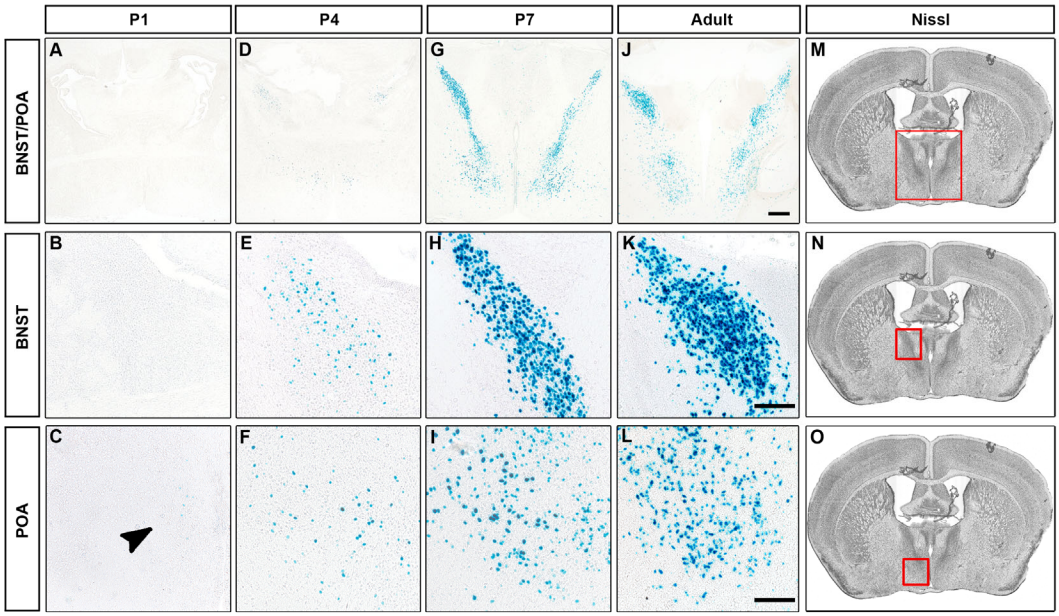


**Figure 3.3: Sexual dimorphism in AR expression**

(A-D) Coronal sections through the BNST and POA of P7 male and female mice bearing the AR-IPIN allele immunolabeled for βgal.

(E) There are more AR expressing cells in males compared to females in the BNST and POA.

Mean and standard error of the mean (SEM) are shown; n = 4 for each genotype, * $p \leq 0.005$. Scale bar equals 100 μm.

**Figure 3.3**

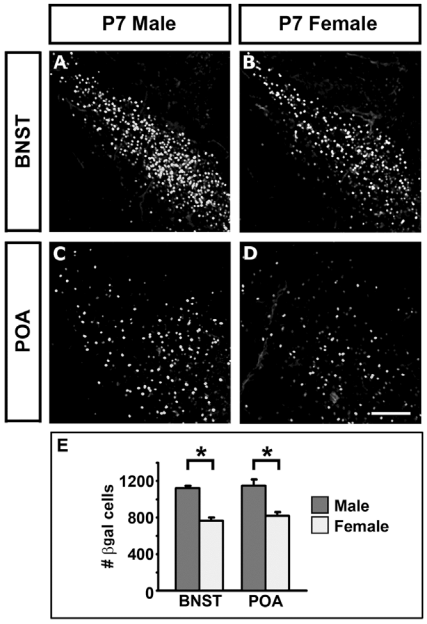

P7 Male
P7 Female
A
B
BNST
C
D
POA
E
*
*
# βgal cells
1200
800
400
0
BNST
POA
Male
Female

**Figure 3.4: Estrogen masculinizes AR expression**

(A-F) Representative coronal sections through the BNST and POA of P1 males processed for in situ hybridization to label ERα, ERβ, and aromatase mRNA.

(G-O) There are more $\beta gal^{+}$ cells in the BNST and POA of P7 females bearing the AR-IPIN allele administered testosterone (TP female) or estrogen (EB female) at P1 compared with age matched control females administered sesame oil vehicle at P1 (SO female) and $aromatase^{-/-}$ (AroKO) males. Horizontal dashed lines represent the mean value in WT males as obtained from the data in Figure 3.3. Mean ± SEM; n = 4 for each group of mice, * $p \leq 0.01$ by Tukey's posthoc test subsequent to a one-way ANOVA. Scale bar equals 200 μm (A-F) and 100 μm (H-O).

**Figure 3.4**

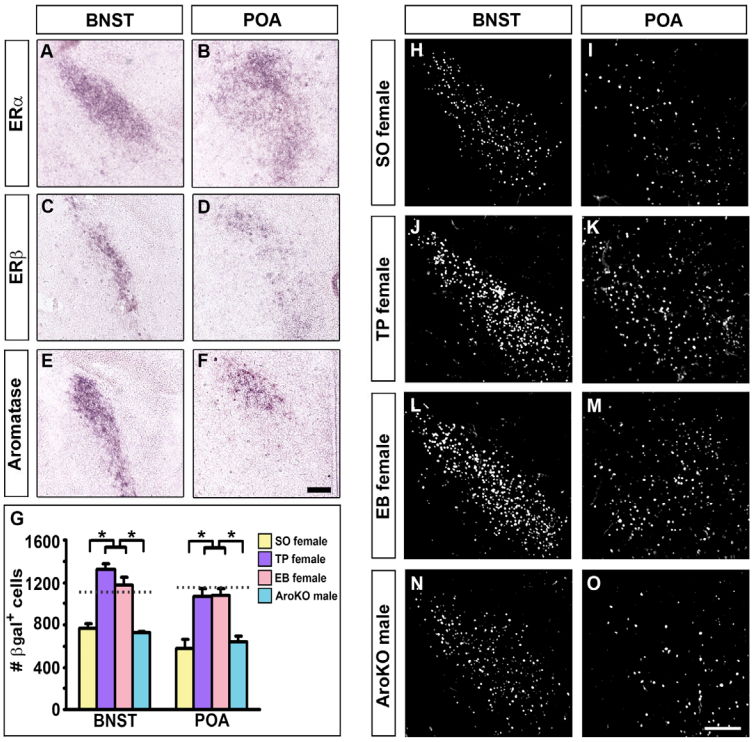

BNST
POA
ERα
ERβ
Aromatase
A
B
C
D
E
F
G
1600
1200
800
400
0
# βgal+ cells
BNST
POA
SO female
TP female
EB female
AroKO male
SO female
TP female
EB female
AroKO male
H
I
J
K
L
M
N
O

**Figure 3.5: Targeted deletion of AR in the nervous system**

(A) Genetic strategy to delete AR in the nervous system.

(B-D) There is masculinization of the external genitalia and the milk line in adult control and $AR^{NsDel}$ males compared to the feminized external morphology of an adult male carrying a constitutive null allele of AR.

(E) Males of the various genotypes had similar levels of serum testosterone and estrogen ($n \geq 12$ per genotype).

(F) The testes and seminal vesicles of all males were similar in weight ($n \geq 7$ per genotype).

(G) The body length (snout to base of tail, $n \geq 4$ per genotype) and weight ($n \geq 12$ per genotype) were similar between males of all four genotypes.

(H) There is no difference in the time to fall from the rotarod between control and $AR^{NsDel}$ males ($n \geq 4$ per genotype).

(I, J) Normalized AR transcript levels in $AR^{NsDel}$ males as a percentage of the levels in control male mice. There is a reduction in AR mRNA in the brain (Hypo., hypothalamus; Cing. Cx., cingulate cortex; Rost. Hypo., rostral hypothalamus; Caud. Hypo., caudal hypothalamus; Hippoc., hippocampus) of P1 (I), P7 (I), and adult (J) $AR^{NsDel}$ males compared to controls. There are no differences in AR transcript levels between $AR^{NsDel}$ and control males in skeletal muscle, pituitary and testes. Mean ± SEM; ‡ mRNA < 0.5% of control, * $p < 5x10^{-4}$, $n = 4$ for each genotype.

(K-V) Representative coronal sections through the septum, POA, BNST, VMH, MeA and ventral premamillary nucleus (PMV) immunolabeled for AR reveal fewer AR positive

cells in each of these regions in $AR^{NsDel}$ males compared to control males. Scale bar equals 100 μm.

## Figure 3.5

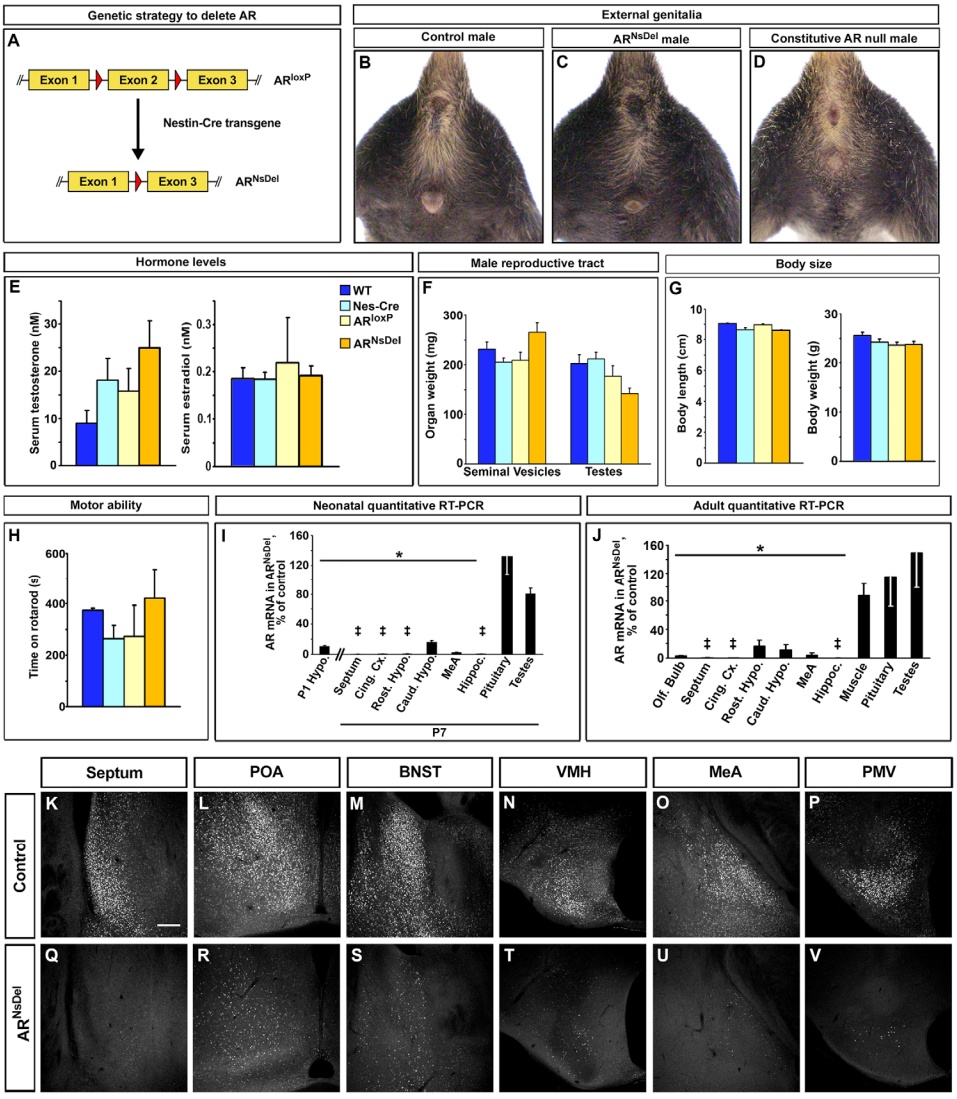

Genetic strategy to delete AR
A
Exon 1
Exon 2
Exon 3
AR^loxP
Nestin-Cre transgene
Exon 1
Exon 3
AR^NsDel
External genitalia
Control male
AR^NsDel male
Constitutive AR null male
B
C
D
Hormone levels
E
Serum testosterone (nM)
Serum estradiol (nM)
WT
Nes-Cre
AR^loxP
AR^NsDel
Male reproductive tract
F
Organ weight (mg)
Seminal Vesicles
Testes
Body size
G
Body length (cm)
Body weight (g)
Motor ability
H
Time on rotarod (s)
Neonatal quantitative RT-PCR
I
AR mRNA in AR^NsDel -/- % of control
P1 Hypo.
Septum
Cing. Cx.
Rost. Hypo.
Caud. Hypo.
MeA
Hippoc.
Pituitary
Testes
P7
Adult quantitative RT-PCR
J
AR mRNA in AR^NsDel -/- % of control
Olf. Bulb
Septum
Cing. Cx.
Rost. Hypo.
Caud. Hypo.
MeA
Hippoc.
Muscle
Pituitary
Testes
Septum
POA
BNST
VMH
MeA
PMV
Control
AR^NsDel
K
L
M
N
O
P
Q
R
S
T
U
V

**Figure 3.6: AR increases the frequency of male mating**

(A) $AR^{NsDel}$ and control males emit more ultrasonic vocalizations to female than male intruders. (B) $AR^{NsDel}$ males mount and intromit females in fewer assays than controls.

(C) As a group, $AR^{NsDel}$ males exhibit fewer mounts and intromissions towards females compared to control males.

(D) There is no statistical difference between $AR^{NsDel}$ and control males in the time spent mounting females, but as a group, $AR^{NsDel}$ mice intromit females for a shorter duration than controls.

(E, F) Once male mating is initiated, there is no difference between $AR^{NsDel}$ males and their control cohorts in the total number (E) or duration (F) of mounts and intromissions displayed towards females.

Mean ± SEM; * $p < 0.033$, $n \geq 3$ for each genotype; ** $p < 0.05$, post hoc Bonferroni's correction for Fisher's exact test, $n \geq 12$ for each genotype; *** $p < 0.05$, Tukey's test after Kruskal-Wallis comparison for multiple groups, $n \geq 12$ for each genotype. In all experiments, we deemed an effect of the $AR^{loxP/Y}$; Nes-Cre ($AR^{NsDel}$) genotype to be statistically significant only if this genotype differed from each of the 3 control cohorts (WT, $AR^{loxP/Y}$, and Nes-Cre).

## Figure 3.6

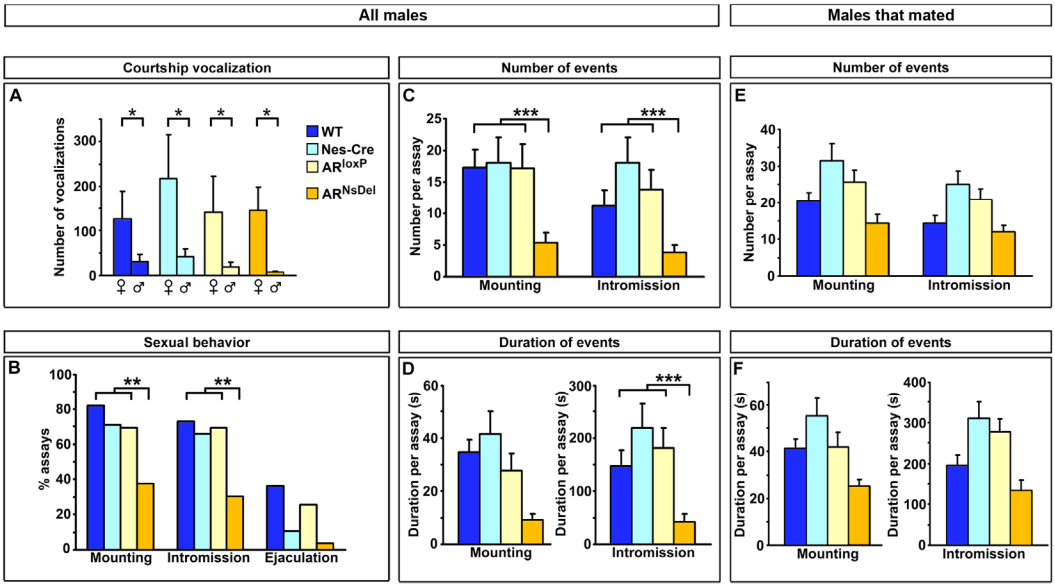

All males
Males that mated
Courtship vocalization
Number of events
Number of events
A
C
E
Number of vocalizations
Number per assay
Number per assay
WT
Nes-Cre
AR^loxP
AR^NsDel
♀ ♂
Mounting
Intromission
Sexual behavior
Duration of events
Duration of events
B
D
F
% assays
Duration per assay (s)
Duration per assay (s)
Mounting
Intromission
Ejaculation

**Figure 3.7: AR increases the levels of male territorial displays**

(A) There is no statistical difference between $AR^{NsDel}$ and control residents in the percent of assays in which aggression is observed.

(B) As a group, $AR^{NsDel}$ residents attack WT male intruders for a shorter duration compared with control residents.

(C) When $AR^{NsDel}$ residents fight, they do so for a shorter duration compared to control males.

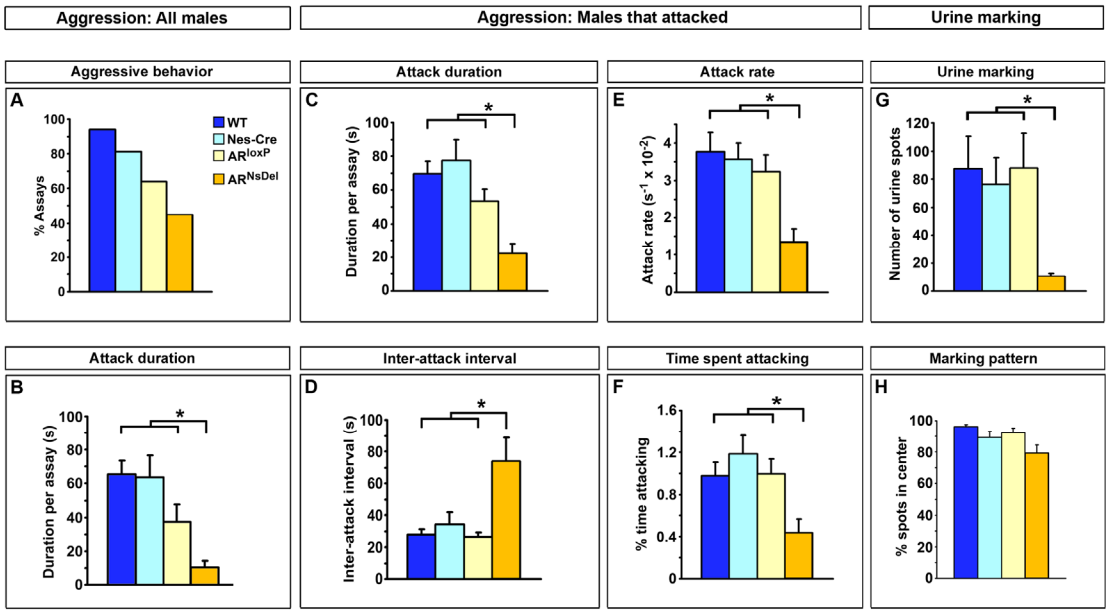
(D-F) In assays with aggression, $AR^{NsDel}$ residents exhibit an increase in time between fights (D), attack at a slower rate (E), and for a smaller percentage of the duration of the assay (F) compared to control males.

(G) $AR^{NsDel}$ males deposit fewer urine marks compared to control males.

(H) There is no difference between $AR^{NsDel}$ and control males in the percentage of urine marks away from the cage perimeter (% center spots).

Mean ± SEM; * $p < 0.05$, Tukey's test subsequent to the Kruskal-Wallis comparison for multiple groups, $n \geq 12$ for each genotype.

## Figure 3.7


Aggression: All males
Aggression: Males that attacked
Urine marking
Aggressive behavior
Attack duration
Attack rate
Urine marking
A
% Assays
WT
Nes-Cre
AR loxP
AR NsDel
C
Duration per assay (s)
E
Attack rate (s-1 x 10-2)
G
Number of urine spots
Attack duration
Inter-attack interval
Time spent attacking
Marking pattern
B
Duration per assay (s)
D
Inter-attack interval (s)
F
% time attacking
H
% spots in center
*

## SUPPLEMENTAL TEXT: EXPERIMENTAL PROCEDURES

### Genotyping animals

Animals were genotyped for the $AR^{loxP}$ allele

(5' primer: AATGCATCACATTAAGTTGATACC;

3' primer: TCAGAATTCTACGGTCTTCTGAG) and for the Nes-Cre transgene

(5' primer: CGCTTCCGCTGGGTCACTGTCG;

3' primer: TCGTTGCATCGACCGGTAATGCAGGC).

### Behavioral assays

We used 9-16 week old male mice in behavioral assays, which were initiated ≥ 1 hour after the onset of the dark cycle. After being singly housed for 2-7 days, mice were first tested for urine marking, in which the animal was allowed to explore a fresh cage lined with Whatman filter paper for 1 hour, and then returned to its homecage. The urine marks were imaged with UV trans-illumination, and the number of spots was enumerated. The proportion of urine marks not abutting a cage wall was also determined (% center spots). The males were subsequently tested for male sexual behavior in their homecage as described previously (Wu et al., 2009). Briefly, a female primed to be in estrus was introduced into the male's cage and animals were allowed to interact for 30 minutes. Following the mating assay, mice were tested for aggression in the resident-intruder test, using group housed adult 129/SvEv (Taconic Farms) males as intruders. A single intruder was inserted into the resident's cage and the males were allowed to interact for 15 minutes. An aggressive interaction was defined as containing one or more instances of biting, tumbling, wrestling, and chasing. In tests of mating or aggression, a

behavioral interaction was scored as "social interaction" when the resident initiated contact with the intruder; such interactions included grooming as well as non-anogenital chemoinvestigation. Various parameters of mating or fighting were scored as such rather than as general "social interaction". Following the resident-intruder test, the mice were tested for ultrasonic vocalizations produced in response to an intruder in their cage. A bat detector tuned to detect sound at 60-80 kHz (Mini-3, Noldus) was placed above the animal's cage and an intruder was introduced into the cage for 3 minutes. Each resident was tested for vocalization separately with a male and a female intruder. All animals were tested 2-3 times each in assays of sexual behavior and aggression, and once each for urine marking and ultrasonic vocalization. The experimental animals were always exposed to intruder mice they had not encountered previously, and each assay was separated by $\geq$ 2 days. Mating and aggression tests were recorded using an infrared sensitive video camera. All tests were scored by an experimenter blind to the genotype of the resident with a software package we have developed in Matlab. The mice were sacrificed 3-7 days following their last behavioral assay, their serum was processed to determine hormone titers, and their brains were analyzed for AR expression using immunolabeling or qPCR.

**Histology**

Brains were dissected from paraformaldehyde-perfused postnatal animals, and fixed for an additional 2 hours (adult) or 45 minutes (P1, P4, P7) at 4$^{o}$C. Freshly dissected heads of E13.5, E15.5 and E17.5 embryos were immediately rinsed in cold phosphate-buffered saline and fixed in 4% paraformaldehyde for 45 minutes at 4$^{o}$C. Embryonic and

postnatal brains were cryoprotected overnight in 20% sucrose at 4°C and embedded in 1:1 TissueTek OCT (Sakura) and Aquamount (VWR). Sections were obtained from these animals at 20 μm thickness, and serially adjacent sections from various brain regions were collected on sets of three slides. These slides were either stored frozen at -80°C for subsequent histological analyses or immediately processed for staining for βgal activity or immunolabeling as described previously (Shah et al., 2004; Wu et al., 2009). To examine residual AR expression in the adult brain of $AR^{NsDel}$ mice, the brains of these males and their controls were fixed as described above, embedded in 3% bacto-agar, and sectioned at 65 μm thickness, using a Leica vibrating microtome; these slices were processed as described previously (Shah et al., 2004; Wu et al., 2009).

The Epitomics anti-AR antibody recognizes an epitope on the C-terminal 20 residues of AR. These final 20 amino acids are extremely unlikely to be translated from the $AR^{NsDel}$ mRNA because deletion of the second exon in the AR gene leads to a frame-shift, generating multiple premature stop codons. Thus, the antibody is likely to recognize only the unrecombined, native AR, barring an unusual, aberrant splicing event that somehow generates a second frame-shift and permits translation of the native epitope even in cells that deleted exon 2. The mutation in Tfm mice also leads to a shift in the open reading frame in AR such that there are several stop codons prior to the C-terminal 20 amino acids, and we did not observe any AR+ cells in various brain regions in these mutants. Thus, any labeled cells we observe in the brain of $AR^{NsDel}$ mice are likely to express native AR protein. Indeed, the strong reduction in AR labeling observed in the brains of $AR^{NsDel}$ mice resembles the diminution in intact AR message detected by qPCR.

The fluorophore conjugated secondary antisera are Cy3 donkey anti-rabbit (1:800, Jackson ImmunoResearch) and AlexaFluor 488 donkey anti-mouse (1:300, Invitrogen). The sections were exposed overnight at 4$^{o}$C to primary antisera and for two hours at room temperature to secondary antibodies. The buffers, washes, and mounting media used in these studies have been described previously (Shah et al., 2004; Wu et al., 2009) . In situ hybridization for aromatase, ERα and ERβ was performed as described previously (Wu et al., 2009).

To quantitate AR expressing cells at P1, we enumerated cells labeled for βgal activity in every third section through the entire BNST, POA, and MeA using brightfield optics and a 20X objective lens. To estimate the size of the sex difference in AR positive cells within the BNST and POA at P7, we imaged every third histological section (20 μm thickness) immunolabeled for βgal through the entire extent of the left BNST and POA. This strategy yielded 5-6 imaged sections per animal. Each of these histological sections was imaged with a 10X objective lens using a Nikon C1si confocal microscope, and image stacks were generated with five 4μm thick optical slices through the section. The images were subjected to despeckling and background noise subtraction using ImageJ (NIH) as described previously (Shah et al., 2004; Wu et al., 2009). The βgal is targeted to the nucleus and we enumerated all labeled nuclei in the central optical slice of each histological section, and these counts are reported in Figures 3.3 and 3.4. We therefore effectively enumerated βgal positive nuclear figures in optical slices separated by 56 μm. The diameter of βgal labeled nuclei is significantly smaller than 56 μm and does not exhibit a sex difference (POA: male, 6.1 ± 0.04 μm and female, POA 6.1 ± 0.03 μm;

BNST: male, 6.4 ± 0.03 μm and female, 6.2 ± 0.05 μm; n = 3 animals with 30 labeled nuclei measured in each region per animal, $p > 0.32$), indicating that our estimate of the size of the sexual dimorphism in the BNST and POA is likely to be unbiased. Indeed, we observed a similar sexual dimorphism in the BNST and POA (JT, unpublished observations), using unbiased stereology with an Optical Fractionator probe whose parameters were set per the manufacturer's instructions (StereoInvestigator, MicroBrightField) and published protocols (Keuker et al., 2001).

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## SUPPLEMENTARY FIGURE LEGENDS

**Figure 3.8: Limited expression of AR in the newborn brain**

(A-F) Representative coronal sections through the MeA and VMH of P1, P4, P7 male mice bearing the AR-IPIN allele stained for βgal activity. There are few AR expressing cells in the MeA at birth, and progressively more AR positive cells are observed at P4 and P7. By contrast, there is a collection of AR expressing cells in the vicinity of the VMH and the arcuate nucleus (ArcN/VMH) at each of these postnatal ages. n = 3 at each age. Scale bar equals 100 μm.

**Figure 3.8**

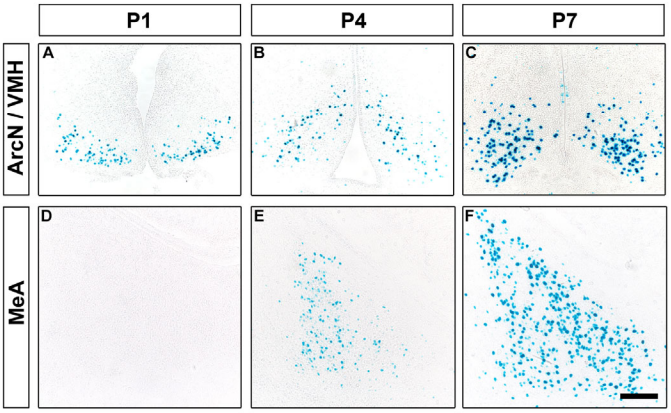


**Figure 3.9: Sparse expression of AR in the prenatal brain**

(A-L) Representative coronal sections through the POA, BNST, MeA, and ArcN/VMH of E13.5, E15.5, E17.5 male mice bearing the AR-IPIN allele labeled for βgal activity. We failed to detect AR positive cells in these regions at E13.5; note that at this age, panel B shows the brain region containing the presumptive BNST. At E15.5 and E17.5, AR expressing cells (arrowheads, E, I) appear limited to the ArcN/VMH region. The area circumscribed by the dashed line delineates the lateral (B, F, J) and third (A, C, E, G, I, K) ventricles. The dotted line (D, H, L) marks the optic tract. Dorsal is at the top. The midline is in the center (A, E, I) or to the right (B-D, F-H, J-L). $n \geq 2$ at each age. Scale bar equals 200 μm.

**Figure 3.9**

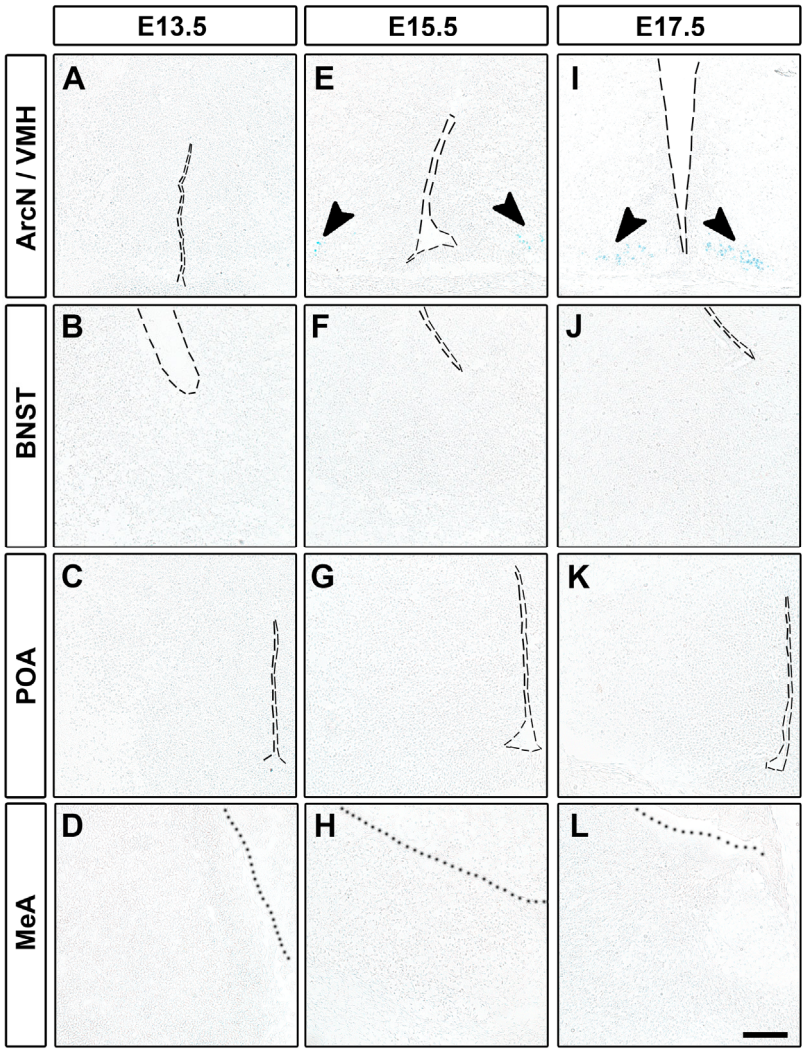


**Figure 3.10: AR is not required in the nervous system for locomotor behavior and social interactions**

(A) In mating tests, there is no difference in the number of midline crosses in $AR^{NsDel}$ and control males (n = 5 for each genotype).

(B) In mating tests, there is no difference between $AR^{NsDel}$ and control males in the latency to first interact with the female, and in the number and duration of social interactions with the female intruder ($n \geq 12$ for each genotype).

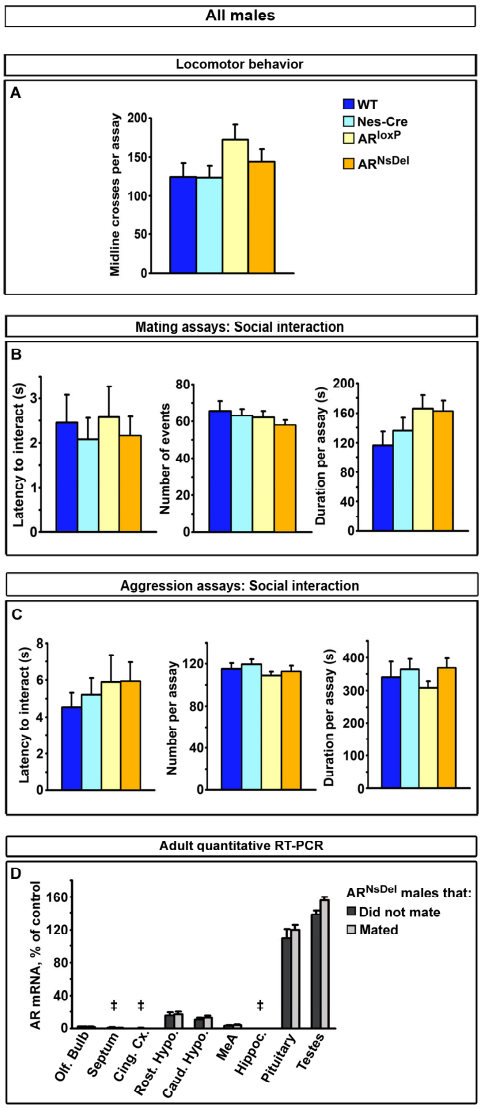
(C) In the resident intruder assay, there is no difference between $AR^{NsDel}$ and control residents in the latency to first interact with the WT male intruder, and in the number and duration of social interactions with the intruder ($n \geq 12$ for each genotype).

(D) Normalized AR transcript levels in adult $AR^{NsDel}$ males as a percentage of the levels in control male mice. There is no difference in the residual AR mRNA in various brain regions between $AR^{NsDel}$ males that mated and $AR^{NsDel}$ males that did not. We re-analyzed the results in the main Figure 3.5J with additional $AR^{NsDel}$ mice to generate the comparison shown in this panel. ‡ mRNA < 0.5% of control. $n \geq 3$ per group. Mean ± SEM. (A-C) One-way ANOVA or Kruskal-Wallis test: $p > 0.05$ for all parameters. (D) KS test: $p \geq 0.15$ for each pairwise comparison.

(E-X) Coronal sections through the brain of adult WT ($AR^{+/Y}$) and Tfm ($AR^{Tfm/Y}$) male mice immunolabeled with a rabbit monoclonal anti-AR antibody reveal AR+ cells in WT but not in mutant males. Cells expressing AR are easily visualized in the BNST, VMH, PMV, MeA and POA of the WT brain (E-I). By contrast, there are essentially no AR labeled cells in these regions in the Tfm animal (J-N). The immunolabeled sections in the WT and Tfm animals have also been co-stained with DAPI (O-S, T-X, respectively)

to enable visualization of all cells in these regions. Solid yellow lines indicate the ventral surface of the brain, dashed yellow lines indicate the edge of the lateral (J) or the third (K, L, N) ventricle, and the dotted yellow line indicates the ventrolateral edge of the optic tract. Similar results were observed in 3 animals of each genotype. Scale bar equals 200 μm.

## Figure 3.10


All males
Locomotor behavior
A
Midline crosses per assay
WT
Nes-Cre
AR^loxP
AR^NsDel
Mating assays: Social interaction
B
Latency to interact (s)
Number of events
Duration per assay (s)
Aggression assays: Social interaction
C
Latency to interact (s)
Number per assay
Duration per assay (s)
Adult quantitative RT-PCR
D
AR mRNA, % of control
AR^NsDel males that:
Did not mate
Mated
Olf. Bulb
Septum
Cing. Cx.
Rost. Hypo.
Caud. Hypo.
MeA
Hippoc.
Pituitary
Testes

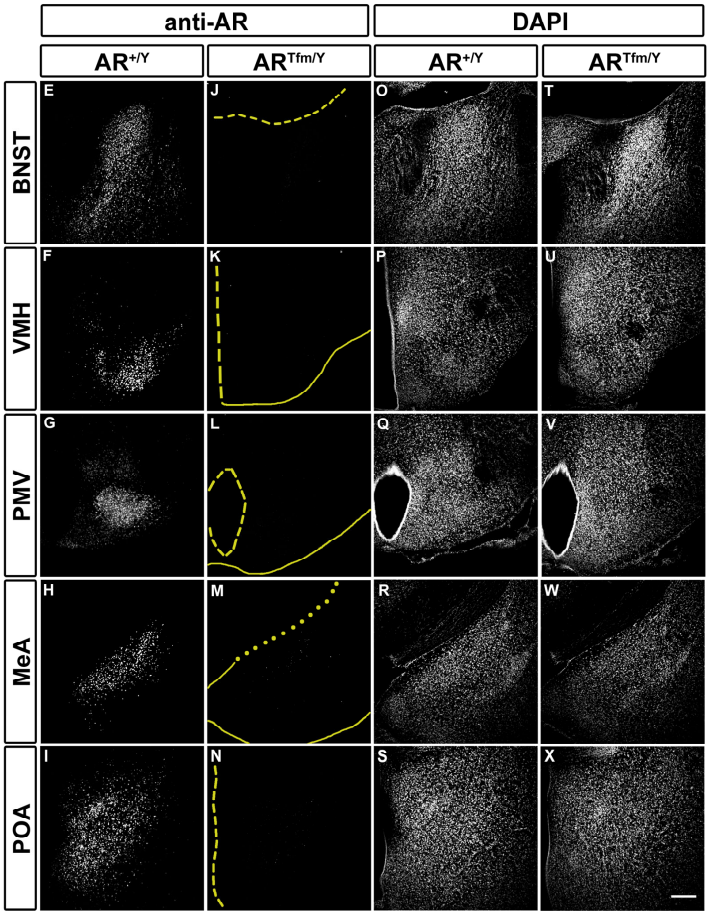

anti-AR
DAPI
AR+/Y
ARTfm/Y
AR+/Y
ARTfm/Y
BNST
VMH
PMV
MeA
POA
E
J
O
T
F
K
P
U
G
L
Q
V
H
M
R
W
I
N
S
X

**Figure 3.11: AR is not essential in the nervous system for many parameters of male sexual behavior**

(A) There is no statistical difference in the percentage of $AR^{NsDel}$ and control males initiating male mating. Fisher's Exact Test: Mounting, $p < 0.025$ for $AR^{NsDel}$ vs. WT and $AR^{NsDel}$ vs. Nes-Cre, $p > 0.1$ for $AR^{NsDel}$ vs. $AR^{loxP/Y}$; intromission, $p < 0.025$ for $AR^{NsDel}$ vs. WT and $AR^{NsDel}$ vs. Nes-Cre, $p > 0.05$ for $AR^{NsDel}$ vs. $AR^{loxP/Y}$; ejaculation, $p < 0.025$ for $AR^{NsDel}$ vs. WT and $AR^{NsDel}$ vs. $AR^{loxP/Y}$, $p > 0.15$ for $AR^{NsDel}$ vs. Nes-Cre.

(B) There is no difference between $AR^{NsDel}$ and control males in the latency to first chemoinvestigate females and the total number and duration of such female-directed chemoinvestigations. One-way ANOVA or Kruskal-Wallis test: $p > 0.05$ for all parameters.

(C) In assays where the males displayed sexual behavior, there is no difference between $AR^{NsDel}$ and control males in the latency to first mount or intromit the female. One-way ANOVA: $p > 0.05$.

(D) There is no statistical difference between $AR^{NsDel}$ and control males in the time between mounts or intromissions. Tukey's test subsequent to Kruskal-Wallis test: Mounting, $p < 0.05$ for $AR^{NsDel}$ vs. Nes-Cre, $p > 0.1$ for $AR^{NsDel}$ vs. WT and $AR^{NsDel}$ vs. $AR^{loxP/Y}$. Kruskal-Wallis test: Intromission, $p > 0.05$.

(E) Once mating is initiated in a test, there is no statistical difference between $AR^{NsDel}$ and control males in the rate of these behavioral displays. Tukey's test subsequent to Kruskal-Wallis test: Mounting, $p < 0.05$ for $AR^{NsDel}$ vs. Nes-Cre, $p > 0.2$ for $AR^{NsDel}$ vs. WT and $AR^{NsDel}$ vs. $AR^{loxP/Y}$; intromission, $p < 0.05$ for $AR^{NsDel}$ vs. Nes-Cre, $p > 0.5$ for $AR^{NsDel}$ vs. WT and $AR^{NsDel}$ vs. $AR^{loxP/Y}$.

(F) Once mating is initiated in a test, there is no statistical difference between $AR^{NsDel}$ and control males in the percent of the assay duration engaged in mounting or intromission. Tukey's test subsequent to Kruskal-Wallis test: Mounting, $p < 0.05$ for $AR^{NsDel}$ vs. Nes-Cre, $p > 0.05$ for and $AR^{NsDel}$ vs. WT and $AR^{NsDel}$ vs. $AR^{loxP/Y}$; intromission, $p < 0.05$ for $AR^{NsDel}$ vs. Nes-Cre, $p > 0.05$ for $AR^{NsDel}$ vs. WT and $AR^{NsDel}$ vs. $AR^{loxP/Y}$.

(G) For male residents that displayed sexual behavior, there is no difference between $AR^{NsDel}$ and control males in the percent of assays in which they exhibited mounting, intromission, or ejaculation. Kruskal-Wallis test: $p > 0.4$ for each comparison.

Mean ± SEM; $n \geq 12$ for each genotype.

## Figure 3.11

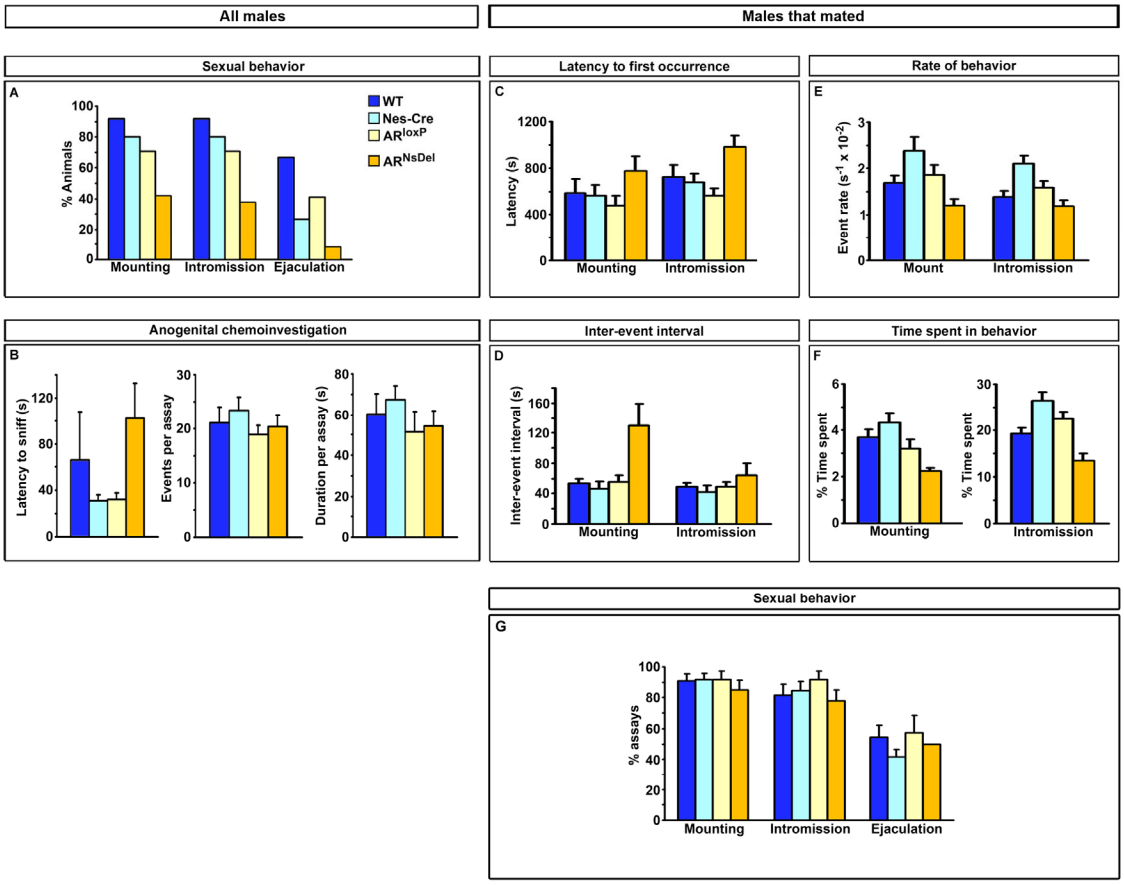

All males
Males that mated
Sexual behavior
Latency to first occurrence
Rate of behavior
A
% Animals
WT
Nes-Cre
AR^loxP
AR^NsDel
Mounting
Intromission
Ejaculation
C
Latency (s)
Mounting
Intromission
E
Event rate (s^-1 x 10^-2)
Mount
Intromission
Anogenital chemoinvestigation
Inter-event interval
Time spent in behavior
B
Latency to sniff (s)
Events per assay
Duration per assay (s)
D
Inter-event interval (s)
Mounting
Intromission
F
% Time spent
Mounting
% Time spent
Intromission
Sexual behavior
G
% assays
Mounting
Intromission
Ejaculation

**Figure 3.12: AR is essential in the nervous system for experiential changes in aggression**

(A) There is no statistical difference between $AR^{NsDel}$ and control residents in the percentage of animals attacking a WT male intruder. Fisher's Exact Test: % animals attacking, $p < 0.001$ for $AR^{NsDel}$ vs. WT and $AR^{NsDel}$ vs. Nes-Cre, $p > 0.05$ for $AR^{NsDel}$ vs. $AR^{loxP/Y}$.

(B) There is no statistical difference in the number of attacks initiated by $AR^{NsDel}$ and control resident males towards WT male intruders. Tukey's test subsequent to Kruskal-Wallis test: $p < 0.05$ for $AR^{NsDel}$ vs. WT and $AR^{NsDel}$ vs. Nes-Cre, $p > 0.05$ for $AR^{NsDel}$ vs. $AR^{loxP/Y}$.

(C) There is no difference between $AR^{NsDel}$ and control residents in the latency to first chemoinvestigate a WT male intruder or in the number and duration of intruder-directed chemoinvestigations. One-way ANOVA or Kruskal-Wallis test: $p > 0.05$ for all parameters.

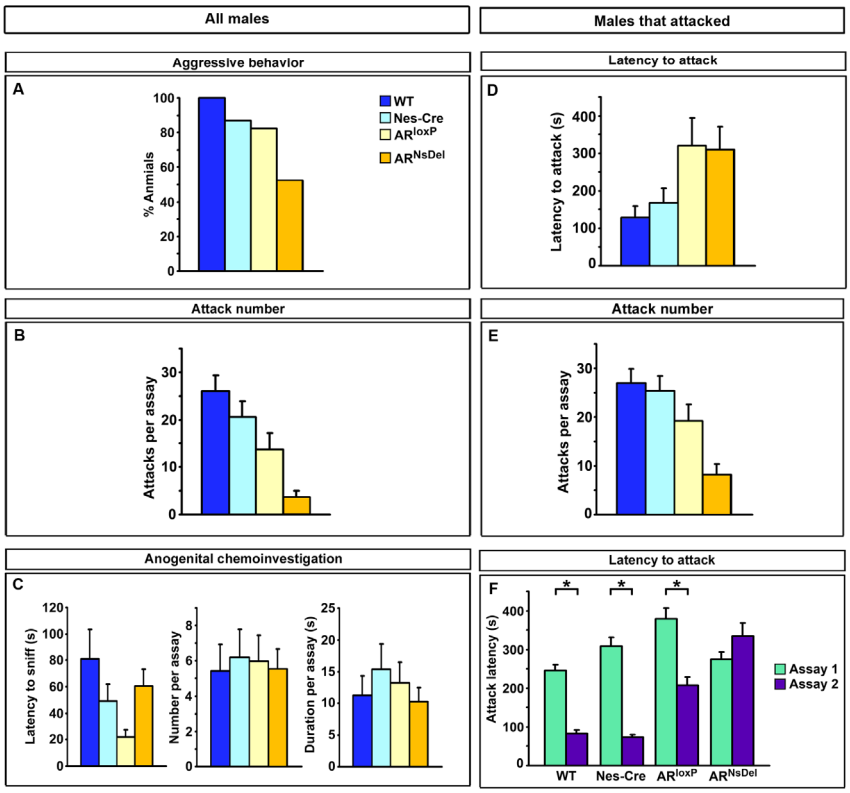
(D) In assays with fighting, there is no difference between $AR^{NsDel}$ mutants and controls in the latency to the first attack. Kruskal-Wallis comparison: $p > 0.05$.

(E) Once fighting is initiated, there is no statistical difference in the number of attacks by $AR^{NsDel}$ and control residents towards WT male intruders. Tukey's test subsequent to Kruskal-Wallis test: $p < 0.05$ for $AR^{NsDel}$ vs. WT and $AR^{NsDel}$ vs. Nes-Cre, $p > 0.05$ for $AR^{NsDel}$ vs. $AR^{loxP/Y}$.

(F) Control, but not $AR^{NsDel}$, residents attack intruder males with a shorter latency in the second assay compared to the first test. * KS test: $p \leq 0.036$.

Mean ± SEM; $n \geq 12$ for each genotype.

**Figure 3.12**


All males
Males that attacked
Aggressive behavior
Latency to attack
A
% Animals
WT
Nes-Cre
AR loxP
AR NsDel
D
Latency to attack (s)
Attack number
Attack number
B
Attacks per assay
E
Attacks per assay
Anogenital chemoinvestigation
Latency to attack
C
Latency to sniff (s)
Number per assay
Duration per assay (s)
F
Attack latency (s)
Assay 1
Assay 2
WT
Nes-Cre
AR loxP
AR NsDel

# CHAPTER 4

# Conclusions

## AR controls sexual and territorial behavior in the mouse

Although a role for testosterone in male-typical behaviors was demonstrated over 60 years ago, how this hormone controls these behaviors has remained elusive. In particular, uncertainty over the relative roles of testosterone and its metabolites, especially estrogen, has prevented a complete understanding of how male behavior is controlled. The cognate receptor for testosterone, AR, is necessary for male mating and aggression, but due to pleitropic effects in the reproductive tract, AR mutant mice have very low testosterone levels. This finding raises the possibility that the deficit in male behaviors is due to low availability of testosterone for conversion to an active metabolite in the brain. Indeed, as estrogen is necessary and sufficient for male mating and aggression, this metabolite is viewed by some as the sole agent of masculinization in the brain. However, an independent role for AR in controlling male behavior could not be ruled out by previous studies. In the course of my thesis work, I have sought to establish directly the function of AR, if any, in controlling male mating and territorial behaviors. By utilizing a genetic approach, we have generated a mouse which lacks a functional AR in the nervous system ($AR^{NsDel}$ mice), but retains a masculinized reproductive tract and produces normal levels of testosterone, the substrate for estrogen. Behavioral tests reveal that these mice exhibit reduced levels of male sexual and territorial behaviors. However, when the behavior does occur, it appears normal. For male mating, AR in the brain is specifically required for the initiation of mounting. Once mounting has begun, the progression through the

rest of the mating routine proceeds independent of AR. In contrast, the probability of initiating intermale aggression is unchanged in $AR^{NsDel}$ males. When analyzing those animals that do initiate aggressive behavior, striking differences in the timing of attacks emerge. These animals initiate fewer attacks, spread more sparsely throughout the course of the assay. In tests of territorial marking behavior, $AR^{NsDel}$ males mark in the center of their cages, similar to control males, but they deposit far fewer spots. Thus, the function of AR in the nervous system appears restricted to controlling the extent of male-typical behavior, but it does not program its male quality.

The qualitatively male nature of behavior in $AR^{NsDel}$ mice indicates that AR is not necessary for the masculinization of the circuits controlling male-typical behaviors. This finding is in accord with the dearth of AR-expressing neurons in the brain during the perinatal testosterone surge. In the perinatal mouse brain, regions known to control sexually dimorphic behaviors such as the preoptic hypothalamus (POA), bed nucleus of the stria terminalis (BNST), and the medial amygdala contain only a handful of cells expressing AR at the time of the perinatal testosterone surge. This suggests that the male differentiation of the brain normally occurs via estrogen signaling. The late onset of AR expression in these regions, only after the critical period for male differentiation of the brain, suggests that the function of AR is restricted to later time points, most likely in adulthood.

**Sexual dimorphism in AR+ neurons controlled by estrogen**

Estrogen signaling has been shown to control the early sexual differentiation of numerous anatomical and molecular dimorphisms. Our findings add AR to this list. Male mice have more AR+ cells in the BNST and POA (Shah et al., 2004). We find that estrogen is necessary and sufficient for this masculinization. This dimorphism may result from increased estrogen-mediated transcription of AR in males. Alternately, estrogen may control the number of cells in these brain regions, while expression of AR expression is regulated independent of estrogen. Estrogen may thus indirectly control the number of AR+ cells.

The relevance of the sex difference in AR+ cells, like most other dimorphisms, is unclear. It is tempting to speculate that the increased number of AR+ neurons may contribute functionally to the initiation or patterning of male behaviors. The increased number of AR+ cells in the BNST and POA of males suggests an increased sensitivity to testosterone in the adult brain, though it is difficult to disentangle an effect of increased AR signaling from increased AR+ cell number. How might an increase in the number AR+ neurons affect male-typical behavior? This may result in changes in the magnitude of output from dimorphic brain regions, thus resulting in differential input to downstream targets. A quantitative increase in AR+ cell number could also reflect the presence of an as-yet unidentified male-specific cell type. Such a subset of cells may have different physiological properties, or a different pattern of connectivity. This subset of cells could modulate the signal processed by the neural circuits for male behaviors, or process a different stream of information.

How can we test the function of a dimorphic set of neurons? Blocking apoptosis by genetically deleting the proapoptotic gene *Bax* results in the loss of the sexual dimorphism in AR+ neurons in the BNST (Holmes et al., 2009). However, blocking apoptosis altogether affects all signaling pathways converging on Bax, resulting in a greater number of cells in the BNST than that observed in wildtype males. To selectively control the effects of estrogen on apoptosis, a more restricted approach is required. Estrogen signaling has been shown to regulate the apoptotic pathway in neurons via the anti-apoptotic gene *Bcl-2* (Dubal et al., 1999; Fan et al., 2008), and there is an estrogen response element in the *Bcl-2* locus (Perillo et al., 2008). Deleting the estrogen response element in *Bcl-2* (or other genes in the apoptotic pathway) would enable us to test the role of estrogen-modulated cell death in sexually dimorphic behaviors, thereby assessing the role of male-specific neurons.

**A model for the hormonal control of male behavior**

These findings, together with previous research, suggest a model in which male differentiation of the brain proceeds primarily under the control of estrogen signaling, while the role for AR is restricted to controlling the extent of male sexual and territorial behaviors. Several lines of evidence support the finding that AR is not necessary to masculinize the brain during the critical perinatal testosterone surge. First, we find that although AR expression is very sparse in the brain during the surge, ERα, ERβ, and aromatase are robustly expressed. This indicates that testosterone produced by $AR^{NsDel}$ males can masculinize the perinatal brain via estrogen signaling. Second, perinatal

estrogen treatment of females masculinizes male-typical sexual and territorial behavior to an extent similar to $AR^{NsDel}$ males (Wu et al., 2009). Even in *Tfm* male mice, which lack a functional AR, perinatal estrogen treatment masculinizes mating and aggression (Olsen, 1992; Sato et al., 2004; Scordalakes and Rissman, 2004). These findings demonstrate the potency of estrogen to masculinize the perinatal mouse brain, and indicate that AR is dispensable for this process. The function of AR appears restricted to the control of male sexual and territorial behavior later in life. $AR^{NsDel}$ males exhibit mating, intermale aggression, and territorial marking at a reduced level relative to controls. The qualitative patterns of these behaviors are male in nature, consistent with the role of estrogen in masculinizing the circuits underlying these behaviors. Estrogen signaling is also critical for the display of male behaviors in adulthood. Male mice doubly mutant for ERα and ERβ do not display male mating or aggression, despite high levels of testosterone in circulation (Dupont et al., 2000; Ogawa et al., 2000). A full restoration of sexual and territorial behaviors in castrated males requires treatment with estrogen and the non-aromatizable androgen DHT (Finney and Erpino, 1976; Wallis and Luttge, 1975). These findings suggest that estrogen and AR signaling cooperate in adulthood to control male behavior.

How does AR control the levels of male mating and aggression? Presumably, AR signaling controls the firing properties of the neurons in the circuits controlling male behavior, causes changes in the structure of those circuits, or both. At a molecular level, future experiments will identify direct and indirect targets of AR through chromatin immunoprecipitation and gene expression profiling. In previous studies AR has been

suggested to control the dimorphic expression of two genes that could affect social behavior, arginine vasopressin and calbindin (Bodo and Rissman, 2008; Han and De Vries, 2003), and these or other genes could directly regulate the output of the neural circuits for sexually dimorphic behavior. Alternately, the interaction between AR and the estrogen receptors may turn out to be more complex than expected. We have shown that the sexual differentiation of AR expression is downstream of estrogen signaling. It remains possible that the expression of genes in the estrogen signaling pathway (e.g. ERα itself) is modulated by AR signaling. As a result, the more proximal effector of increased male sexual and territorial behaviors may be estrogen signaling. These molecular effects downstream of AR signaling will provide insight into the circuit-level changes that occur, and how they control the gain on male behavior.

**Relevance to other species**

It is tempting to speculate on how these results may extend to other species, especially humans. Primates and mice depend on AR and ER signaling to different extents in controlling male-typical behaviors. Mice depend on estrogen signaling to establish and activate the circuits for male behavior, while AR signaling plays a role restricted to controlling the levels of these behaviors. In contrast, primates seem to depend exclusively on AR signaling. Human males with a null mutation in aromatase, who therefore cannot produce estrogen, or in ERα, report heterosexual orientation and normal sexual drive, suggesting that estrogen is not necessary for masculinization of the brain (Carani et al., 1999; Grumbach and Auchus, 1999). Estrogen treatment is also not sufficient to elicit male-typical sexual behavior in castrated male rhesus monkeys

(Thornton and Goy, 1986), nor in human subjects with a non-functional AR, who routinely receive estrogen supplements (Wilson, 2001). In contrast, AR signaling is sufficient to elicit male sexual behavior in rhesus monkeys (Thornton et al., 2009). These results indicate that estrogen does not play a major role in the masculinization of male behavior in primates. How have these two sets of transcription factors, AR and the ERs, evolved to regulate the same behavioral processes? Whether the ERs in mice regulate the same genes in the mouse as does AR in primates is an interesting question that will be answered through future chromatin immunoprecipitation and transcript profiling experiments.

**Circuit-level challenges**

A major challenge in neuroscience is to define circuits controlling specific behaviors. The neural circuits for male-typical behaviors are beginning to be defined by genetically by labeling cell bodies and projections of neurons that express genes controlling these behaviors (Manoli et al., 2005; Rideout et al., 2010; Shah et al., 2004; Stockinger et al., 2005; Wu et al., 2009). Understanding how AR regulates the activity of these circuits will require a molecular analysis of target genes, and investigation of which subsets of AR+ neurons control each component of behavior. Mapping these AR+ neurons requires a spatially- or cell-type-restricted manipulation of gene function or neuronal activity. By deleting AR in defined populations of cells through the use of mouse lines expressing Cre in restricted cell types or by injecting Cre-expressing viruses into selected brain regions, experimenters can refine the location of neurons where AR is necessary for controlling

the extent of male behaviors. These experiments will allow the identification of neurons that regulate the circuits for sexually dimorphic behaviors.

The limited expression of AR in the perinatal brain suggests that estrogen signaling controls the organization of male behavior, while the role for AR is limited to activation. AR is expressed at puberty, and it is possible that AR could play a role in developmental processes during this period. This can be directly tested by using temporally controlled deletion of AR in the brain prior to, or subsequent to puberty. Such experiments will further refine the temporal requirement for AR in controlling male sexual and territorial behaviors.

How does the nervous system integrate information from its environment, and produce an appropriate behavioral response? Work in my thesis has highlighted the role of AR in controlling the output of the neural circuits for male sexual and territorial behaviors. Future experiments will further define how estrogen establishes this circuit, and how AR signaling regulates its behavioral output.

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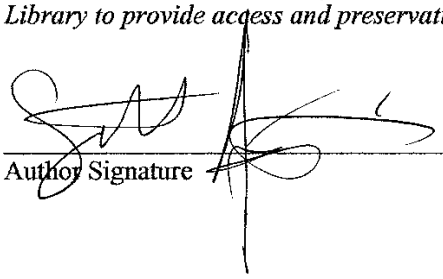

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