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Hormone control of male development and behavior

by

Melody Wu

DISSERTATION

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Hormone control of male development and behavior

Melody Wu

ABSTRACT

Males and females of most species display differences in behavior. The steroid hormones testosterone and estrogen each play integral roles in the regulation of these sex specific behaviors. How these two hormone signaling pathways interact to control differentiation of the male brain and behavior is still unclear. This dissertation discusses the contribution of gonadal steroid hormones to the development of the male brain and male specific behavior.

Testosterone in male circulation is converted, via the enzyme aromatase, to estrogen in the brain to regulate male behavior. In order to visualize where estrogen is synthesized in the brain, we generated mice in which aromatase+ cells also express reporters which allow for labeling of both cell soma and cell membranes. We find that aromatase expression is sexually dimorphic and that male differentiation of aromatase expression is independent of androgen signaling. In fact, we find that neonatal estrogen is sufficient to masculinize aromatase expression in females via estrogen mediated cell survival.

We also find that females given exogenous estrogen at birth display male specific territorial behaviors. These behaviors can also be elicited in males mutant for

the androgen receptor, suggesting that androgen signaling is not necessary for the display of these behaviors. In fact, there is little to no androgen receptor expression in the male brain at birth, suggesting that early in development, testosterone serves as a precursor which is converted to estrogen, which then regulates male behaviors. Estrogen signaling is also required in adulthood for acute activation of male typical behaviors. Testosterone signaling via the androgen receptor appears to control the levels of male typical behavior. We generated mice in which the androgen receptor is deleted specifically in the nervous system and find that these mice exhibit male sexual and territorial behaviors but at a lower intensity than wildtype males.

Taken together, this thesis presents a model whereby early in development testosterone is converted to estrogen, which signals via the estrogen receptors to masculinize the brain and behavior. Activation of male behaviors also requires estrogen signaling, but the intensity of these displays is regulated by testosterone signaling via the androgen receptor.

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Chapter 1:

Introduction to male specific sexually dimorphic behaviors

Males and females of all vertebrate species display differences in behavior. Sex specific displays are innate and robust behaviors that are thought to promote propagation of the species. Examples of these sex typical behaviors include courtship and mating, territorial behaviors, and parental care. These differences in behavior are conserved across many species including mice and humans. Mating behaviors are distinct between males and females of a species. Males will also display territorial marking and aggressive behaviors which are absent in females. Parental care, on the other hand, is usually the domain of females. Male sexual and territorial behaviors consist of several stereotyped routines. Upon encountering a female, a male mouse will display a sequence of male typical mating subroutines which includes mounting, intromission, and ejaculation (McGill, 1962). Additionally, male mice will court females by vocalizing towards them in the ultrasonic range (Nyby, et al., 1977). Males also display territorial behaviors, both by depositing urine spots to mark their territory (Desjardins, et al., 1973) and by attacking intruder males that enter into this marked region (Beeman, 1947).

Appropriate display of mating, ultrasonic vocalization, and aggression also requires correct identification of the conspecific encountered. Mice depend on olfactory cues for identification of conspecific individuals. These cues are processed by either of two olfactory sensory organs in rodents: the main olfactory epithelium (MOE) or the vomeronasal organ (VNO), both of which are required for normal display of sexually dimorphic behaviors (Dulac and Wagner, 2006). The MOE detects volatile cues and innervates the main olfactory bulb (MOB) while the VNO senses non-volatile olfactory

stimuli and sends projections to the accessory olfactory bulb (AOB). Males lacking a functional CNGA2 (the cyclic nucleotide gated channel required for MOE signaling) show a severe deficit in both mating and aggressive behaviors (Mandiyan, et al., 2005; Yoon, et al., 2005). Genetic ablation of TrpC2 (which encodes the cation channel activated by olfactory cues in the VNO) results in inappropriate sex specific behavior. Males mutant for TRPC2 do not display aggressive behavior, but mount both male and female intruders indiscriminately (Leypold, et al., 2002; Stowers, et al., 2002). Both the MOE and the VNO relay information to the hypothalamus albeit through different pathways (Dulac and Wagner, 2006). Although it is still unclear how olfactory information from both the main olfactory and vomeronasal pathways is integrated to direct behavior, it is certain that olfactory cues are necessary for correct display of sex-specific behaviors.

Sexually dimorphic behaviors are known to be under the control of gonadal steroid hormones and their respective hormone receptors. For example, progesterone, estradiol, progesterone receptor (PR), and the estrogen receptors ($ER\alpha$ and $ER\beta$) are required for the display of female typical behaviors (Beach, 1976; Mani, et al., 1997; Ogawa, et al., 1998a). Male typical displays, on the other hand, require testosterone and its cognate receptor, the androgen receptor (AR) (Sato, et al., 2004). In addition, male specific behaviors are under the control of estradiol and the ERs (Ogawa, et al., 2000). How testosterone and estrogen signaling pathways are both required for male behaviors is a complex problem that is still actively being studied. This thesis explores how these two hormonal pathways interact to regulate differentiation of the male brain and male typical behaviors in the mouse.

NEURAL CORRELATES OF MALE SPECIFIC BEHAVIORS

Previous studies using electrolytic lesions as well as detection of immediate early gene (such as c-fos) expression have implicated multiple regions of the brain in sexually dimorphic behaviors. Many of these brain regions are themselves also sexually dimorphic. These regions include the medial amygdala (MeA), the bed nucleus of the stria terminalis (BNST), the preoptic area of the hypothalamus (POA), the ventromedial hypothalamus (VMH), and the ventral region of the premammillary nucleus (PMV).

Medial amygdala

The medial amygdala receives olfactory sensory input directly from both the main and accessory olfactory bulbs. Neurons in the MeA send projections to the BNST, POA, PMV, and the VMH (Canteras, et al., 1995). The regional volume of the posterodorsal portion of the MeA (MePD) is larger in male rats than in females, and there is an increase in both neuronal cell size as well as the number of neurons in the male MePD compared to the female (Morris, et al., 2008; Rasia-Filho, et al., 2004). The number of synapses terminating on dendritic shafts is also greater in the male MeA as compared to the female (Nishizuka and Arai, 1981b). Lesions of the medial amygdala result in deficits in mounting, intromission, and non-contact erection in rats as well as a complete loss of ejaculatory behavior (Kondo, et al., 1998). Dramatic fos induction has been seen in the posterodorsal region of the MeA (MePD) following mating to ejaculation (Baum and Everitt, 1992; Coolen, et al., 1996) and also following male-male aggressive bouts in the Syrian hamster (Kollack-Walker and Newman, 1995). These data suggest that the MeA is involved in first pass processing of olfactory cues to regulate mating to ejaculation as well as aggression.

Bed nucleus of the stria terminalis

The principal nucleus of the BNST (BNSTp) receives direct projections from the MeA and sends reciprocal connections back to the MeA in addition to innervating the POA, VMH, PMV, and other hypothalamic areas (Dong and Swanson, 2004). The volume and the number of cells in the BNST are both larger in the male rat compared to the female (del Abril, et al., 1987). Lesions of the BNST result in an increase in the latency to ejaculate (Claro, et al., 1995), increased numbers of intromissions before ejaculation, and increased duration between intromissions (Emery and Sachs, 1976). c-Fos induction has been shown in the male BNST following either chemoinvestigation of females or ejaculation (Baum and Everitt, 1992; Coolen, et al., 1996). Fos induction is also higher in the BNST of golden hamsters following attacks directed towards male intruders (Delville, et al., 2000). The BNST thus appears to play a role in the timing of male mating behaviors.

Preoptic area of the hypothalamus

The POA sends projections to the VMH and the PMV (Simerly and Swanson, 1988). In rats, the regional volume of and the number of cells in the POA is larger in males than in females (Gorski, et al., 1980). Lesions of the entire POA result in a significant loss of male mounting, intromission, and ejaculation (Christensen, et al., 1977). On the other hand, microstimulation of the POA reduces the latency to mount as well as the number of intromissions required before ejaculation (Malsbury, 1971). Fos induction has been shown in the male POA following male mounting of females (Baum and Everitt, 1992). Taken together, these studies suggest that the POA, in combinations with the BNST, regulates the initiation and timing of male intromission and ejaculation.

Ventromedial hypothalamus

Projections from the VMH innervate the POA and the VMH (Canteras, et al., 1994) and the volume of the VMH is larger in male rats compared to females (Matsumoto and Arai, 1983). The ventrolateral division of the VMH (VMHvl) appears to inhibit male mating behaviors as lesions increase the frequency of both mounts and intromissions (Christensen, et al., 1977). On the other hand, lesions of the dorsomedial partition of the VMH (VMHdm) impair ultrasonic vocalization (Harding and McGinnis, 2005).

Ventral subdivision of the preammillary nucleus

The PMV innervates many major brain regions involved in sexually dimorphic behaviors including the MePD, BNSTp, POA, and VMHvl (Canteras, et al., 1992). Similar to the VMH, lesioning the PMV increases the duration of aggressive attacks in rats (van den Berg, et al., 1983). The PMV may also process information regarding pheromonal cues as exposure to female bedding induces fos expression in the PMV (Yokosuka, et al., 1999).

In summary, each of these brain regions is structurally part of the neural circuit connecting olfactory input with motor output as shown by tracing studies. Many of them, in fact, are strongly interconnected with each other. Additionally, they have each been implicated in sexually dimorphic behaviors either with lesion studies or by fos induction, and many of them are structurally dimorphic as well. Each of these regions also expresses steroid hormone receptors, which play critical roles in sexually dimorphic behaviors.

GONADAL STEROID HORMONES

The sex steroid hormones testosterone and estradiol are both required for the display of male behaviors. Testosterone is the predominant androgen in circulation while estradiol is the major estrogen.

Androgens and the androgen receptor

Testosterone is one of a number of androgens derived from cholesterol through a series of oxidation and reduction steps. In the male, 99% of these androgens are synthesized in the testes and secreted into circulation. There is very little testosterone in female circulation. Testosterone is normally either bound to the steroid hormone binding globulin (SHBG) or be found free in circulation. Unbound testosterone can cross the cell membrane to bind its cognate receptor, the androgen receptor (AR). Unliganded AR is normally bound to chaperone proteins such as hsp90 or hsp70. Once testosterone binds AR, the bound complex can enter the nucleus to bind androgen response elements (AREs) on DNA and act as a transcription factor (reviewed in (Ribeiro, et al., 1995)). AR is expressed in multiple regions of the brain implicated in sexually dimorphic behaviors including the MeA, BNST, POA, and PMV and is expressed in a sexually dimorphic manner in the brain, with more AR in the male BNST and POA compared to females (Shah, et al., 2004).

AR can also bind a metabolite of testosterone, dihydrotestosterone (DHT). Testosterone is converted to DHT via two 5α -reductase isozymes and an increased number of mice lacking both isozymes die before birth (Mahendroo, et al., 1997). Those that survive to adulthood, however, exhibit wildtype male physiology and are fertile (Mahendroo, et al., 2001). As DHT is not aromatizable to estrogen (see below), studying

how DHT regulates male differentiation provides important insight in distinguishing the roles of androgen versus estrogen signaling in male typical behaviors.

Estrogens and the estrogen receptors

In addition to testosterone signaling through the androgen receptor, estrogens and the estrogen receptors are required for regulation of male behavior. While there are low levels of estrogens in male circulation (Hess, 2003), it is known that estradiol is a direct metabolite of testosterone, via conversion by the enzyme aromatase (Naftolin and Ryan, 1975). Similar to testosterone, free estradiol in the cytosol can bind to the estrogen receptors alpha and beta ($ER\alpha$ and $ER\beta$), which then act as nuclear transcription factors. Both ERs are expressed in the hypothalamus and limbic regions including in the MeA, BNST, POA, and VMH (Simerly, et al., 1990).

Previous experiments have demonstrated aromatase conversion of testosterone to estrogen in regions of the rodent brain implicated in sex specific behaviors (Roselli, et al., 1985; Selmanoff, et al., 1977; Wozniak, et al., 1992). RT-PCR and in-situ hybridization studies have also revealed expression of aromatase mRNA in the hypothalamus and limbic regions (Harada and Yamada, 1992; Wagner and Morrell, 1996). Sexual dimorphisms in aromatase expression have been shown, but results from each of these studies are not in agreement (Lauber and Lichtensteiger, 1994; Roselli, et al., 1998; Wagner and Morrell, 1996). Aromatase is a cytochrome P450 and it is difficult to raise antibodies against aromatase that do not cross-react with other members of the CYP450 family. Weak expression of aromatase may also contribute to the inability to localize aromatase using traditional immunocytochemical techniques. Antibody staining has been carried out in the zebra finch and the quail, but these studies have also yielded

conflicting results (Foidart, et al., 1994; Peterson, et al., 2005). To address this problem, we have generated a mouse line with two reporter genes inserted 3' of the untranslated region of the aromatase gene. PLAP, a GPI-linked placental alkaline phosphatase allows for labeling of the membranes, and nuclear lacZ permits visualization of the cell soma. While not disrupting endogenous aromatase expression, this mouse line has allowed us to visualize cellular localization of aromatase in the brain (see Chapter 2).

Testosterone and the AR are thought to be required for male behaviors since both castrated males (who thus have no circulating testosterone) and males with a nonfunctional AR are deficient for these behaviors (Beeman, 1947; McGill and Tucker, 1964; Sato, et al., 2004). Estradiol and the ERs are also necessary for these behaviors. Males harboring a targeted disruption of aromatase (and thus deficient for estradiol synthesis) exhibit severe deficits in male mating behavior (Honda, et al., 1998; Matsumoto, et al., 2003). Aromatase knockout males also display reduced aggressive behavior directed against male intruders (Matsumoto, et al., 2003). ER KO males are also deficient for both mating and aggression (Ogawa, et al., 2000). Both testosterone and estrogen act both early in development to organize the male brain and male typical behaviors and in adulthood to activate male specific behaviors (Goy and McEwen, 1980). However, since testosterone is a precursor of estradiol, the interplay between these two hormones and how they regulate male differentiation becomes more complicated. The remainder of this chapter attempts to synthesize previous experiments which have examined the role of androgen and estrogen signaling in the regulation of male typical differentiation.

DEVELOPMENTAL DIFFERENTIATION OF THE MALE BRAIN AND BEHAVIOR

Organization of the male brain and male typical behaviors by testosterone

Testosterone is required early in life for differentiation of male physiology and behavior. The testes start producing testosterone in the male mouse at about E12.5 (O'Shaughnessy, et al., 1998). Postnatally, there is a surge of testosterone in the first two hours after birth in the male mouse which then subsides to low baseline levels (Corbier, et al., 1992). This early testosterone is required for maintenance of the testes and additional accessory sex organs such as the spinal nucleus of the bulbocavernosus (SNB). Perinatal castration results in degeneration of both the muscles of the SNB as well as the neurons innervating this region (Breedlove and Arnold, 1983). Loss of early testosterone also results in testicular atrophy.

In addition, perinatal testosterone is required for the display of both adult male typical sexual and aggressive behaviors. Rats castrated at birth cease to ejaculate when tested in adulthood and this loss cannot be rescued with exogenous testosterone supplementation later on in life (Gerall, et al., 1967). These males also lose mounting behavior. However this mounting can be re-established, albeit at a much lower extent, with continued testosterone treatment in adulthood (Gerall, et al., 1967). Neonatal castration of mice also results in a significant reduction in male sexual behavior (Quadagno, et al., 1975). Adult aggressive behavior is also under the control of early testosterone as mice castrated neonatally never attack intruder males (Motelica-Heino, et al., 1993) although 20-30% of the mice castrated on the first day after birth will eventually attack opponents when given exogenous testosterone as adults (Bronson and Desjardins, 1969; Motelica-Heino, et al., 1993).

Perinatal testosterone is also sufficient to induce differentiation of male genitalia and elicit male typical behavior. Females normally have no circulating sex steroid hormones at birth. Administration of testosterone to pregnant dams will result in masculinization of both external and internal genitalia of female pups, including an increase in the anogenital distance, increased phallus length, and the induction of prostatic tissue and seminal vesicles (Wolf, et al., 2002). The number of neurons in the SNB is also masculinized in females given testosterone on the second day after birth (Breedlove, et al., 1982). Additionally, females given perinatal testosterone will display both male specific territorial behaviors as well as male typical mounting and intromissive behaviors in adulthood. Females given a single injection of testosterone at birth displayed both mounting and intromission behaviors when presented with another female in estrus (Manning and McGill, 1974). A single dose of testosterone to at birth is also sufficient to elicit attacking behavior in adult female mice towards either male or female intruders (Bronson and Desjardins, 1968). Effects of neonatal testosterone also include repression of female specific physiology and behaviors such as ovulation and female sexual receptivity (Edwards, 1970; Edwards and Thompson, 1970).

Neonatal testosterone is also both necessary and sufficient for sexual differentiation of the brain. Neonatal castration of males leads to a 50% decrease in the volume of the adult SDN-POA (Jacobson, et al., 1981). The volume of the BNST in neonatally castrated males also decreases to that seen in wildtype females (del Abril, et al., 1987). Neonatal castration also decreases the number of dendritic shaft synapses in the male MeA to the number observed in wildtype females (Nishizuka and Arai, 1981b). Conversely, neonatal treatment of females with testosterone increases the volume of the SDN-POA (Jacobson, et al., 1981). However, both pre and postnatal treatment with

testosterone is required for full masculinization of the female SDN-POA (Dohler, et al., 1986). Postnatal testosterone treatment alone is sufficient to increase the volume of the BNST in females to wildtype male levels (del Abril, et al., 1987). Postnatal treatment of testosterone is also sufficient to increase the number of dendritic spine shafts in the female MeA to numbers comparable to the wildtype male (Nishizuka and Arai, 1981b).

Neonatal DHT also plays a role in masculinization of the male brain and behavior. Combined pre- and postnatal DHT treatment alone to female rats is sufficient to masculinize the number of neurons in the SNB as well as cell size and the connections of these neurons in adulthood (Goldstein and Sengelaub, 1992). Administration of neonatal DHT is sufficient to fully rescue mounting behavior and partially rescue intromission but cannot rescue ejaculation (Hart, 1977). Intromission is fully rescued with a combination of neonatal DHT and adult testosterone treatment (Hart, 1979). Taken together, these studies indicate that testosterone is necessary early in development for male differentiation of both brain structures and behavior.

Organization of the male brain male typical behaviors by estrogen

While there is low circulating estrogen in the neonatal rodent, aromatase catalyzed conversion of testosterone to estrogen in the brain has been shown by 2 hours after birth (Amateau, et al., 2004). As stated above, testes start producing testosterone as early as E12.5. The ovaries, on the other hand, are quiescent early in development and there are no circulating gonadal hormones in neonatal females. While females can be exposed to prenatal estrogen secreted by the mother, this estrogen is bound by the plasma glycoprotein alpha-fetoprotein (AFP). AFP has been shown to protect the neonatal female brain from estrogen exposure (Bakker, et al., 2006).

Neonatal estrogen plays a critical role in the regulation of male typical mating. Rats castrated perinatally and then supplemented with one dose of exogenous estrogen regain mounting behavior (Hart, 1977). However, this treatment only partially rescues intromission and does not restore ejaculatory behaviors (Hart, 1977). Perinatal estrogen treatment is sufficient to rescue fertility as well as aggressive behavior in aromatase knockout mice (Toda, et al., 2001a; Toda, et al., 2001b). Neonatal estrogen supplementation to female mice can masculinize aggressive behavior towards intruder males if these females are also treated with hormones in adulthood (Simon and Gandelman, 1978; Simon and Whalen, 1987). Female mice treated with estrogen at birth will also display aggressive behavior towards intruder females if given neonatal estrogen (Bronson and Desjardins, 1968). Neonatal estrogen treatment can also defeminize lordotic behavior in adult females (Edwards and Thompson, 1970; Vale, et al., 1973). These studies demonstrate that estrogen signaling is both necessary and sufficient for male typical behaviors.

Masculine differentiation of the brain is also dependent on neonatal estrogens. Perinatal treatment of female rats with the synthetic estrogen diethylstilbestrol (DES) is sufficient to masculinize the volume of the SDN-POA (Dohler, et al., 1984; Tarttelin and Gorski, 1988). The mechanism for this masculinization appears to be an estrogen mediated increase in cell survival (Arai, et al., 1996), a process first shown in the songbird (Konishi, 1989). The regional volume of the MeA in females treated with estrogen on the day of birth is increased to that of males (Mizukami, et al., 1983). The number of dendritic shaft synapses is also masculinized with neonatal estrogen treatment (Nishizuka and Arai, 1981a). These studies indicate that estrogen signaling

early in development is sufficient to elicit male typical differentiation of the brain and behavior.

ACUTE ACTIVATION OF MALE BEHAVIOR

Activation of male behavior by testosterone

In addition to neonatal effects, testosterone also regulates male typical sexual and aggressive behaviors acutely in adulthood. Male mice castrated in adulthood do not ejaculate (McGill and Tucker, 1964). Additionally, there is a dramatic drop in aggressive behavior in these adult castrated males (Beeman, 1947). This deficit in mating and aggression can be rescued by exogenous testosterone supplementation (Ogawa, et al., 1996; Whalen and Luttge, 1971). Upon cessation of testosterone supplementation, these castrated males no longer exhibit aggressive behavior (Beeman, 1947). Castration also leads to deficits in urine marking behavior that can be rescued with acute exogenous testosterone (Kimura and Hagiwara, 1985).

Adult testosterone can also elicit male typical behaviors in females. Female mice ovariectomized postpubertally and then supplemented with exogenous testosterone display a significant increase in the number of ultrasonic vocalizations and the number of mounts directed towards estrus females (Nyby, et al., 1992). As this mating and vocalization behavior can be elicited in females solely with adult testosterone, this suggests that neonatal testosterone is not required for these behaviors. Circulating testosterone also regulates sexual dimorphisms in the brain. Adult castration reduces the volume of the MePD as well as neuronal size in this region in males. This loss can be rescued with exogenous testosterone treatment (Morris, et al., 2008). Testosterone

treatment of adult females also masculinizes these parameters (Morris, et al., 2008) indicating that acute testosterone is sufficient for these behaviors.

Acute DHT, however, is not sufficient for the display of mating behavior, as males castrated in adulthood and supplemented with DHT alone do not regain mounting, intromissive, or ejaculatory behavior (Whalen and Luttge, 1971). DHT supplementation alone to adult castrated mice is similarly not effective in restoring aggressive behavior (Finney and Erpino, 1976), urine marking behavior (Kimura and Hagiwara, 1985), or ultrasonic vocalizations (Nunez, et al., 1978).

Activation of male behavior by estrogen

Estrogen appears to also elicit male typical behaviors in adulthood. Male rats castrated in adulthood will begin to mount, intromit, and ejaculate after daily supplementation with estrogen (Sodersten, 1973). While this estrogen rescue is dose dependent, estrogen is never as effective as testosterone in restoring male behavior (Sodersten, et al., 1986). Acute activation of male behavior by estrogen may be acting through a transcription independent mechanism as mounting behavior can be elicited in less than an hour (Cross and Roselli, 1999). In fact, acute estrogen is required for male sexual behavior as males castrated and treated with both testosterone and fadrozole, an aromatase inhibitor, exhibit deficits in both intromission and ejaculation (Bonsall, et al., 1992). Adult estrogen treatment is also sufficient to recapitulate aggressive behavior in castrated mice, but not to rescue this behavior to male levels (Finney and Erpino, 1976). Acute estrogen treatment following castration can fully rescue territorial marking behavior (Kimura and Hagiwara, 1985) as well as ultrasonic vocalization (Nunez, et al., 1978).

Regulation of male behavior by both androgen and estrogen signaling

As described above, estrogen or dihydrotestosterone alone do not completely rescue mating or aggression in adult castrate males (Finney and Erpino, 1976; Nunez, et al., 1978; Whalen and Luttge, 1971). However, there are synergistic effects of androgen and estrogen signaling such that DHT and estrogen together fully rescue these behaviors (Feder, et al., 1974; Sodersten, 1973; Whalen and Luttge, 1971). Combined treatment with both estrogen and DHT also rescues aggressive behavior in adult castrated mice to wildtype levels (Finney and Erpino, 1976). Similarly, supplementation with both DHT and estrogen is sufficient to rescue ultrasonic vocalizations to a level indistinguishable from testosterone rescue alone (Nunez, et al., 1978). Taken together, these studies indicate that testosterone is required acutely in adulthood for the activation of male specific behaviors and additionally, that estrogen and DHT in combination is sufficient to elicit these behaviors.

REGULATION OF MALE BEHAVIOR BY HORMONE RECEPTORS

Androgen receptor knockout mice

Like testosterone, the androgen receptor (the cognate receptor for testosterone) is also necessary for male behaviors. Mice bearing the testicular feminization (*tfm*) allele, which is a spontaneous mutation in AR, are completely androgen insensitive (Lyon and Hawkes, 1970). Males bearing the *tfm* mutation have female external genitalia, have testicular atrophy, and lack accessory sex organs such as vas deferens and epididymi (Charest, et al., 1991). *Tfm* males do not display any mating or aggressive behaviors (Ohno, et al., 1974). Genetic AR knockout males have also been generated that recapitulate both the physiological seen in *Tfm* males (Sato, et al., 2004). These males

exhibit no mating behaviors and are severely deficient in aggressive behaviors. One caveat to these AR knockout males is that these males exhibit undescended testes and testicular atrophy, leading to low levels of circulating testosterone (Kerkhofs, et al., 2009; Sato, et al., 2004) and thus low levels of the testosterone metabolites 5 α -dihydrotestosterone (DHT) and estradiol.

Estrogen receptor knockout mice

Estrogen regulates male behaviors by signaling via the estrogen receptors ER α , ER β , and GPR30. Gpr30 knockout mice are fertile (Martensson, et al., 2009; Otto, et al., 2009) but more detailed assays of sex typical behaviors have yet to be assayed. ER α knockout males display a severe deficit in mounting and intromissive behaviors (Ogawa, et al., 1998b). These males never ejaculate in the presence of an estrus female and also never attack an intruder male (Ogawa, et al., 1998b). Although mating behavior could be restored to a low intensity in these mice by exogenous testosterone supplementation, aggression was never elicited (Ogawa, et al., 1998b). ER β knockout males, on the other hand, behave mostly normally. There are no deficits in any parameter of mating or aggressive behavior in these knockout animals (Ogawa, et al., 1999). Mice bearing null alleles of both ER α and ER β (ER $\alpha\beta$ knockout mice) lose all male sexual typical behaviors. They do not mount, intromit, or ejaculate nor do they vocalize towards females (Ogawa, et al., 2000). Similar to ER α knockout animals, ER α,β double knockout males also do not attack intruder males (Ogawa, et al., 2000).

Estrogen and androgen signaling also interact by regulating expression of either receptors or synthetic enzymes in the other pathway. Testosterone can both increase

the number of aromatase expressing cells (Hutchison, et al., 1999) and alter the morphology of these cells (Beyer and Hutchison, 1997) in cultures of fetal mouse hypothalami. Aromatase activity also appears to be regulated by testosterone in the adult rat. Testosterone administration to male rats increases conversion of testosterone to estrogen in wildtype but not Tfm mutant rats (Roselli, et al., 1987). Conversely, sex dimorphic expression of the androgen receptor is under the control of neonatal estrogen in rats (McAbee and DonCarlos, 1999) and mice (see Chapter 3). It remains to be determined whether AR expression is altered in aromatase knockout mice.

Taken together, these data suggest that while testosterone signaling appears to be necessary for male typical behaviors, much of this regulation is mediated by estrogen signaling via the estrogen receptors following conversion of testosterone to estrogen. It does appear that adult AR signaling may regulate some aspects of mating and aggressive behavior, but it may modulate rather than dictate these behaviors. In this thesis, I present data that supports the model that estrogen, which has been synthesized from testosterone, signals via the estrogen receptors to organize masculine differentiation of the brain and behavior. In addition to the necessity for estrogen for acute activation of these behaviors, I also show that testosterone signaling via the androgen receptors serves to modulate the intensity of these behaviors in adulthood.

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Chapter 2

Estrogen masculinizes neural pathways and sex-specific behaviors

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SUMMARY

Sex hormones are essential for neural circuit development and sex-specific behaviors. Male behaviors require both testosterone and estrogen, but it is unclear how the two hormonal pathways intersect. Circulating testosterone activates the androgen receptor (AR) and is also converted into estrogen in the brain via aromatase. We demonstrate extensive sexual dimorphism in the number and projections of aromatase-expressing neurons. The masculinization of these cells is independent of AR but can be induced in females by either testosterone or estrogen, indicating a role for aromatase in sexual differentiation of these neurons. We provide evidence suggesting that aromatase is also important in activating male-specific aggression and urine marking because these behaviors can be elicited by testosterone in males mutant for AR and in females subjected to neonatal estrogen exposure. Our results suggest that aromatization of testosterone into estrogen is important for the development and activation of neural circuits that control male territorial behaviors.

INTRODUCTION

All sexually reproducing species exhibit gender dimorphisms in behavior. Such sex differences can be observed in various displays, including in mating, aggression, territorial marking, and parental care. Behavioral dimorphisms can be observed in socially naive animals, suggesting that sexual differentiation of the underlying neural circuits is tightly controlled by internal physiological regulators. In vertebrates, gonadal steroid hormones play a central role in the development and function of these neural

circuits (Arnold et al., 2003; Goy and McEwen, 1980; Morris et al., 2004). Both testosterone and estrogen are required for male behaviors in many vertebrates, including mammals. It remains to be determined how these two hormonal pathways intersect to control dimorphic behaviors in males (Juntti et al., 2008).

Testosterone is required for male behaviors in most vertebrates, including mice and humans. Testosterone mediates its effects by activating AR and male mice mutant for this receptor do not display sexual behavior or aggression (Ohno et al., 1974). Testosterone is essential in newborn and adult male mice for the display of sex specific behaviors such as aggression (Finney and Erpino, 1976; Peters et al., 1972; Wallis and Luttge, 1975). This testicular hormone is thought to masculinize neural circuits in neonatal rodents, and to act upon these pathways in adult males to permit the display of dimorphic behaviors (Phoenix et al., 1959).

Estrogen is also essential for male behaviors. The requirement for estrogen to masculinize behavior seems counterintuitive because this ovarian hormone is essentially undetectable in the male circulation. Estrogenic steroids are synthesized *in vivo* from testosterone or related androgens in a reaction catalyzed by aromatase. Aromatase-expressing cells in the brain convert circulating testosterone into estrogen, and it is this local estrogen that is thought to control dimorphic behaviors in males (Figure 1A) (MacLusky and Naftolin, 1981; Naftolin and Ryan, 1975). Consistent with a requirement for estrogen in male behaviors, aromatase activity is essential for male behaviors. Mice mutant for aromatase exhibit a profound reduction in male sexual behavior and

aggression (Honda et al., 1998; Toda et al., 2001). Similar to testosterone, estrogen is essential in neonates and adults for the display of dimorphic behaviors in males (Finney and Erpino, 1976; McCarthy, 2008; Scordalakes and Rissman, 2004; Toda et al., 2001; Wallis and Luttge, 1975). Estrogen mediates many of its effects by signaling through the estrogen receptors ER α and ER β , which exhibit overlapping expression patterns, and regulate masculinization of the brain and behavior in a complex, redundant manner (Bodo et al., 2006; Ogawa et al., 1999; Ogawa et al., 2000; Ogawa et al., 2004; Perez et al., 2003; Rissman et al., 1997). The role of a third estrogen receptor, GPR30, in male behaviors is presently unknown (Revankar et al., 2005).

The dual requirement for testosterone and estrogen signaling in male behaviors suggests that these two pathways might interact genetically to control these dimorphic displays. One potential site of interaction is the control of aromatase expression. We have therefore sought to determine whether aromatase expression is regulated by testosterone or estrogen signaling. To visualize aromatase expression at cellular resolution, we have genetically modified the aromatase locus such that all cells expressing this enzyme co-express two reporters, nuclear targeted LacZ and placental alkaline phosphatase, thereby labeling the cell bodies and projections of aromatase-positive neurons.

We find extensive, previously uncharacterized sexual dimorphisms in both the number and projections of neurons expressing aromatase. The masculinization of these neural pathways is independent of AR but can be induced in neonatal females by

testosterone or estrogen, indicating that aromatase plays an important role in the sexual differentiation of these neurons. In addition, testosterone activates male typical fighting and urine marking independent of AR, demonstrating that the differentiation and function of the neural circuits underlying these behaviors are governed by testosterone, at least in part, after its conversion into estrogen. Finally, our results show that adult gonadal hormones of either sex can support male territorial marking and fighting provided estrogen has neonatally masculinized the underlying neural circuitry.

RESULTS

Aromatase is expressed in a sparse manner in the mouse brain

To define where testosterone may be converted into estrogen in the mouse brain, we sought to characterize the expression of aromatase at cellular resolution. We used homologous recombination in ES cells to insert an *IRES-plap-IRES-nuclear lacZ reporter* cassette into the 3' untranslated region (UTR) of the aromatase locus (Figure 1B). The use of IRES elements permits faithful expression of PLAP and nuclear β -galactosidase (β gal) in cells transcribing the targeted allele (Shah et al., 2004). This strategy maintains the expression and function of aromatase, thereby permitting the examination of neural pathways expressing this enzyme in otherwise wildtype (WT) animals. In sharp contrast to *aromatase*^{-/-} animals, mice homozygous for the *aromatase-IRES-plap-IRES-nuclear lacZ* (aromatase-IPIN) allele are fertile and behaviorally similar to their WT littermates, and they have normal levels of serum testosterone and estrogen (Supplemental Text).

Analysis of β gal activity in the brain reveals small pools of cells that account for less than 0.5% of neurons in the brain (Figures 1C-1F). We observe β gal labeled cells in discrete locations, including the posteromedial component of the bed nucleus of the stria terminalis (BNST), posterodorsal component of the medial amygdala (MeA), preoptic hypothalamus (POA), and lateral septum. The expression of β gal mirrors the distribution of aromatase mRNA as revealed by in situ hybridization (Figure S1) and RT-PCR (Harada and Yamada, 1992). The absence of sensitive, specific antibodies to aromatase precludes colocalization studies with β gal in single cells. Although in situ hybridization (ISH) reveals low levels of aromatase mRNA with poor signal:noise, the activity of our reporters is robust and offers superior cellular resolution (Figure S2). In addition, labeling the brain for PLAP activity reveals the soma and projection fibers of neurons expressing aromatase (Figure S3).

Aromatase-expressing neurons display extensive sexual dimorphism

A comparison of aromatase-positive cells in adult male and female mice bearing the aromatase-IPIN allele reveals several previously undescribed sexual dimorphisms. We find sex differences in both the number and projection patterns of aromatase expressing neurons. We find more aromatase-positive cells in the BNST and MeA in males compared to females (Figures 2A-2D and 2P). These two regions have previously been shown to regulate sexual and aggressive behaviors (Albert and Walsh, 1984; Kondo et al., 1998; Liu et al., 1997). We also observe sexual dimorphism in cell number in the caudal hypothalamus, where we observe more aromatase-expressing cells in females

compared with males (Figures 2E, 2F, and 2P). The presence of more aromatase-positive neurons in the female caudal hypothalamus is surprising as there is little testosterone in the female circulation that could serve as a substrate for conversion to estrogen. In any case, most β gal positive cells ($> 95\%$; $n \geq 3$) in these dimorphic clusters express the pan-neuronal marker NeuN, indicating that they are neurons. A similar proportion of NeuN-positive cells in the BNST and MeA expresses β gal in both sexes, indicating an absolute increase in the total number of neurons in these regions in males (Supplemental Text).

Labeling for PLAP activity also revealed sex differences in the fibers of aromatase-expressing neurons. Consistent with the dimorphisms in cell number, PLAP labeling reveals a richer plexus of fibers in the BNST and MeA in males and in the caudal hypothalamus in females (Figure S3). We also observe previously undescribed dimorphic processes in the anterior hypothalamic (AH) region and the ventromedial hypothalamus (VMH), with PLAP labeled fibers occupying a larger volume in males compared with females (Figures 2G-2J and 2P). In contrast to the poorly characterized anterior hypothalamic region, the VMH is known to regulate feeding and sexual behaviors (Musatov et al., 2006; Musatov et al., 2007). Within the limits of detection, the dimorphisms in PLAP activity are unlikely to reflect differences in aromatase expression levels as extended staining did not reveal additional fibers in the female brain. Both the anterior and ventromedial hypothalamic regions contain a few β gal-positive cells in both sexes (Supplemental Text), indicating that the dimorphisms may reflect an increase in arborization of local neurons or projections from distant aromatase expressing neurons. There are more synapses on VMH neurons in males (Matsumoto and Arai, 1986), and

our findings suggest that aromatase positive neurons may contribute to this dimorphic innervation. The pattern of PLAP-labeled fibers surrounding the VMH resembles afferent input to this structure from the BNST, MeA, POA, and septum (Canteras et al., 1995; Choi et al., 2005; Dong and Swanson, 2004; Millhouse, 1973; Simerly and Swanson, 1988; Varoqueaux and Poulain, 1999). Each of these afferent regions expresses aromatase (Figures 1 and S1), and might contribute to the dimorphic PLAP labeling in the VMH. Taken together, our studies using genetically encoded reporters reveal previously undescribed sex differences in aromatase-expressing neural pathways.

Sexual differentiation of aromatase-expressing neurons is independent of AR

We tested whether masculinization of aromatase-positive neural pathways requires the activity of androgens such as testosterone signaling through AR. We crossed mice carrying the aromatase-IPIN allele to animals harboring the *tfm* allele, a loss-of-function allele of the X-linked AR (Charest et al., 1991). We find male typical differentiation of aromatase-positive neurons in *Tfm* mutant males. The number of β gal-positive neurons in the BNST, MeA, and caudal hypothalamus is similar between *Tfm* and WT males (Figures 3A-3F and K). Staining for PLAP activity labels male typical projection patterns in the VMH and the anterior hypothalamic area of *Tfm* mutant males (Figures 3G-3K). These findings demonstrate that masculinization of the number and projections of aromatase-expressing neurons is largely independent of testosterone signaling through AR.

Estrogen masculinizes aromatase-expressing neurons

We next sought to determine the influence of estrogen in establishing the sexual dimorphisms in aromatase-positive neurons. The complexity and redundancy within estrogen signaling pathways makes it difficult to test the relevance of individual ERs in establishing dimorphisms in aromatase expression. We therefore asked if estrogen is sufficient to masculinize aromatase expression in females. Because estrogen influences sexual differentiation of neural pathways in the early neonatal period in male rodents (McCarthy, 2008), we treated female pups bearing the aromatase-IPIN allele with estrogen at P1 (postnatal day 1, the day of birth), P8, and P15. A cohort of control females was administered vehicle (NV) at these time points. The dosage and injection schedule were chosen as being the most effective at masculinizing aromatase positive neurons. We find that in females administered estrogen neonatally (NE), aromatase expressing neural pathways appear indistinguishable from those observed in WT males (Figure 4). These data demonstrate that neonatally administered estrogen can masculinize both the cell number and projection patterns of aromatase-expressing neurons.

Our results show that the masculinization of aromatase-positive neurons is independent of AR (Figure 3) and can be induced in females by neonatal estrogen (Figure 4). In neonates, the ovaries are quiescent whereas the testes generate a surge in circulating testosterone immediately after birth (McCarthy, 2008). We therefore hypothesized that aromatization of testosterone into estrogen masculinizes aromatase-positive neurons during this period. To test this hypothesis, we provided testosterone to neonatal females. We find that testosterone administration is equivalent to estrogen supplementation in masculinizing aromatase expressing neural pathways (Figure S6A),

suggesting that testosterone induces male differentiation of these neurons after its conversion into estrogen *in vivo*.

Estrogen promotes cell survival in the neonatal BNST and MeA

We wished to uncover the mechanism whereby estrogen drives sexual differentiation of aromatase-positive neurons. An equivalent number of cells expresses aromatase in both sexes in the BNST and MeA at P1, whereas there are more β gal-positive cells in these regions in males compared to females at P14 (Figures 5A-5E, and data not shown). Neurons in the BNST and MeA are born prenatally in rodents (al-Shamma and De Vries, 1996; Bayer, 1980), and we therefore asked if the sexual dimorphisms in these regions resulted from sex specific apoptosis. We labeled apoptotic nuclei in the neonatal BNST and MeA with the TUNEL assay. We find more TUNEL-positive cells in the BNST in females compared to males at several time points from P1 to P10, a finding consistent with previous work (Gotsiridze et al., 2007). Moreover, we observe a similar increase in TUNEL positive cells in the MeA in females compared with males at these timepoints (Figures 5F-5I). The most significant sex difference in cell death was observed at P4 in the MeA and P7 in the BNST, with a ~2 fold increase in apoptotic nuclei in females compared with males (Figure 5J). Because the conditions for TUNEL preclude co-labeling for β gal, we immunolabeled sections through the BNST and MeA for β gal and effectors of apoptosis (using a cocktail of antibodies to activated Caspase-3, Caspase-9 and Apaf-1) in P7 mice bearing the aromatase-IPIN allele. In accord with results of the TUNEL assay, these effectors of apoptosis label significantly more cells in females than in males in the BNST and MeA (≥ 2 fold in each region; $n=3$; $p < 0.001$).

We find many apoptotic cells expressing β gal in both sexes, with significantly more double-labeled cells present in females compared to males (% apoptotic cells expressing β gal in BNST: females, 50 ± 1 ; males, 26 ± 2 ; $n = 3$; $p < 0.001$. % apoptotic cells expressing β gal in MeA: females, 54 ± 2 ; males, 35 ± 3 ; $n = 3$; $p < 0.01$).

Because neonatally administered estrogen is sufficient to masculinize aromatase-positive neurons in the female BNST and MeA, we asked whether such supplementation promoted the survival of cells fated to die in these regions. Neonatal estrogen treatment promotes cell survival such that the number of TUNEL-positive nuclei in the BNST and MeA is indistinguishable between NE females and WT males (Figure 5J). Such cell survival promoting effects of estrogen are reminiscent of the known neuroprotective effects of this hormone (Arai et al., 1996). Is estrogen synthesis essential for cell survival in the BNST and MeA? To address this issue, we analyzed cell death in male mice null for aromatase (Honda et al., 1998). In these males, the number of apoptotic figures is comparable to that observed in WT females, and significantly different from WT males (Figure 5J). In summary, we observe more cell death in the BNST and MeA in females than in males, with an increase in the survival of aromatase expressing cells in males compared to females. Further, administration of estrogen to female pups reduces cell death whereas abrogation of estrogen synthesis increases apoptosis in males, demonstrating that estrogen is necessary and sufficient to promote cell survival in the BNST and MeA.

Neonatal estrogen exposure masculinizes territorial but not sexual behavior

We tested adult NE females bearing the aromatase-IPIN allele to determine whether sex specific behaviors were also masculinized. Males reliably mate with females at high frequency whereas females exhibit male pattern sexual behavior at a low frequency towards females (Baum et al., 1974; Jyotika et al., 2007; Spors and Sobel, 2007). NE and NV females were individually housed as adults and presented with a WT female in estrus. Some resident females, regardless of hormone treatment, mated with the intruder, showing no apparent differences in mounting or pelvic thrusts, which are indicative of intromission (penetration) in males. In sharp contrast to resident males, both NE and NV females mated with intruders in fewer assays, consistent with the notion that neonatal estrogen does not alter the low frequency of male sexual behavior in females (Figure 6C) (Burns-Cusato et al., 2004; Vale et al., 1973).

We next examined NE females for male territorial behaviors. Individually housed male but not female residents attack male intruders (resident intruder aggression test) (Miczek et al., 2001). In striking contrast to NV females who never displayed aggression, we find that, like males, most NE female residents attack a male intruder (Figure 6A; Movies S1 and S2). Similar to male residents, NE females initiated bouts of biting, chasing, wrestling, and tumbling. The aggression displayed by NE animals is not a response to mating attempts by the male: in most assays (73%; n = 11 assays) the fighting preceded any mating attempt, and most individual attacks (93%; n = 90 events) were not preceded by sexual behavior. As part of territorial defense, resident male mice mark their territory by scattering many urine drops across the cage floor, whereas females pool their urine at the cage perimeter (Desjardins et al., 1973; Kimura and Hagiwara, 1985). NE females deposit urine in a pattern resembling that of males

(Figures 6D-6G). Unlike NV females who pool urine, NE mice scatter significantly more urine drops, with a large fraction of such drops deposited away from the perimeter. Because neonatal testosterone exposure mimicked neonatal estrogen exposure in masculinizing aromatase-expressing circuits, we wished to test if neonatal testosterone would also mimic the effects of neonatal estrogen on territorial behaviors. Our data show that neonatal testosterone and estrogen are equivalent in eliciting male territorial behaviors in females (Figures S6B-S6D). Taken together, these results suggest that neonatal testosterone masculinizes territorial marking and aggression, at least in part, after its aromatization into estrogen *in vivo*.

We next asked whether NE females are sexually receptive to males. The standard test of female mating involves surgical removal of the ovaries (ovariectomy) followed by estrogen and progesterone injections to induce estrus on the day of testing (Beach, 1976). Accordingly, we hormonally primed ovariectomized females treated neonatally with estrogen (NEAOP) or vehicle (NVAOP), and presented them to male residents. Male mice vigorously mount females, but only receptive females permit such mounts to proceed to intromission. Thus, one measure of female receptivity is the ratio of intromissions to the total number of mounts (receptivity index). There was a large reduction in the receptivity index and the duration of intromissions in NEAOP females compared to NVAOP controls (Figures 6I and 6J), consistent with the notion that neonatal estrogen defeminizes sexual receptivity (Whalen and Nadler, 1963). Unlike NVAOP mice, NEAOP females actively rejected mounts, often attacking and chasing the male (Figure 6I). This reduction in sexual behavior does not reflect a lack of interest from males because these residents attempted to mate with NEAOP or NVAOP females

in most assays (Figure 6H). Moreover, the low receptivity index of NEAOP females resulted from an absolute increase in the number of mounts (Figure 6J), demonstrating the males' interest in mating with these mice. Thus, neonatal estrogen treatment appears to permanently defeminize sexual receptivity, even under estrus-inducing hormonal conditions.

Gonadal hormones are required to activate the display of sex specific behaviors such as aggression in adults (Beeman, 1947; Goy and McEwen, 1980; Morris et al., 2004). However, we observe male type fighting and urine marking in NE females in the absence of adult supplements of sex steroids. We hypothesized that male-type fighting and urine marking may be activated by ovarian hormones. We directly tested this by ovariectomizing a cohort of adult NE animals: such females did not fight (0/5 females attacked males) or scatter urine (5/5 females pooled urine), demonstrating that the masculinized brain in NE animals utilizes ovarian hormones to activate these male behaviors. In summary, NE females exhibit a dissociation of sex typical behaviors: they do not mate like females or males but they display masculinized patterns of aggression and urine marking in the presence of ovarian hormones.

Neonatal estrogen exposure masculinizes the behavioral response to testosterone independent of AR

There are significant quantitative differences in fighting and urine marking between NE females and WT males (Figures 6B and 6G). We hypothesized that such differences reflect the low levels of circulating testosterone in NE mice. Testosterone titers are

equivalent between NE and NV females, and > 10 fold lower than male titers. By contrast, all three groups of animals have similar, low baseline levels of estrogen, with periodic elevations of estrogen in the females that presumably accompany estrus (Supplemental Text). We masculinized circulating testosterone levels by providing this hormone to adult, ovariectomized females that were treated neonatally with estrogen (NEAOT) or vehicle (NVAOT) (Serum testosterone: males, 5.8 ± 1.4 nM; NEAOT females, 8.6 ± 3 nM; NVAOT females, 9.1 ± 4.3 nM; $n = 4$; $p > 0.42$). In resident intruder aggression tests, we find that NEAOT but not NVAOT females attack male intruders (Figure 7A; Movies S3, S4). Moreover, the pattern, frequency, and duration of attacks were similar between NEAOT and male residents (Figure 7B). The number and pattern of urine marks were also indistinguishable between NEAOT females and males (Figure 7C). These findings demonstrate that neonatal estrogen exposure masculinizes the response to testosterone in adults, and that male typical levels of testosterone augment the degree of male territorial displays without substantially altering the nature of these behaviors.

These data show that adult testosterone administration is sufficient to activate male territorial behaviors in NE females, consistent with a functional role for aromatase and, potentially, aromatase-expressing neural circuits in these mice. It is also possible, however, that neonatal estrogen treatment masculinizes AR positive pathways in the brain, which in turn respond to adult testosterone and activate male territorial displays. We therefore examined the behavioral response of adult AR mutant (Tfm) males to testosterone administration. At birth, AR mutants have normal titers of testosterone (Sato et al., 2004), thereby leading to the development of a male pattern of aromatase-

expressing neurons following local conversion into estrogen (Figure 3). However, these mutants subsequently develop testicular atrophy, resulting in extremely low levels of circulating testosterone in adult life (Sato et al., 2004). As adults, these mutants do not attack intruders and they pool urine at the cage perimeter (Ohno et al., 1974) (Scott Juntti and NMS, unpublished observations). We find that provision of testosterone to adult Tfm males (TfmAT) significantly increases the number of urine marks compared to mutants administered vehicle (TfmAV) (Figure 7F). The number of urine spots was lower compared to WT males, indicating that a male typical frequency of urine marking may require additional contributions from AR signaling. Although TfmAT males deposited more urine spots in the cage center compared to TfmAV controls, this trend did not reach statistical significance, suggesting AR signaling is essential for this component of territorial marking. In contrast to this complex control of male urine marking, we find that TfmAT mice attack intruders in the resident intruder assay similar to WT residents. Indeed, all TfmAT males attacked the intruder in most assays, whereas none of the TfmAV residents initiated attacks (Figure 7D). Moreover, both the frequency and duration of the attacks were similar between WT and TfmAT males (Figure 7E). Taken together, these results provide evidence that testosterone elicits many components of male territorial behaviors in adult animals independent of AR.

DISCUSSION

We have used genetic reporters to visualize aromatase-expressing neural pathways. Our reporters reveal aromatase expression at cellular resolution in discrete pools in

regions previously shown to express aromatase (Roselli et al., 1998; Wagner and Morrell, 1996). This small set of aromatase-positive neurons is therefore likely to influence the diverse neural circuits that utilize estrogen signaling to control male behaviors. The sensitive nature of the reporters reveals previously unreported sex differences in the number and projections of aromatase positive neurons. Even within regions such as the BNST, only a subset of cells expresses aromatase, suggesting functional specialization within these large dimorphic neuronal pools (Hines et al., 1992; Morris et al., 2008; Shah et al., 2004). Indeed, most aromatase positive cells in the BNST ($98\% \pm 0.5$; $n = 3$) express AR, whereas only a subset of AR positive neurons co-labels with aromatase ($35\% \pm 2$; $n = 3$). The reciprocal connectivity between the aromatase expressing regions we have identified suggests the interesting possibility that aromatase positive neurons might form an interconnected network that regulates sexually dimorphic behaviors.

Our results show that aromatization of testosterone into estrogen plays an important role in the development of aromatase expressing neural pathways and in activating male territorial behaviors. However, the relevance of the dimorphisms in aromatase-positive neurons to male territorial behaviors remains to be determined. These neurons may serve as a dimorphic neuroendocrine source of estrogen or they may directly participate in the circuits that control male behaviors. It is unlikely that such dimorphisms are required solely to provide a dimorphic source of local estrogen because NE females exhibit male territorial displays in response to circulating ovarian hormones. Consistent with the notion that these cells might function within neural circuits that mediate dimorphic behaviors, many aromatase positive neurons also express ER α in the

BNST and MeA (Figure S4 and data not shown). It will be important, in future studies, to understand the functional significance of the sex differences in cell number and connectivity that we have identified in this study.

Masculinization of aromatase positive neural pathways and territorial behavior is governed by aromatase

We find that neonatal estrogen exposure masculinizes aromatase-expressing neurons and territorial behaviors in females. These results seem counterintuitive because one would expect that estrogen produced by the neonatal ovaries should induce male typical differentiation in all WT females. In fact, the ovaries are quiescent in neonates (McCarthy, 2008). By contrast, males experience a neonatal surge in circulating testosterone, leading to a corresponding increase in estrogen in the brain via local aromatization (Amateau et al., 2004). Our provision of estrogen to female pups therefore exposes their brains to this hormone during a period when only males would experience a local rise in estrogen. Such plasticity of the female brain to the masculinizing effects of estrogen is transient because adult females do not have a male pattern of aromatase expression despite the spikes in estrogen within the ~4 day estrous cycle. Indeed, we find that estrogen administration to adult WT females does not masculinize aromatase-positive neurons and behavior (Figure S5 and data not shown). In addition, our finding that neonatal testosterone functions to masculinize aromatase positive neural pathways and territorial behaviors (Figure S6) suggests that the masculinizing effects of neonatal estrogen treatment are unlikely to be a gain-of-function of estrogen signaling, but rather reflect, at least in part, the physiological conversion of testosterone into estrogen by aromatase. Such locally derived estrogen may also

influence the differentiation of other, aromatase-negative neuronal pools, which might play an important role in the subsequent display of sex-typical behaviors.

Our results do not exclude the possibility that androgen and estrogen signaling masculinize aromatase-expressing neurons in a redundant manner. Previous work in rats indicates that testosterone signaling can upregulate aromatase activity (Roselli et al., 1987). These biochemical studies are not incompatible with our data as the expression of β gal and PLAP report cell number and fiber projections of neurons expressing aromatase and not aromatase activity. Nevertheless, we find that restoring circulating testosterone titers to WT male levels in AR mutants is sufficient to elicit male typical aggression and some components of urine marking. Taken together, our results demonstrate that estrogen synthesis in neonatal and adult life is sufficient to masculinize aromatase-expressing neurons and territorial behaviors independent of AR.

The cellular mechanism of estrogen action

The dimorphic projections in the anterior hypothalamus and VMH could arise from sex-specific neurite outgrowth or retraction. However, both regions contain aromatase-expressing neurons in the postnatal period, making it difficult to distinguish the sexually dimorphic fibers from the processes of local neurons. The small number of aromatase-expressing cells in the caudal hypothalamus makes it difficult to elucidate the mechanism underlying the sex difference in this region. Increased survival of aromatase-positive cells in the neonatal male BNST and MeA likely accounts for the dimorphism in cell number in these regions. Moreover, estrogen is necessary and

sufficient to promote such cell survival in vivo. Most aromatase-expressing cells in the neonatal BNST and MeA also express ER α (Figure S4). Both regions also express ER β (Figure S4), suggesting that estrogen may mediate cell survival of aromatase-positive cells by signaling through one or more classes of receptors in a cell-autonomous manner. Irrespective of the mechanism underlying cell survival, our study demonstrates that estrogen ultimately acts on the very cells that synthesize this hormone to promote their sexual differentiation in a positive feedback manner.

Separable components of gender related behaviors

Several research groups have recently provided insight into the molecular mechanisms underlying sex-specific behaviors in fruit flies (Manoli et al., 2005; Stockinger et al., 2005; Vrontou et al., 2006). These studies show that the repertoire of sexually dimorphic displays in *Drosophila* appears to be regulated in an unitary manner by Fruitless, a putative transcription factor. In contrast to flies, and similar to humans (Byne, 2006; Hines, 2006), we find that dimorphic behaviors can be dissociated in mice: neonatal estrogen exposure masculinizes territorial but not sexual behaviors. The male typical fighting displayed by females treated neonatally with estrogen is unlikely to result from altered gender discrimination as these mice direct their aggression, like WT males, exclusively towards male intruders. What controls male sexual behavior? Previous work has demonstrated that testosterone supplementation to adult female mice is sufficient to elicit male mating behavior (Edwards and Burge, 1971). Consistent with these studies, we find that the majority of NEAOT and NVAOT residents exhibit male sexual behavior towards estrus intruders (5/5 NEAOT and 4/5 NVAOT females mated with estrous mice; $p = 0.29$, Chi-squared test). Such mating attempts were displayed in

≥ 70% of assays by both cohorts of females, a frequency that is comparable to WT males. These findings further underscore the notion that the neural circuits that mediate sexual and territorial behaviors are regulated by distinct hormonal and temporal mechanisms.

Previous work has demonstrated that females treated neonatally with estrogen fight with males in resident intruder assays (Simon et al., 1984). Such studies coupled neonatal and adult hormonal interventions, making it difficult to understand the long-term behavioral consequences of neonatal estrogen exposure. We find that females treated solely with neonatal estrogen display masculinized patterns of fighting and urine marking in the presence of sex hormones produced by the ovaries. Administration of testosterone to these females to mimic normal male circulating titers of this hormone increases these behavioral displays to approximate the levels observed in males. These results indicate that the adult hormonal profile produced by the testes may not be instructive for male territorial behaviors: hormones produced by the adult gonads of either sex support male patterns of fighting and territorial marking provided that neonatal estrogen has masculinized the underlying neural circuits.

Marking behavior defines a range within which the animal will defend resources and advertise its social and reproductive status (Ralls, 1971). Sex differences in territorial marking appear to be innate and mice display dimorphic urine marking patterns even in social isolation (Desjardins et al., 1973; Kimura and Hagiwara, 1985), providing an objective assessment of what appears to be an internal representation of sexual

differentiation of the brain. Females treated neonatally with estrogen fight and mark territory like males, demonstrating masculinization of social and solitary sex-typical behaviors.

Circulating testosterone and locally derived estrogen in the brain are critical for the expression of male behaviors. It has been difficult to determine the individual contributions of these two hormones to masculinization of the brain and behavior. Our gene targeting strategy has allowed us to identify at cellular resolution the small population of aromatase expressing neurons that can synthesize estrogen from testosterone. Testosterone appears to serve, at least in part, as a pro-hormone for estrogen for the male typical differentiation of aromatase-positive neurons and for masculinization of territorial behaviors. The genetic marking of this discrete set of aromatase-expressing neural pathways should ultimately permit us to functionally link them with distinct sex-specific behavioral outcomes.

EXPERIMENTAL PROCEDURES

Generation of mice bearing a modified aromatase allele

The *IRES-plap-IRES-nuclear lacZ* reporter was inserted into the 3' UTR of the aromatase locus using previously described strategies (Supplemental Experimental Procedures) (Shah et al., 2004). All experiments involving animals were in accordance with IACUC protocols at UCSF.

Hormone supplementation

We injected steroid hormones into all females within a litter to test the effects of these steroids on neuronal differentiation and behavior. We injected pups subcutaneously with 5 µg of 17β-estradiol benzoate (EB) (Sigma) or 100 µg of testosterone propionate (TP) (Sigma) dissolved in 50 µL of sterile sesame oil (Sigma) at P1, P8, and P15. Control females in other litters were injected with 50 µL vehicle at the same time points.

To generate NEAOT and NVAOT mice, we ovariectomized adult NE or NV females and allowed them to recover for three weeks. We injected 100 µg of TP dissolved in 50 µL sesame oil subcutaneously on alternate days in these animals. Such animals were used for behavioral testing ≥ 3 weeks after initiating TP injections. The same injection regimen was used to generate TfmAT and TfmAV males. Hormone titers were assayed with kits from Cayman Chemicals (estradiol) and DRG International (testosterone).

Histology

Sexually naive, group-housed, age-matched mice were used in all histological studies. PLAP or β gal activity was visualized in 80 μ m (adult) or 12 μ m (neonate) thick brain sections obtained from mice homozygous for the aromatase-IPIN allele. Immunolabeling was performed on 65 μ m (adult) or 20 μ m (neonate) thick brain sections obtained from mice heterozygous for the aromatase-IPIN allele. We used previously described protocols to process these sections for histochemistry or immunolabeling (Shah et al., 2004). Message for aromatase, ER α , and ER β was localized by ISH as described in Supplemental Experimental Procedures. Sections of 16 μ m thickness were processed for TUNEL according to the manufacturer's instructions (Chemicon). In these studies, we processed in parallel at least one animal of each sex or experimental manipulation (WT and Tfm males; NE and NV females; NT and NV females) bearing the aromatase-IPIN allele and one control animal with an unmodified aromatase locus. Quantitation of cell numbers and fiber innervation was performed using unbiased stereology and other approaches (Supplemental Experimental Procedures). All histological analysis was performed by an investigator blind to sex, age, genotype, and hormone treatment.

Behavioral Assays

We used 10 - 24 week old singly housed mice in behavioral tests, which were done \geq 1 hr after lights were switched off. Mice were first tested for male sexual behavior in their home cage in a 30 min assay with an estrous female. The residents were subsequently tested for territorial marking. Mice were allowed to explore a fresh cage lined with

Whatman filter paper for one hr, and then returned to their homecage. The marking pattern was visualized with UV transillumination. Residents were subsequently tested for aggression directed towards a WT intruder male for 15 min. NE and NV females were then ovariectomized and tested for female sexual behavior after estrus induction in the homecage of a sexually experienced male for 30 min. Each animal was tested twice for sexual behavior and aggression, allowing us to analyze the total fraction of assays in which these behaviors were observed. We always exposed the experimental animals to mice they had previously not encountered, and individual assays were separated by ≥ 2 -3 days. A separate cohort of NE and NV females was used to generate NEAOT and NVAOT mice. All tests were scored by an experimenter blind to the sex, genotype, and hormone treatment of mice, using a software package we developed in Matlab.

Statistical Analysis

We used the chi-square test to determine whether the proportion of experimental animals exhibiting a particular behavior was significantly different from control subjects. All other experimental comparisons were analyzed using both parametric (Student's t test) and non-parametric (Kolmogorov-Smirnov, ks-test) tests of significance. All statistically significant results presented in the text ($p < 0.05$) using the Student's t test were also determined to be statistically significant with the ks-test.

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FIGURE LEGENDS

Figure 1. Visualizing aromatase expressing neurons in the mouse brain

(A) Sex steroid hormone control of male behaviors. **(B)** Schematic of genetic modification of the aromatase locus. **(C-F)** Coronal sections through the forebrain of an adult male homozygous for the aromatase-IPIN allele stained for β gal activity. Aromatase is expressed in a sparse manner in discrete regions including the lateral septum (C), preoptic area (POA) (D), bed nucleus of the stria terminalis (BNST) (E), and medial amygdala (MeA) (F). Scale bar equals 2.5 mm. Inset scale bars equal 200 μ m (C, D) and 50 μ m (E, F).

Figure 2. Extensive sexual dimorphism in aromatase-expressing neural pathways

(A-J) Coronal sections through the adult brain of male and female mice harboring the aromatase-IPIN allele stained for β gal (A-F) or PLAP activity (G-J). There are more aromatase+ cells in the male BNST and MeA, and in the female caudal hypothalamus (CH). PLAP+ fibers occupy a larger volume in the male AH (white arrow) and in the region (arrows) surrounding the VMH (dashed outline). Scale bars equal 500 μ m (A, B) and 250 μ m (C-J). **(K-O)** Nissl stained sections depicting locations of the BNST, MeA, CH, AH, and VMH. **(P)** Quantitation of sexual dimorphism in cell number (β gal+) and fiber tracts (PLAP+) of neurons expressing aromatase. Mean and standard error (SEM) are shown; $n \geq 3$; * $p \leq 0.015$, ** $p \leq 0.005$.

Figure 3. Masculinization of aromatase-expressing neurons is independent of AR

(A-J) Coronal sections through the adult brain of males bearing the aromatase-IPIN allele and a WT or a loss-of-function allele (*tfm*) of AR stained for β gal (A-F) or PLAP activity (G-J). Scale bars equal 500 μ m (A, B) and 250 μ m (C-J). **(K)** There is no significant difference in the number of β gal⁺ cells or in the volume occupied by PLAP⁺ fibers between WT and Tfm males. Mean \pm SEM; n \geq 3; p \geq 0.26.

Figure 4. Estrogen masculinizes aromatase-expressing neural pathways

(A-J) Coronal sections through the brain of adult NE and NV females bearing the aromatase-IPIN allele stained for β gal (A-F) or PLAP activity (G-J). Scale bars equal 500 μ m (A, B) and 250 μ m (C-J). **(K)** Increase in β gal⁺ cell number and volume occupied by PLAP⁺ fibers in NE females. Horizontal dashed lines represent the mean values in males as determined from the data in Figures 2 and 3. Mean \pm SEM; n \geq 3; * p \leq 0.028, ** p \leq 0.003; these p values were obtained for the comparisons between NE and NV females.

Figure 5. Estrogen promotes cell survival in the neonatal BNST and MeA

(A-D) Coronal sections through the brain of P1 male and female mice bearing the aromatase-IPIN allele stained for β gal activity. Scale bars equal 250 μ m. **(E)** No sex difference in the number of β gal⁺ cells at P1. Mean \pm SEM; n = 3; p \geq 0.63. **(F-I)** Adjacent coronal sections through the MeA of P4 male and female mice bearing the aromatase-IPIN allele immunolabeled for β gal or stained for apoptosis with TUNEL (arrows). Arrowhead (I) indicates autofluorescent material. Scale bars equal and 100

μm (F-I). **(J)** There are more apoptotic cells in the BNST and MeA in control (NV) females and *aromatase*^{-/-} (KO) males compared to control (WT) males and estrogen treated (NE) females. Mean \pm SEM; n = 3; * p \leq 0.004.

Figure 6. Estrogen masculinizes territorial but not sexual behavior

(A and B) WT males and NE females attack intruders in resident intruder aggression tests. Resident males fight with greater frequency and for longer duration (B). **(C)** WT males and NE and NV females exhibit male sexual behavior towards estrous intruders. Males mate in more assays than females. **(D-G)** WT males and NE females scatter many urine drops, the majority of which are located away from the cage perimeter (% central spots). NV females deposit urine in one or a few large pools (arrow) near cage walls. **(H)** WT males mount and intromit (Introm.) both NEAOP and NVAOP females equivalently (n \geq 6; p \geq 0.121). **(I)** NEAOP females have a low receptivity index and attack resident males in many assays. **(J)** Resident males mount NEAOP females more frequently and for a longer duration but intromit for a shorter duration, consistent with lowered sexual receptivity. Mean \pm SEM; n \geq 6; * p \leq 0.037, ** p \leq 0.022, *** p \leq 0.009.

Figure 7. Neonatal estrogen masculinizes the response to adult testosterone administration

(A and B) NEAOT resident females attack intruder males equivalently to WT male residents. NVAOT females do not initiate attacks. **(C)** NEAOT females deposit many urine drops away from the cage perimeter, in a manner equivalent to WT males. **(D and E)** TfmAT residents attack intruder males in a manner similar to WT male residents.

TfmAV residents do not initiate attacks. **(F)** TfmAT males deposit significantly more urine drops than TfmAV males. Horizontal dashed lines denote the mean values of these behavioral displays in WT males. Mean \pm SEM; $n \geq 5$; * $p \leq 0.031$, ** $p \leq 0.002$; these p values were obtained for the comparisons between NEAOT and NVAOT females, and TfmAT and TfmAV mutant males.

Figure 1

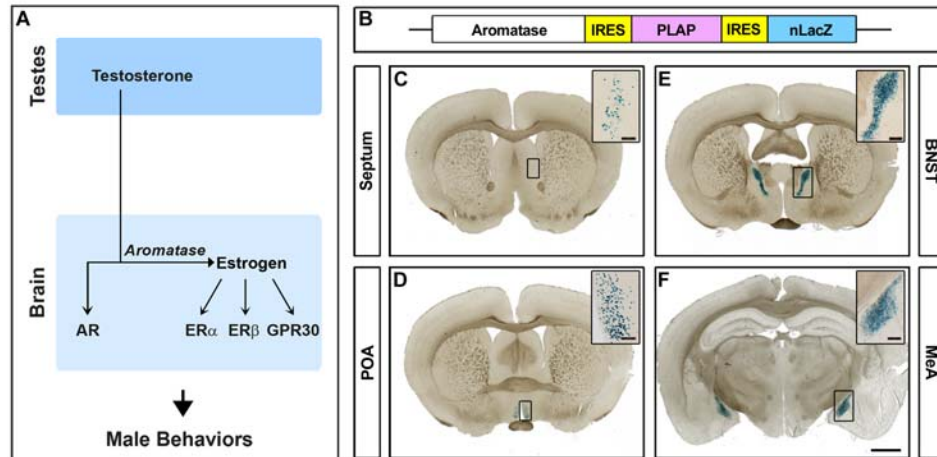


Figure 2

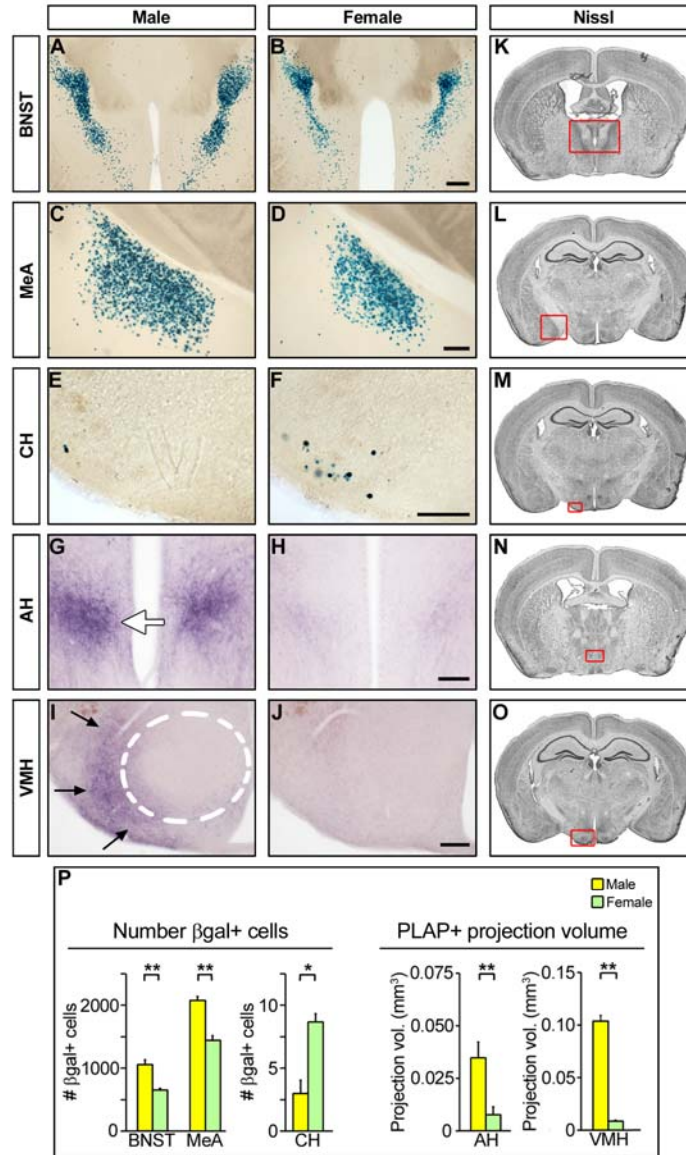


Figure 3

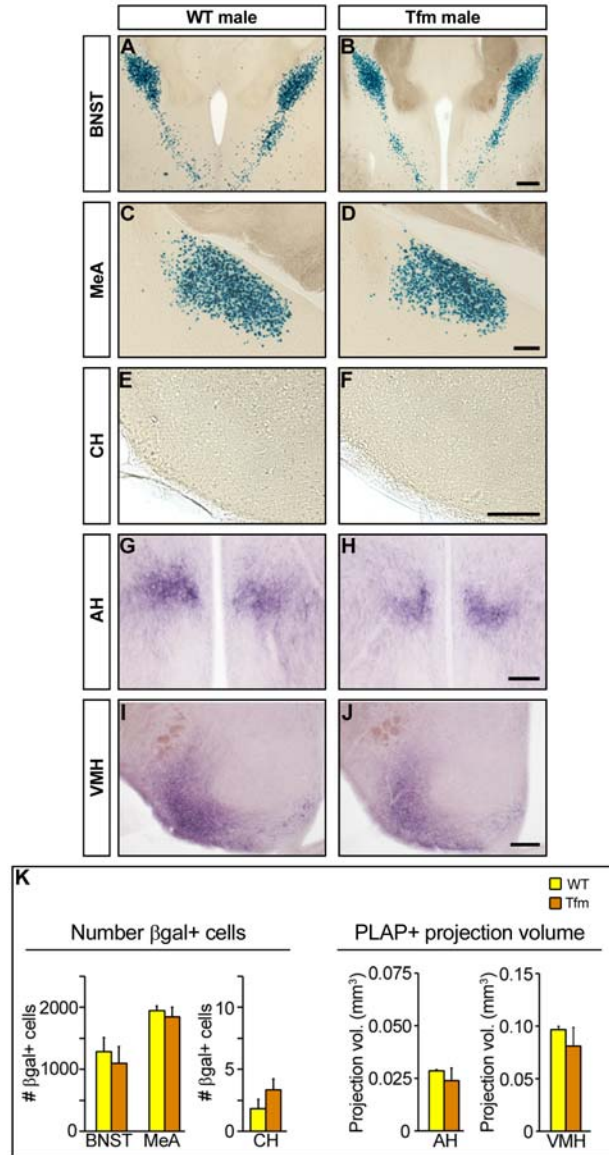


Figure 4

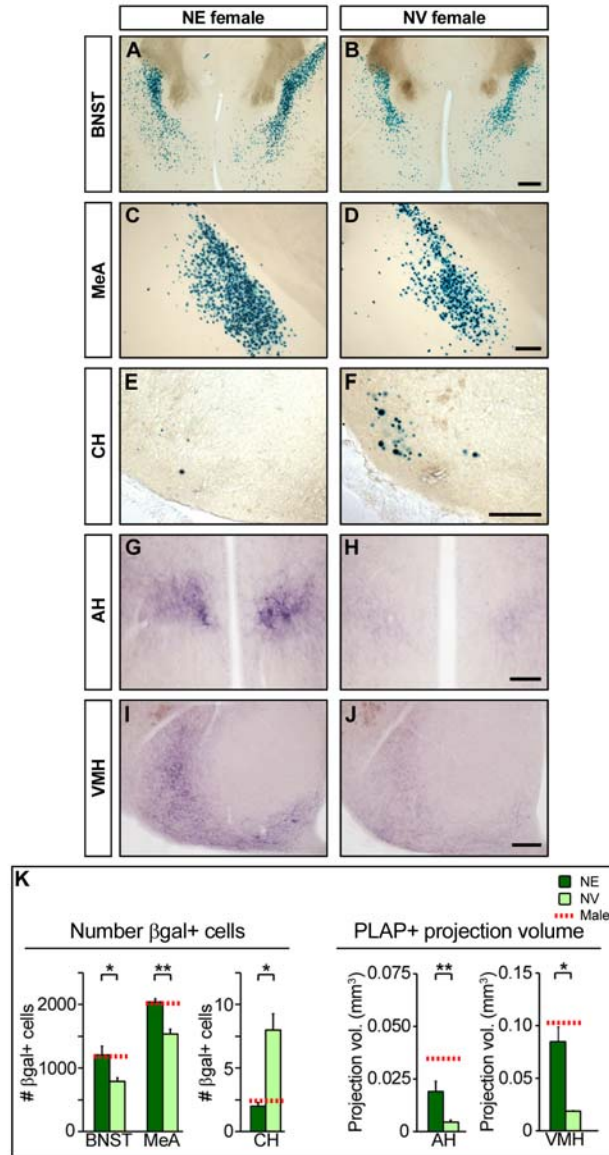


Figure 5

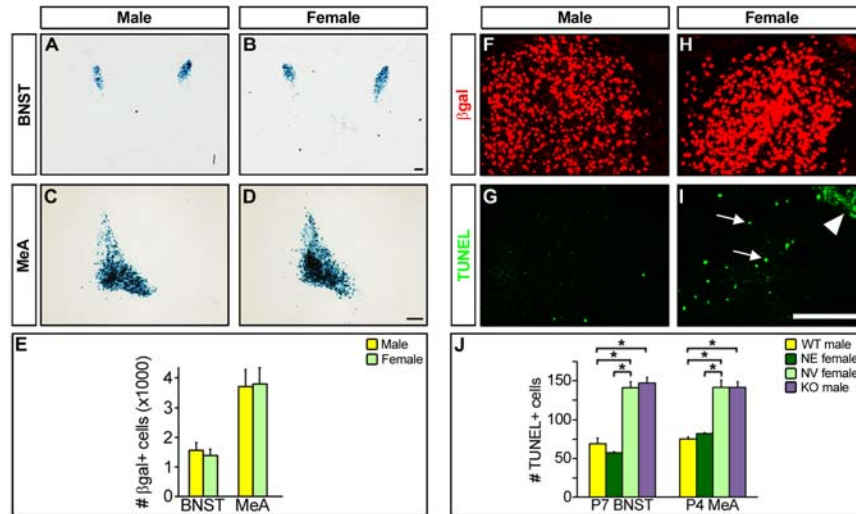


Figure 6

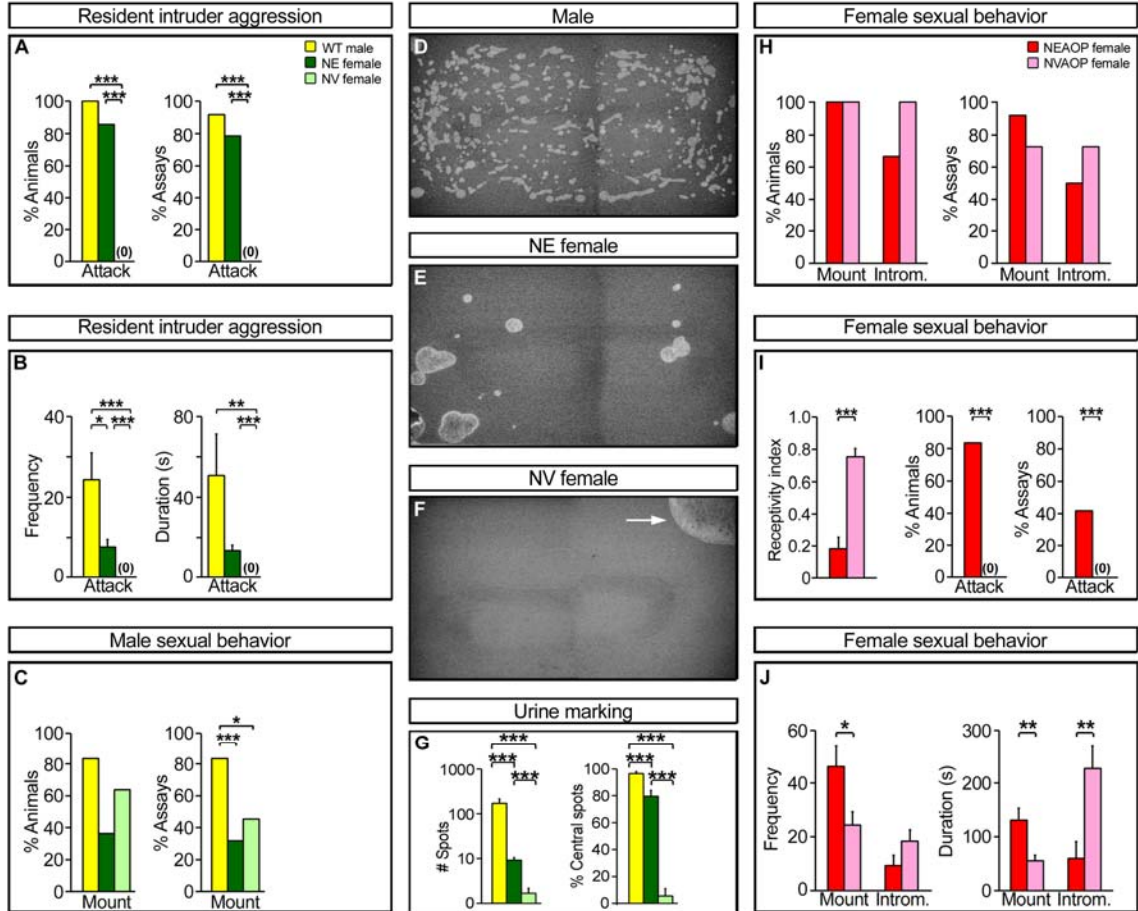
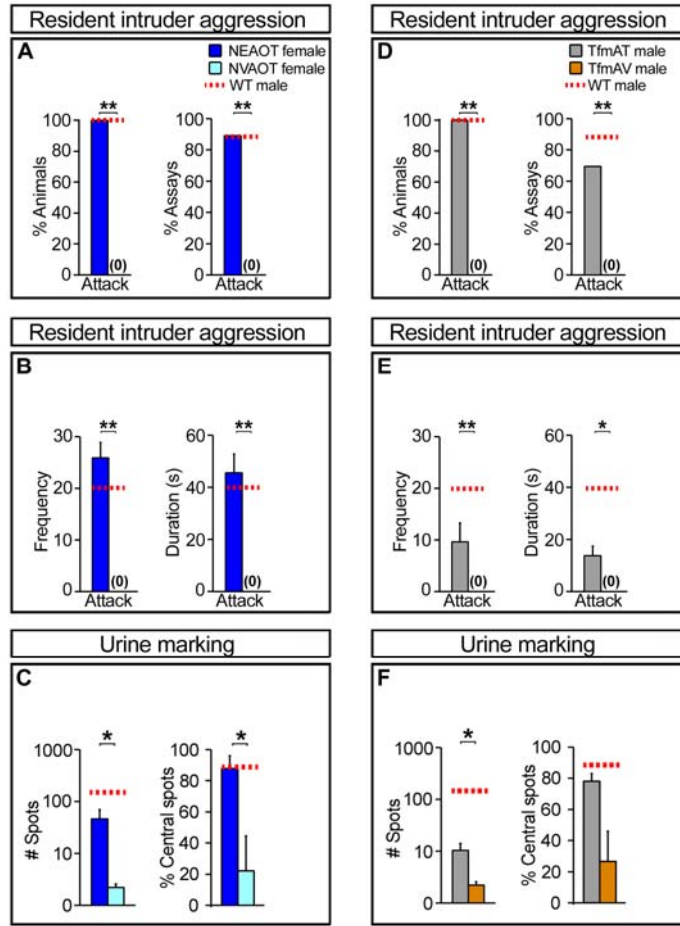


Figure 7



SUPPLEMENTAL TEXT

Serum estrogen and testosterone are unchanged in mice bearing the aromatase-IPIN allele

A comparison of serum hormone levels for testosterone and estrogen did not reveal significant differences between wildtype (WT) animals and mice homozygous for the aromatase-IPIN allele. (Testosterone: WT males, 5.75 ± 1.4 nM; *aromatase*^{IPIN/IPIN} males, 5.43 ± 1.2 nM; WT females, 0.54 ± 0.2 nM; *aromatase*^{IPIN/IPIN} females, 0.5 ± 0.17 nM. Estrogen: WT males, 0.12 ± 0.02 nM; *aromatase*^{IPIN/IPIN} males, 0.15 ± 0.02 nM; WT females, 0.14 ± 0.03 nM; *aromatase*^{IPIN/IPIN} females, 0.17 ± 0.02 nM; Mean \pm SEM; $n \geq 4$; $p > 0.29$ for within sex and hormone comparisons.) Individual females of either genotype often had 2 - 3 fold elevations above the mean in their serum estrogen at the time of sacrifice, presumably reflecting the rise in estrogen preceding estrus.

Similar proportions of neurons express aromatase in the BNST and MeA in both sexes

The dimorphisms in aromatase expression could reflect an absolute difference in cell number in these regions between the sexes. Such dimorphisms could also arise from an increase in the fraction of neurons expressing aromatase in males. We find a similar proportion of NeuN positive cells co-labeled with β gal in the two sexes in the BNST (males, $36.57 \pm 1.3\%$; females, $28.2 \pm 5.9\%$; $n = 3$; $p = 0.17$, Student's t test) as well as the MeA (males, $40.4 \pm 2.3\%$; females, $41.4 \pm 2.6\%$; $n = 3$; $p = 0.78$; Student's t test), indicating an increase in the total number of neurons in both regions in males compared to females.

The anterior and ventromedial hypothalamic regions contain a small, equivalent number of aromatase expressing cells in both sexes

We examined aromatase expression in the anterior and ventromedial hypothalamic regions of adult, age matched male and female mice bearing the aromatase-IPIN allele. We find a small, equivalent number of cells labeled for β gal activity in these regions in both sexes (Anterior hypothalamus: Male, 25 ± 2 ; Female, 24 ± 1 ; $n = 4$; $p > 0.5$, Student's t test; Ventromedial hypothalamus: Male, 21 ± 4 ; Female 20 ± 3 ; $n = 4$; $p > 0.9$, Student's t test).

Serum estrogen and testosterone are similar between adult females treated neonatally with estrogen or vehicle

A comparison of serum hormone levels for testosterone and estrogen did not reveal significant differences between adult females treated neonatally with estrogen (NE) or vehicle (NV). (Testosterone: NV, 0.33 ± 0.17 nM; NE, 0.38 ± 0.17 nM. Estrogen: NV, 0.23 ± 0.06 nM; NE, 0.24 ± 0.06 nM; $n \geq 5$; $p \geq 0.848$.) Individual NE and NV females often had 2 - 3 fold elevations above the mean in their serum estrogen at the time of sacrifice, presumably reflecting the rise in estrogen preceding estrus. We present serum estrogen levels of eight adult individuals of each treatment group to illustrate such elevations above the mean in some females. (Serum estrogen in nM: NV, 0.07, 0.09, 0.12, 0.14, 0.17, 0.33, 0.42, 0.49; NE, 0.06, 0.08, 0.1, 0.13, 0.26, 0.42, 0.42, 0.46.)

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Generation of mice bearing the aromatase-IRES-PLAP-IRES-nLacZ allele

Genomic clones containing the last exon of *aromatase* were obtained by screening a 129/SvJ lambda phage library from Stratagene. A ~5.8 kb *PmlI* genomic clone containing the last two exons of the *aromatase* gene was used to design the targeting vector. An *Ascl* restriction site was inserted 3 bp 3' of the stop codon of the *aromatase* gene using site-directed mutagenesis (Stratagene). This mutagenized targeting vector has 4.5 kb and 1.3 kb of homology 5' and 3' of the *Ascl* restriction site, respectively. As described previously, we utilized the self-excising neomycin cassette, ACN, which was inserted 3' of IRES-PLAP-IRES-nLacZ (Bunting et al., 1999; Shah et al., 2004). This IRES-PLAP-IRES-nLacZ-ACN cassette was inserted into the targeting vector as an *Ascl* fragment. The aromatase targeting vector containing the reporters and the neomycin selection cassette was electroporated into a 129/SvEv mouse ES cell line. We obtained a targeting frequency of 5% for homologous recombinants, which were detected using PCR for the 3' arm for the targeting vector. We used a primer (5'-CATCGCCTTCTATCGCCTTCTTGAC) that was complementary to the ACN cassette and an external primer (5'-CTTGATCATTGGAGCCAAATCTGGATG) that was complementary to genomic sequence located 3' of the 3' homology arm of the targeting vector. A subset of positive clones was tested by PCR for homologous targeting of the 5' arm using an external primer (5'-CCAGCTGGATTTCTTGGGATCAAATTCAGG) and a primer unique to the modified allele (5'-GAATTCGGCGCGCCTCTTCACTGTTG). ES clones harboring the homologously recombined modified aromatase allele were injected into blastocysts to obtain chimeric mice which were crossed to C57Bl/6J females to obtain germline transmission. Chimeric mice that transmitted the aromatase-IRES-PLAP-IRES-nLacZ allele

were obtained from one positive clone. ACN contains a *neomycin^R* gene that is self-excised upon passage through the male germline, and F1 progeny obtained by crossing the chimeric males to C57Bl/6J females had deleted ACN as determined by PCR. The resulting progeny (backcrossed > 3 generations in C57Bl/6J) were used for experimental analysis.

Other mouse strains

Mice bearing the *tfm* allele were obtained from Jackson Laboratories and backcrossed extensively to the C57Bl/6J strain (> 10 generations) prior to experimental use. Mice bearing a null allele of aromatase (deletion of exons 1 and 2) have been described previously (Honda et al., 1998).

Estrus induction

Females aged 8 - 16 weeks were ovariectomized, and allowed to recover from the surgery for one week. Estrus was induced by sequential daily injections of 10 μ g and 5 μ g of EB followed by 50 μ g of progesterone. The females were used for sexual behavior 4 - 6 hours after progesterone injection. Females were allowed to recover for \geq 1 week between assays.

Immunostaining

The primary antisera used in this study are: rabbit anti- β gal (Cortex Biochem, 1:2000 or ICL, 1:5000), mouse anti- β gal (Promega, 1:2500), mouse anti-NeuN (Chemicon, 1:300), rabbit anti-ER α (Upstate, 1:50000), rabbit anti-activated caspase-3 (Cell Signaling Tech, 1:100; Chemicon, 1:100; R&D Systems, 1:200), rabbit anti-activated caspase-9 (Cell Signaling Tech, 1:50), and rabbit anti-Apaf-1 (Epitomics, 1:5000). The fluorophore conjugated secondary antisera are: Cy3 donkey anti-rabbit, Cy3 donkey anti-mouse (Jackson ImmunoResearch, 1:800), AlexaFluor 488 donkey anti-mouse, and AlexaFluor 488 donkey anti-rabbit (Invitrogen, 1:300). Sections were exposed to the primary antisera for 12 - 16 hours at 4°C, and to the secondary antisera for 2 hours at room temperature. The buffers, washes, and mounting media used in these experiments have been described previously (Shah et al., 2004).

TUNEL

Brains were dissected from paraformaldehyde - perfused animals and fixed for an additional 30 minutes at 4°C. The brains were incubated in 20% sucrose for 12 - 18 hours at 4°C, and embedded in 1:1 mix of Tissue-Tek OCT (Sakura) and Aquamount (VWR). The brains were sectioned at 16 μ m, and adjacent sections were collected on alternating slides. One set of slides was immunolabeled for β gal as described above. The second set was stained for apoptotic cells using the ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit (Chemicon) in accordance with the manufacturer's protocol. These TUNEL stained sections were imaged using an upright epifluorescence microscope. For every animal, TUNEL positive cells were enumerated bilaterally in 6 - 7 representative sections containing the BNST or MeA each. An identical approach was

used to enumerate the apoptotic cells (labeled with antibodies to Caspase-3, 9 and Apaf-1) that also expressed aromatase.

In situ hybridization

We performed in situ hybridization (ISH) using RNA probes to detect expression of ER α and ER β mRNA in the neonatal brain, and aromatase message in the adult brain. The probes used to detect ER α and ER β mRNA correspond to those utilized by the Allen Institute for Brain Science for the mouse brain (Lein et al., 2007). We prepared RNA sense and anti-sense probes corresponding to 396 - 728 bp of aromatase mRNA (accession D00659). ISH on neonatal brains using digoxigenin labeled RNA probes was performed as described previously (Kurrasch et al., 2007). For ISH on adult animals, we dissected the brain of mice perfused with 4% paraformaldehyde (PFA), and fixed it for an additional 14 - 18 hours at 4°C in 4% PFA. The brains were embedded in 3% bacto-agar and sectioned at 90 μ m. These sections were treated with proteinase K (10 μ g/mL, Roche) for 30 minutes, rinsed, and fixed in 4% PFA for 30 minutes at room temperature. We treated these sections with acetylation buffer for 10 minutes and equilibrated them in hybridization solution for 2 - 5 hours at 65°C. These sections were subsequently incubated for 14 - 18 hours at 65°C in fresh hybridization buffer containing 0.5 μ g/mL RNA probe. The sections were subsequently washed in high stringency buffers and subjected to electrophoresis to remove unhybridized probe as described previously (Kobayashi et al., 1994). These washes were followed with incubation for 12 - 18 hours at 4°C in buffer containing alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (1:2000, Roche). After extensive washing, we incubated the sections for 6

hours at 37°C in staining solution containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Roche). The sections were subsequently washed, fixed in 4% PFA, and mounted on glass slides. Detailed protocols are available upon request.

Quantitation of histological data

We estimated the cell number and volume for both sides of the brain (left and right) individually, and obtained the mean for each animal. All imaging and data analysis were performed by an experimenter blind to sex, age, genotype, and hormone treatment. When using unbiased stereology based methods for quantitation, cells were enumerated with an Optical Fractionator probe, and projection fiber volume was estimated with a Cavalieri Estimator probe, using parameters set per the manufacturer's instructions (Stereoinvestigator, MicroBrightField) and published stereology protocols (Keuker et al., 2001).

To enumerate immuno-labeled βgal^+ cells in adult animals, 65 μm thick sections encompassing the BNST and MeA were imaged with confocal microscopy to collect Z-stacks (3.9 μm step) through the entire section, and processed as described previously (Shah et al., 2004). All immunolabeled cells in the central three optical slices in each histological section were enumerated through the rostrocaudal extent of the BNST and MeA. Similar results were also obtained using unbiased stereology as follows. In each Z-stack, the region of interest was outlined, 4 μm guard zones were established to preclude overcounting, and βgal^+ cells were enumerated with a 20x20 μm^2 counting box in a 70x70 μm^2 sampling grid. To determine whether βgal^+ cells expressed NeuN,

images of the sections were collected as Z-stacks (3.4 μm steps) using a 63X lens. Guard zones of 3 μm were established, and a 20x20 μm^2 counting box was used in a 45x45 μm^2 grid to enumerate cells. The same parameters were used to determine whether βgal^+ cells expressed $\text{ER}\alpha$ or the androgen receptor.

Sections through the entire BNST and MeA of P1 animals were collected at 12 μm , stained for βgal activity, and viewed with brightfield optics (10X) on an upright microscope with a motorized stage. We used 1 μm guard zones and a 20x20 μm^2 counting box in a 40x40 μm^2 sampling grid to enumerate βgal^+ cells in alternate sections.

PLAP stained sections spanning the rostrocaudal extent of the anterior hypothalamus or the VMH were imaged using 5X brightfield optics on an upright microscope. A lattice with nodes spaced 12 μm apart was superimposed on the images, and each node that overlapped PLAP⁺ fibers was manually marked to determine the area occupied by these projections. The software estimated the volume filled by PLAP⁺ fibers as we used sections of defined thickness (80 μm).

Behavioral Assays

We used 10 - 24 week old mice in behavioral assays. Mice were group housed by sex after weaning, and were sexually naive when the behavioral testing was initiated. Animals were tested at least one hour after the lights were switched off. The behaviors were recorded using an infrared sensitive video recorder. Animals were isolated two

days before testing started and maintained in individual housing for the duration of the tests. The animals were rested for ≥ 2 days between behavioral tests, and residents were always exposed to a novel intruder.

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SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Expression of β -galactosidase mirrors the distribution of aromatase mRNA

(A-L) Coronal sections through the adult BNST, MeA, septum (Sep), POA, anterior olfactory nucleus (AON), and thalamus (Thal) of a mouse heterozygous for the aromatase-IPIN allele. Adjacent sections were processed for in situ hybridization to reveal aromatase mRNA (A-F) or β gal activity (G-L). The expression of β gal mirrors that of aromatase message. There are no β gal positive cells in the AON and the thalamus, regions with no detectable aromatase message. There are small clusters of aromatase mRNA expressing and β gal positive cells in adjacent sections through the BNST, MeA, septum, and POA. **(S)** There is no sex difference in the number of β gal expressing cells in the POA or septum. **(M-R)** Nissl stained (grayscale) sections depicting locations of the BNST, MeA, septum, POA, AON, and thalamus. Error bars represent SEM; $n = 4$; $p \geq 0.164$. Scale bars equal 250 μ m.

Figure S2. β -galactosidase and PLAP activity offer excellent resolution of the distribution of aromatase positive neurons and their fiber tracts

(A, B, D, E) Coronal sections through the adult brain of male mice bearing the WT (B, E) or genetically modified aromatase (aromatase-IPIN) (A, D) allele stained for β gal (A, B) or PLAP (D, E) activity. These representative sections through the BNST (A, B) and VMH (D, E) demonstrate robust labeling of the cell bodies and fibers of aromatase expressing neurons in mice bearing the aromatase-IPIN allele. There is no visible

background labeling for β gal or PLAP activity in the WT brain. Note that sections from the WT and genetically modified mice were processed in parallel for the histochemical stainings in this figure and for all the studies presented in this study. **(C, F)** Nissl stained (grayscale) sections depicting locations of the BNST and VMH. Scale bars equal 500 μ m (A, B) and 250 μ m (D, E).

Figure S3. Sexual dimorphism in aromatase expressing neural pathways

Coronal sections through the adult brain of male and female mice bearing the aromatase-IPIN allele stained for PLAP activity. Adjacent sections through the BNST (A-D), MeA (E-H), and caudal hypothalamus (CH) (I-L) are shown, with the top panel in each instance representing the more rostral section. PLAP stained fibers occupy a larger volume in the male BNST and MeA. There are more PLAP labeled fibers in the female caudal hypothalamus. Note that PLAP not only labels the cell bodies that express aromatase, but also the fibers emanating from these neurons. The arrow (F) shows the PLAP labeled stria terminalis, a fiber tract that contains projections from the BNST and MeA, both of which contain aromatase expressing neurons. Scale bars equal 250 μ m.

Figure S4. ER α and ER β are expressed in the neonatal BNST and MeA

(A-H) Coronal sections through the BNST and MeA of WT newborn male and female mice processed for in situ hybridization to reveal ER α or ER β mRNA. There are ER α and ER β expressing cells in the BNST and MeA in both sexes. **(I-T)** Representative

coronal sections through the BNST and MeA of newborn male and female mice bearing the aromatase-IPIN allele immunostained for β gal (red) and ER α (green). The merged images show that individual cells co-express (yellow) aromatase (β gal) and ER α in the BNST and MeA. The vast majority (>99%) of β gal positive cells co-express ER α . n \geq 2. Scale bars equal 250 μ m (A-H) and 100 μ m (I-T).

Figure S5. Estrogen administration in adulthood does not masculinize aromatase expressing neurons

Representative coronal sections through the brain of adult females bearing the aromatase-IPIN allele treated with estrogen or vehicle as adults and stained for β gal (A-J) or PLAP activity (K-R). There appears to be no significant difference in aromatase positive neural pathways between estrogen treated females and their controls. Adult females were injected subcutaneously every other day with 5 μ g of estrogen or vehicle for three weeks. These animals were sacrificed and their brains processed for β gal or PLAP activity. Arrows (A-D) indicate the POA visible in these low magnification views of the brain at the level of the BNST. n \geq 2. Scale bars equal 250 μ m.

Figure S6. Testosterone masculinizes aromatase expressing neural pathways and territorial behaviors

(A) Adult females treated neonatally with testosterone (NT) have significantly more aromatase expressing (β gal+) cells in the BNST and the MeA compared to NV controls. The number of cells in these regions is comparable between NT females and WT males.

The volume occupied by PLAP stained fibers in the AH and surrounding the VMH is greater in NT compared to NV females and similar to that observed in WT males. PLAP labeling also revealed the cluster of cells in the caudal hypothalamus in NV but not NT females, indicating a masculinization of this region of the brain (not shown). The horizontal dashed lines (red) represent the mean of the number of β gal+ cells or the volume occupied by PLAP+ fibers in these regions in WT males, and were determined from the data in Figures 2 and 3 in the main Text. **(B-D)** NT females display male pattern aggression (B, C) and urine marking (D). The majority of NT residents attack intruder males in most assays of resident-intruder aggression whereas none of the NV females display aggression (B). The frequency and duration of the attacks initiated by NT females is comparable to that observed in WT males (C). NT females deposit more urine spots than NV mice, with a greater proportion of these urine marks being deposited in the cage center (D). The horizontal dashed lines (red) represent the mean values of such behavioral displays by WT males observed in the data presented in Figure 6 in the main Text. Error bars represent SEM; n = 3 (A), n = 5 (B-D); * p < 0.04, ** p < 0.03, *** p < 0.008; these p values were obtained for the comparisons between NT and NV females.

Figure S1

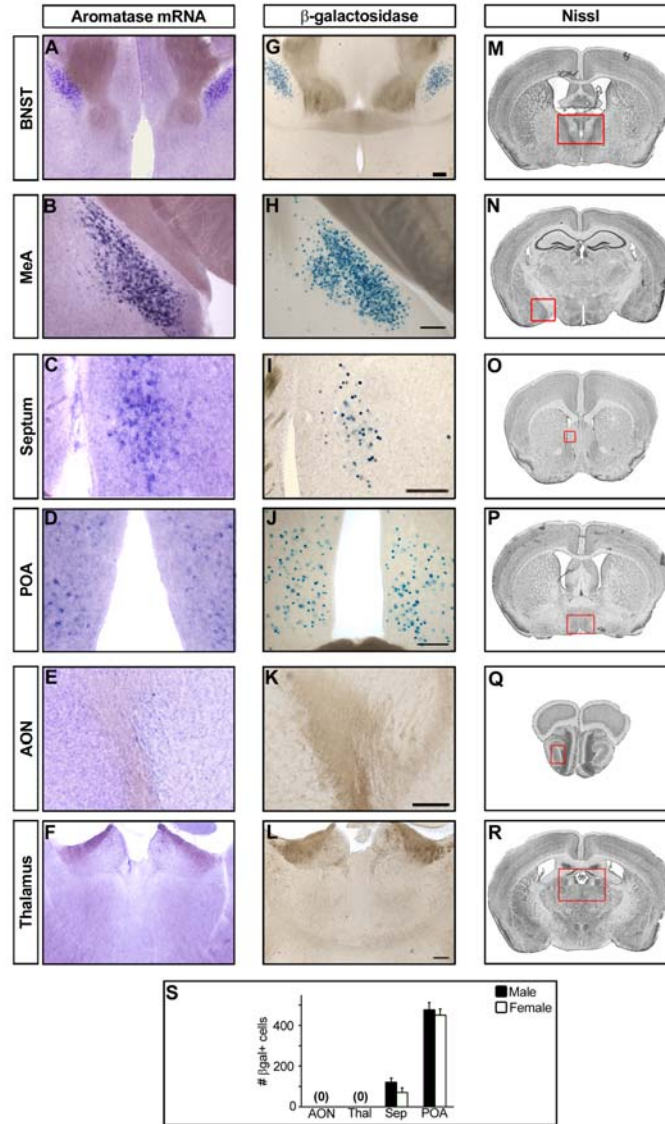


Figure S2

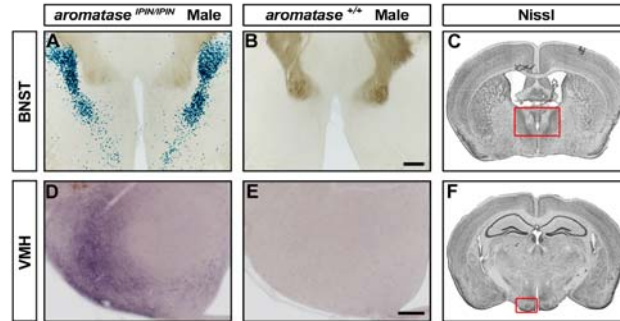


Figure S3

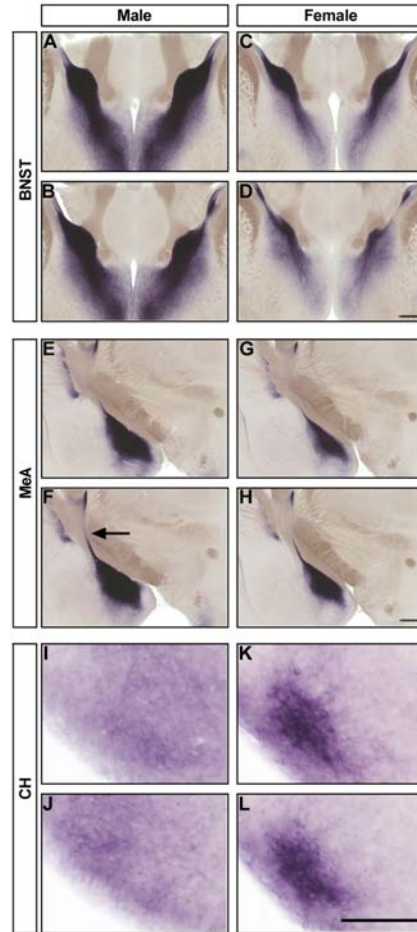


Figure S4

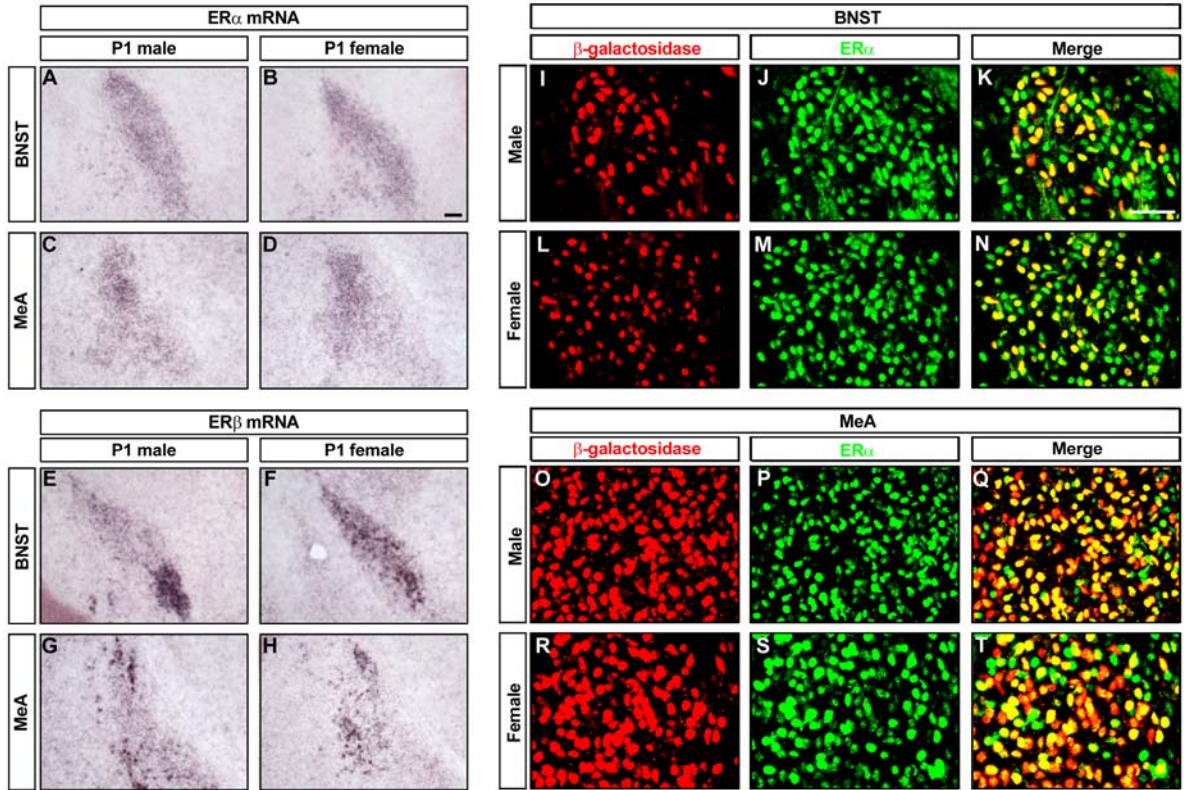


Figure S5

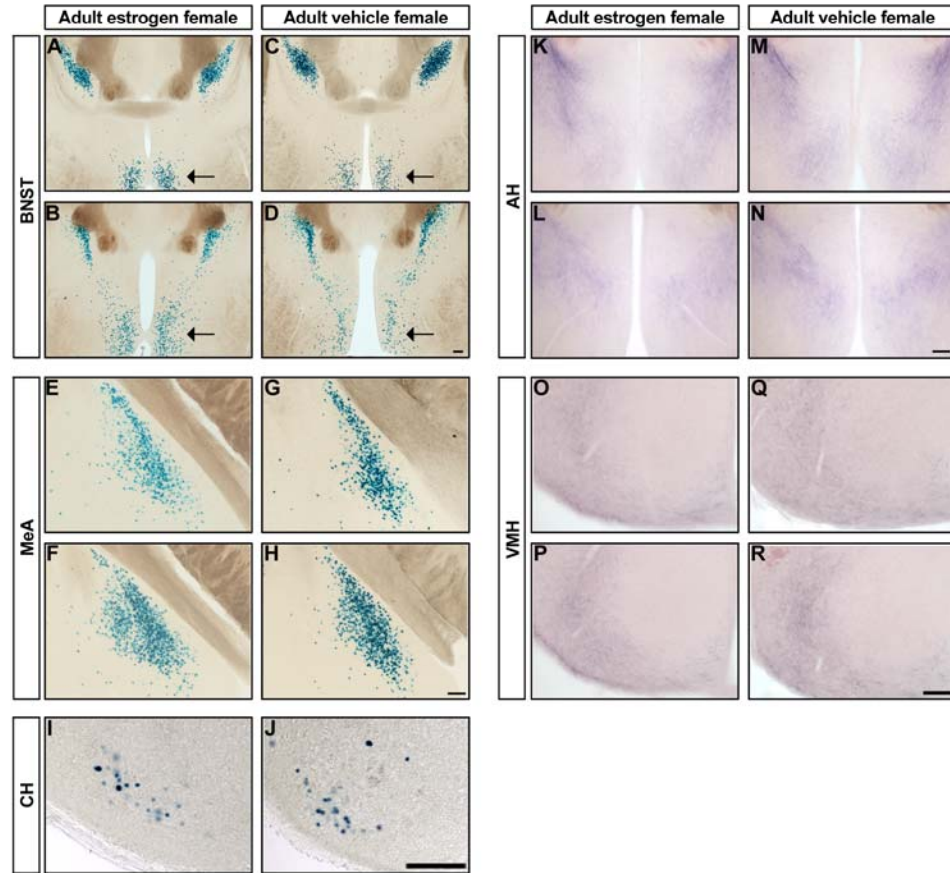
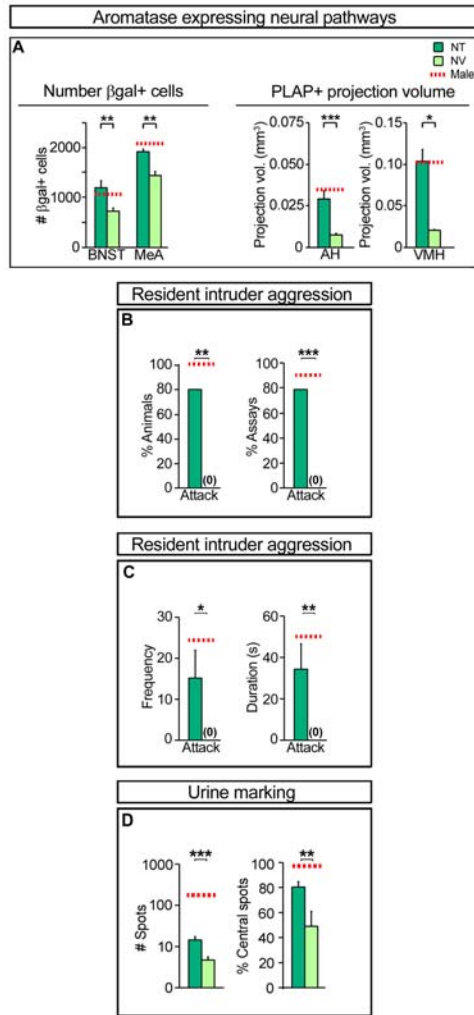


Figure S6



Chapter 3

The androgen receptor governs 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 while AR mutant males exhibit masculine sexual and territorial displays, 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 because 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 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 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 also utilized dihydrotestosterone (DHT) to examine AR function, as this steroid is a non-aromatizable androgen. Recent evidence indicates, however, 3β Adiol, 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 because these animals have low, often undetectable, levels of circulating testosterone (Sato, et al., 2004) (S.A.J., 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 $ER\alpha$ and $ER\beta$ 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) (M.V.W., 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 ER α and ER β are widely expressed in interconnected limbic regions such as the medial amygdala (MeA), the posteromedial component of the medial subdivision of the bed nucleus of the stria terminalis (BNST), and the preoptic hypothalamus (POA). 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 BNST and POA 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 hr, 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 the 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 coexpression of the sensitive, genetically encoded reporter, nuclear β -galactosidase (β gal), in all cells that express AR (Shah, et al., 2004). Previous work has implicated AR-expressing regions such as the MeA, BNST, and the 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 2A-C

and S1D). There were >15- to 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) (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 time point by which the testosterone surge has already subsided (Figures 2D-F and S1E), 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 (Figures S1A and S1B). 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 2G-L, S1, and data not shown).

The fetal testis produces testosterone at 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 β gal to visualize AR-positive cells in mice bearing the *AR-IPIN* allele (Figures S2A-S2D). 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 (Figures S2E-S2L). 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 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 with females (Shah, et al., 2004). AR expression in the BNST and POA in the P7 male resembles that observed in the adult male (Figures 2G-2L), 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 β gal at P7 revealed significantly more AR positive cells in the male BNST and POA than in these regions in the female (Figure 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 because 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 ($ER\alpha$ and $ER\beta$) and 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 $ER\alpha$ and $ER\beta$ and aromatase in the BNST and POA at P1 (Figures 4A-4F), 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 (Figures 4G-4M), 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 is significantly lower than in control males (Figures 4G, 4N, and 4O). 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 simply serves 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 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 (Figures 5B-5D and 5F): 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 a mild elevation in circulating testosterone in AR^{NsDel} mice, this is not statistically different from the titers of this hormone in males of the control genotypes ($p = 0.157$, Kruskal-Wallis test for multiple group comparisons) (Figure 5E). The titers of circulating estrogen in these mutants are also comparable to those of the control males (Figure 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 (Figures 5I and 5J).

Inadvertent deletion of AR in nonneural 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 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 (Figures S3A-S3C). When assayed for motor performance on the rotarod, AR^{NsDel} mice performed equivalently to control males (Figure 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 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 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 and P7 in these mutants compared to that of their control male littermates (Figure 5I). In agreement with these results, immunolabeling reveals very few AR-positive cells in the forebrain of adult AR^{NsDel} males compared to controls (Figures 5K-5V). Taken together, our genetic approach 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 min assay in which a WT estrous female is introduced into the cage of a singly housed WT male (Mandiyan, et al., 2005; McGill, 1962). Forty-two percent of AR^{NsDel} residents (n=24 mice), a percentage statistically similar to that of control residents, mounted at least once with different estrous females when tested in two or three assays for sexual behavior (Figure S4A). When we analyzed the percent of all assays with mounts, we observed mating in fewer assays with AR^{NsDel} residents than in those with males in the control cohort (Figure 6B). Notably, we find that some of the AR^{NsDel} mice never mate, though when they do exhibit sexual behavior, these mutants mate in most assays (85% ± 6%) similarly to control residents (Figure S4G).

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} males (Figures 6C and 6D). Male mice do not always achieve ejaculation within a 30 min assay (McGill, 1962), and we observed ejaculation at

a similar, low frequency in males of all genotypes (Figures 6B and S4A). The deficits in some 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 (Figures 6E, 6F and S4C-S4F; and Movies S1, S2).

Thus, while AR^{NsDel} mutants are less likely to mate, once sexual behavior is initiated, its display appears similar to that of WT 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 with the mutants who do not 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 behavioral performance in mating assays (Figure S3D). Second, when AR^{NsDel} mutants who did not mate in any of the three 30 min mating assays were cohoused with females, they successfully sired litters (S.A.J., unpublished data). Finally, only a subset of constitutive AR mutant males attempted to mate when supplemented with testosterone (or estrogen) at doses that recapitulate WT circulating levels of this steroid hormone (Olsen, 1992; Sato, et al., 2004) (M.V.W.,

unpublished data). 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 critical 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 conspecifics in a manner comparable with that of their control male littermates (Figures S4B and S5C). Previous work shows that, unlike WT males, constitutive AR mutants have a preference for male rather than female odors (Bodo and Rissman, 2007). Chemosensory cues from the two sexes lead 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 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 7A, S5A, and S5B), the mutants spent significantly less time fighting compared with control residents, and this deficit persisted even when we restricted our analysis to the assays in which we observed aggressive interactions (Figures 7B and 7C). 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 S5B-S5E). Rather, our analysis revealed a deficit in the pattern of aggression following the first fight initiated by AR^{NsDel} males (Figures 7D-7F and S5F). Compared with the control residents, AR^{NsDel} spent less time fighting with the intruder and exhibited a longer interval between successive attacks (Figures 7D and 7F). While AR^{NsDel} residents and their controls attacked a similar number of times in an assay (Figure S5E), the mutants exhibited a lower attack rate (Figure 7E), initiating fewer attacks per unit time following the first attack. 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 7H). By contrast,

we find that AR^{NsDel} residents deposit fewer urine marks compared with control resident males (Figure 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 only 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). Differences in the phenotypes reported in that study compared with those in our study likely arise from variations in the experimental design or strain differences. Here, we have significantly refined the analysis of AR^{NsDel} mutants to provide new 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 all mutant phenotypes. We have also developed analytical tools to examine in an extensive manner the behavioral deficits in sexual and territorial behaviors of AR^{NsDel} males. 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 suggests that AR in the brain 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 the brain in controlling the duration and pattern of intermale 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 eliminated 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 time point 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 sexual and territorial behaviors. Because 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 beyond sexual and territorial displays, and it will be important in future studies to determine whether AR function in the nervous system is essential for the appropriate display of such 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 to masculinize 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 1A). Alternatively, testosterone may serve not only as a precursor for estrogen, but it may also signal via

AR to control male behaviors (Figure 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 WT 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 WT 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 because 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 also controlled by estrogen signaling. This postnatal sexual differentiation of AR is unlikely to result from estrogen-regulated neurogenesis because 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 gonadal hormones 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 ER α and ER 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 12:12 hr light:dark cycle. All studies with animals were done in accordance with UCSF IACUC protocols. The AR-IPIN knockin and aromatase knockout mice have been described previously (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 weaned and group-housed by sex at 3 weeks of age.

Behavioral assays

We used adult, singly housed male mice in behavioral assays, which were performed during the dark cycle. The behavioral testing and analysis was done as described previously (Wu, et al., 2009). In brief, the male was first tested for urine marking for 1 hr in a fresh cage, and then returned to the homecage. Following mating tests, mice were tested 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 two or three times each in assays of mating and aggression, and once each for urine marking and ultrasonic vocalization. Experimental animals were always

exposed to intruder mice they had not encountered previously, and each assay was separated by ≥ 2 days.

Histology

We used age-matched mice for all histological experiments. We visualized β gal activity in 20 μ m thick sections using brightfield optics. Fluorescent immunolabeled sections (20 μ m thick, P7; 65 μ m thick, adult) were imaged using confocal microscopy. The primary antisera used in this study are monoclonal rabbit anti-AR (1:750, Epitomics) and mouse anti- β gal (1:2500, Promega). The anti-AR antibody appears specific to AR because we did not observe AR-positive cells in various brain regions in constitutive AR mutant males (Tfm) (Figure S3). Staining for β gal activity and fluorescent immunolabeling was performed as described previously (Shah, et al., 2004; Wu, et al., 2009). Quantitation of cell numbers was performed using stereology and other experimental approaches.

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, β gal-expressing cells in the anterior hypothalamus and BNST were found within the POA and 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 in these regions cannot be accounted for simply by changes in local distribution or cell density. Similar results were obtained when the quantitation was done by a second investigator.

Data analysis

Quantitation of behavioral and histological data was performed blind to relevant variables, including sex, genotype, and hormone treatment. To analyze categorical data, we used Fisher's exact test and a post hoc Bonferroni's correction for multiple group comparisons. For other comparisons, we first analyzed the distribution of the data with the Lilliefors' goodness-of-fit test of normality. Datasets not violating this test were analyzed with parametric tests (Student's t-test for two groups or one-way ANOVA); otherwise we used non-parametric analyses (Kolmogorov-Smirnov [KS] test for two groups or Kruskal-Wallis test). We used Tukey's post hoc test following one-way ANOVA and Kruskal-Wallis tests to determine which groups differed significantly. For 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).

qPCR

At each age, we collected tissue from AR^{NsDel} mice and each control groups to quantitate AR mRNA. Each tissue sample for individual animals was processed separately for RNA extraction, cDNA synthesis, and qPCR. We used separate qPCR reactions to detect AR and the ubiquitous ribosomal protein Rpl32, which was used for normalization of AR expression. Because AR expression was similar across all control genotypes but was significantly different from AR^{NsDel} mice in all regions, the normalized AR mRNA levels from the control cohorts were combined and compared to those of AR^{NsDel} males. For visualization purposes, this data is presented in Figures 5I, 5J and S3D as the

percent of AR mRNA in AR^{NsDel} males in various tissues compared with that of 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 mice with injections of estrogen and progesterone as described previously (Wu, et al., 2009). For hormonal manipulation of neonates, females were treated on the day of birth (P1) with a single 50 μ L subcutaneous injection of hormone or vehicle. We injected either 100 μ g testosterone propionate (Sigma) or 5 μ g of estradiol benzoate dissolved in sesame oil.

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FIGURE LEGENDS

Figure 1: Models for the role of testosterone in masculinizing the brain and behavior

Schematics illustrating possible mechanisms whereby testosterone controls male-typical behaviors are shown. **(A)** Testosterone acts as a circulating prohormone for estrogen synthesis via the action of aromatase in the brain. In this scenario, it is locally derived estrogen that masculinizes the brain and behavior. **(B)** Testosterone is not only 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 2: Limited expression of AR in the newborn brain

(A-L) Coronal sections through the brain of male P1, P4, P7 and adult mice bearing the *AR-IPIN* allele stained for β gal activity. There are few β -gal positive cells in the BNST or POA at P1 (arrowhead, C). There are more β -gal positive cells at P4 in these areas, and by P7 the number of cells approximates that observed in the adult brain. Scale bar equals 500 μ m (top row), and 100 μ m (bottom two rows). **(M-O)** Nissl-stained sections highlighting (red box) the BNST (M and N) and the POA (M and O). $n \geq 3$ at P1, P4, P7. See also Figures S1 and S2.

Figure 3: Sexual dimorphism in AR expression

(A-D) Coronal sections through the BNST and POA of P7 mice bearing the *AR-IPIN* allele immunolabeled for β gal. **(E)** There are more AR expressing cells in the BNST and

POA of $AR^{IPIN/Y}$ males than in $AR^{IPIN/IPIN}$ females. Mean \pm SEM; n = 4 for each genotype, * $p \leq 0.005$. Scale bar equals 100 μ m. See also Figure S2.

Figure 4: Estrogen masculinizes AR expression

(A-F) Coronal sections through the BNST and POA of P1 males labeled $ER\alpha$, $ER\beta$, or aromatase mRNA. **(G-O)** There are more β gal-positive cells in the BNST and POA of $AR^{IPIN/IPIN}$ females treated at P1 with testosterone (TP female) or estrogen (EB female) than those in females treated with vehicle (SO female) and $aromatase^{-/-}; AR^{IPIN/Y}$ (AroKO) males. Horizontal dashed lines represent the mean value in WT males as shown in Figure 3. Mean \pm SEM; n = 4 for each group of mice, * $p \leq 0.01$ by Tukey's posthoc test following one-way ANOVA. Scale bar equals 200 μ m (A-F) and 100 μ m (H-O).

Figure 5: Targeted deletion of AR in the nervous system

(A) Genetic strategy to delete AR in the nervous system. **(B-D)** Adult external genitalia and milk line are masculinized in control and AR^{NsDel} males, but not in constitutively null AR males. **(E)** Similar levels of serum testosterone and estrogen (n ≥ 12 /genotype). **(F)** Similar weight of testes and seminal vesicles in all males (n ≥ 7 /genotype). **(G)** Similar body length (snout to base of tail, n ≥ 4 per genotype) and weight (n ≥ 12 /genotype) in all males. **(H)** No difference in time to fall from rotarod between control and AR^{NsDel} males (n ≥ 4 /genotype). **(I and J)** Reduction in normalized AR mRNA in the brain of P1 (I), P7 (I), and adult (J) AR^{NsDel} males shown as a percent of AR mRNA in controls (Hypo., hypothalamus; Cing. Cx., cingulate cortex; Rost. Hypo., rostral hypothalamus; Caud.

Hypo., caudal hypothalamus; Hippoc., hippocampus). There are similar AR mRNA levels between AR^{NsDel} and control males in other tissues. Mean \pm SEM; ‡ mRNA < 0.5% of control, * $p < 5 \times 10^{-4}$, $n = 4$ for each genotype. **(K-V)** Fewer AR immunolabeled cells are visualized in coronal sections through septum, POA, BNST, VMH, MeA, and ventral premamillary nucleus (PMV) in AR^{NsDel} males than those in control males. Scale bar equals 100 μ m. See also Figure S3

Figure 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 than control males. **(D)** There was no statistical difference between AR^{NsDel} and control males in the time spent mounting, but as a group, AR^{NsDel} mice intromit females for a shorter duration than controls. **(E and 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. Mean \pm SEM; * $p < 0.033$, $n \geq 3$ /genotype; ** $p < 0.05$, post hoc Bonferroni's correction for Fisher's exact test, $n \geq 12$ /genotype; *** $p < 0.05$, Tukey's test following Kruskal-Wallis comparison, $n \geq 12$ /genotype. See also Figure S4 and Movies S1 and S2.

Figure 7: AR increases the levels of male territorial displays

(A) No statistical difference between AR^{NsDel} and control males in percentage of assays containing aggression. **(B)** As a group, AR^{NsDel} residents attack WT male intruders for a shorter duration than controls do. **(C)** When AR^{NsDel} residents fight, they do so for a shorter duration than controls. **(D-F)** In assays with fighting, AR^{NsDel} residents exhibit an increase in time between fights (D), attack at a slower rate (E) and attack for a smaller percent of the duration of the assay (F) compared with controls. **(G)** AR^{NsDel} males deposit fewer urine marks compared to controls. **(H)** No difference between AR^{NsDel} and control males in the percentage of urine marks away from cage perimeter. Mean ± SEM; * p < 0.05, Tukey's test following Kruskal-Wallis comparison, n ≥ 12/genotype. See also Figure S5

Figure 1

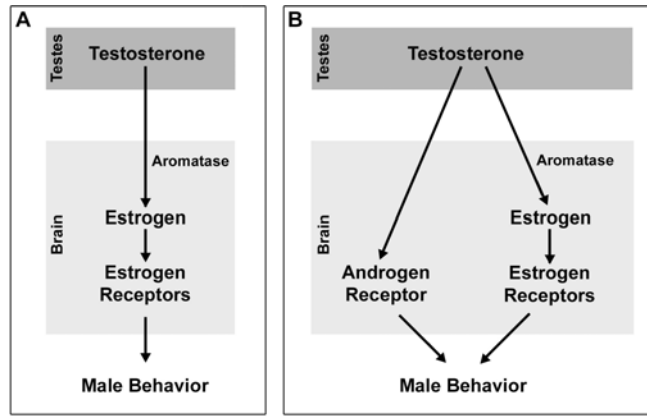


Figure 2

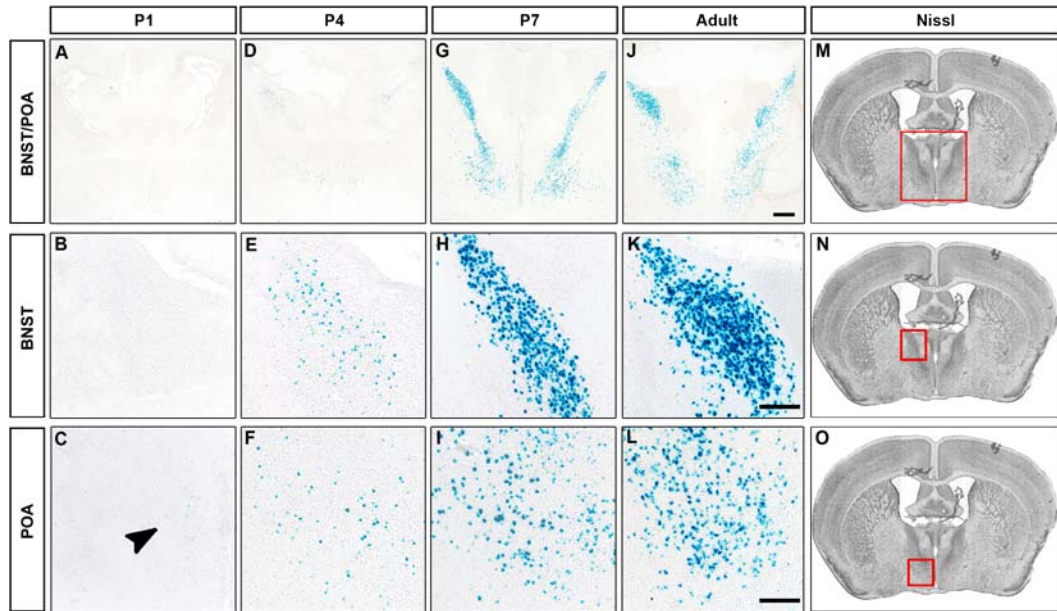


Figure 3

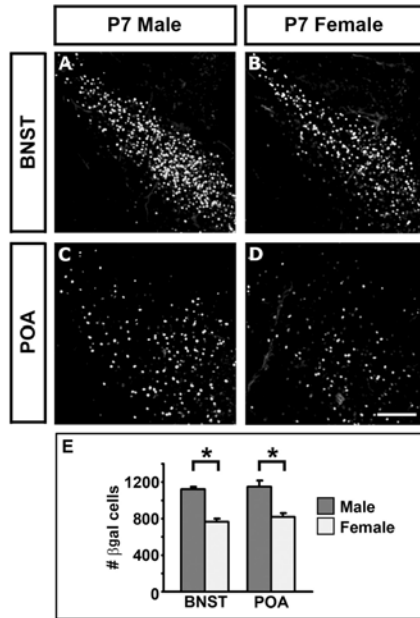


Figure 4

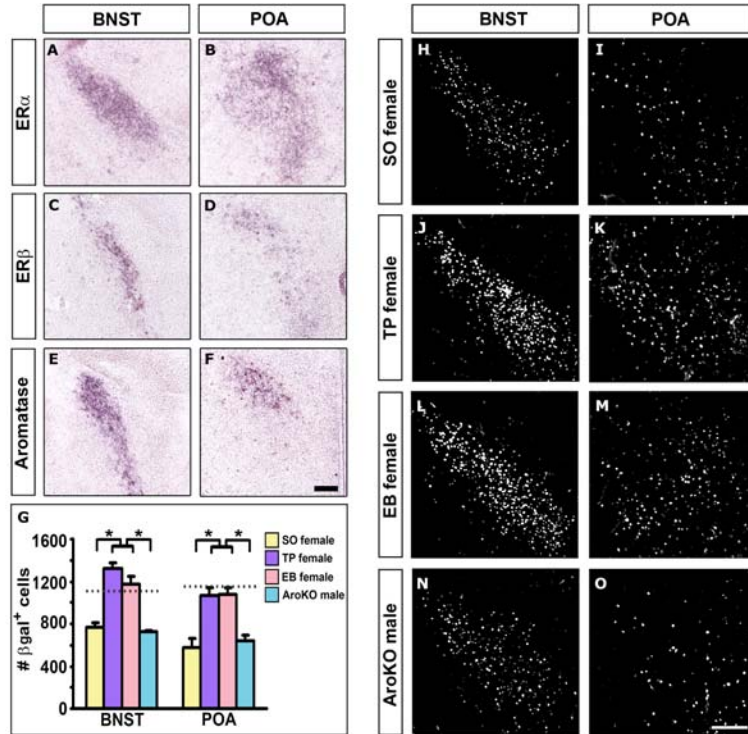


Figure 5

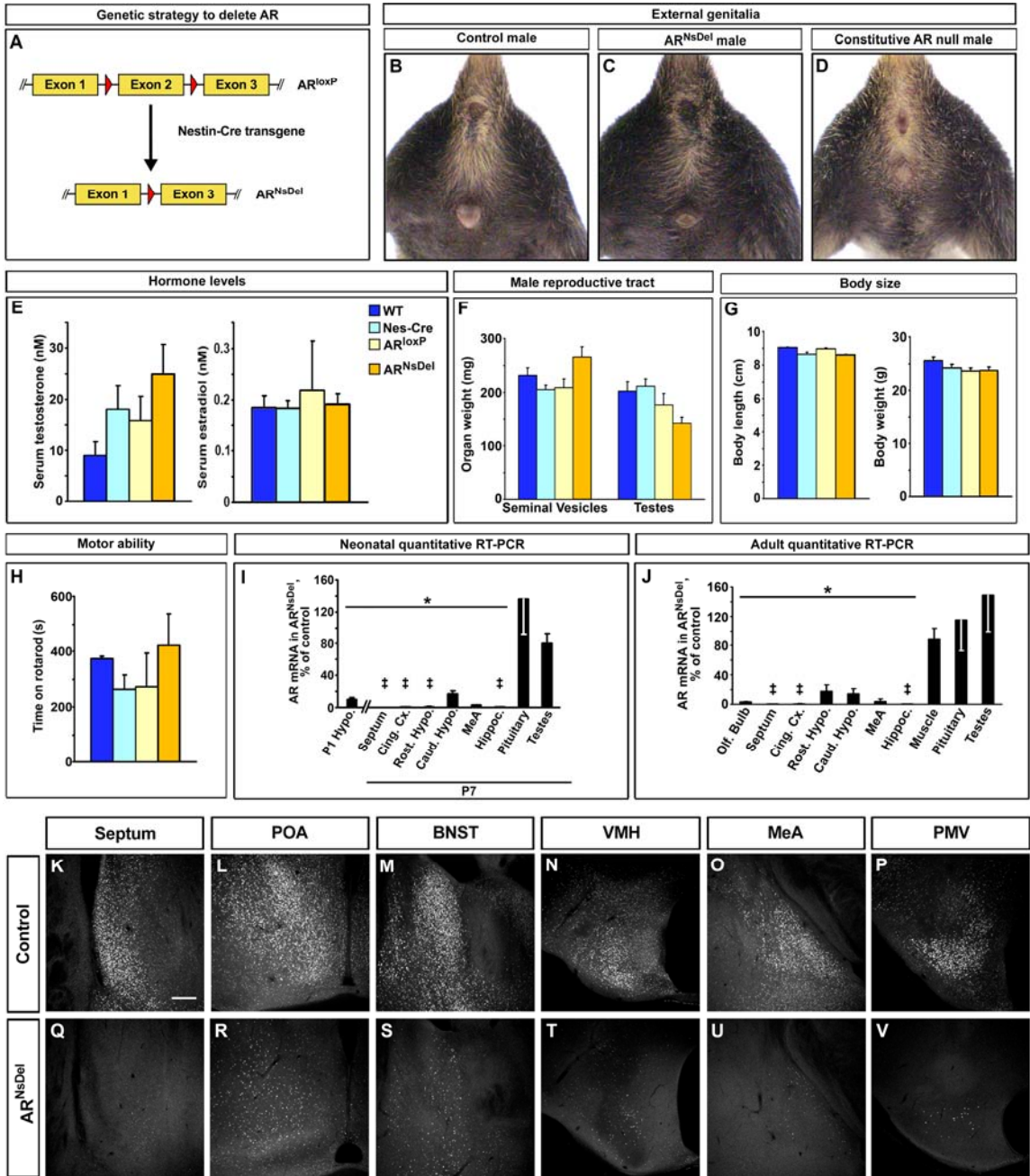


Figure 6

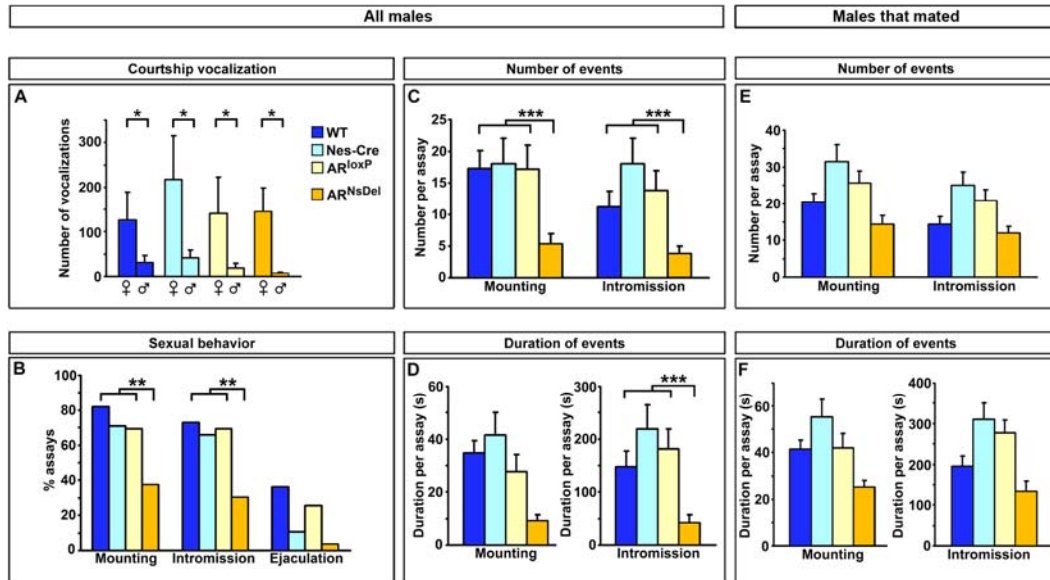
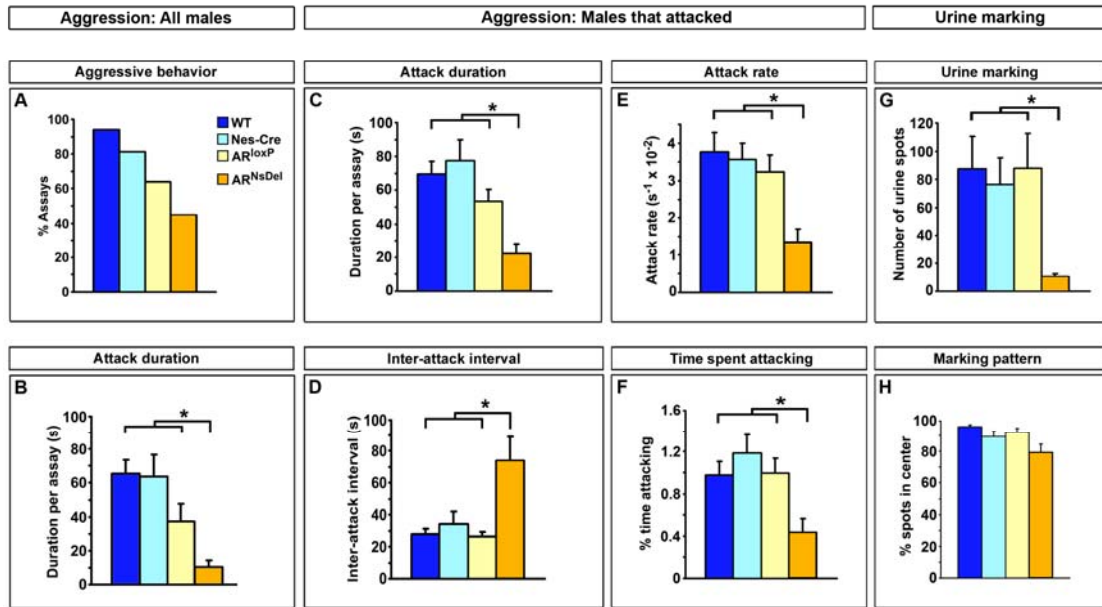


Figure 7



SUPPLEMENTAL 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 ≥ 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°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°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 leading to 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 is similar to 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°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, ER α and ER β was performed as described previously (Wu, et al., 2009).

To quantitate AR expressing cells at P1, we enumerated cells labeled for β gal 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 μ m thickness) immunolabeled for β gal 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 a 4 μ m step size for each optical slice. 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 β gal is targeted to the nucleus and we enumerated all labeled nuclei in the central optical slice (4 μ m thick) of each histological section, and these counts are reported in Figures 3 and 4. We therefore effectively enumerated β gal positive nuclear figures in optical slices separated by 56 μ m. The diameter of β gal labeled nuclei is significantly smaller than 56 μ m and does not exhibit a sex difference (POA: male, 6.1 \pm 0.04 μ m and female, POA 6.1 \pm 0.03 μ m; BNST: male, 6.4 \pm 0.03 μ m and female, 6.2 \pm 0.05 μ m; 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).

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.

Estrus induction

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.

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SUPPLEMENTARY FIGURE LEGENDS

Figure S1: 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 β gal activity. There are few β gal⁺ cells in the MeA at birth, and progressively more β gal⁺ cells are observed at P4 and P7. By contrast, there is a collection of β gal⁺ 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 μ m.

Figure S2: 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 β gal activity. We did not observe β gal⁺ 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, β gal⁺ 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 μ m.

Figure S3: 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 ≥ 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 ≥ 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 5J with additional AR^{NsDel} mice to generate the comparison shown in this panel. ‡ mRNA < 0.5% of control. n ≥ 3 per group. Mean ± SEM. (A-C) One-way ANOVA or Kruskal-Wallis test: p > 0.05 for all parameters. (D) KS test: p ≥ 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 S4: 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 males 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: $p \geq 0.4$ for each comparison.

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

Figure S5: 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 toward 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 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}.

(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 \pm SEM; $n \geq 12$ for each genotype.

Figure S1

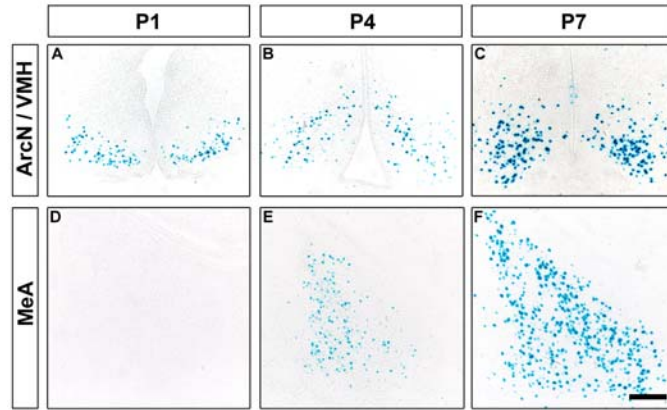


Figure S2

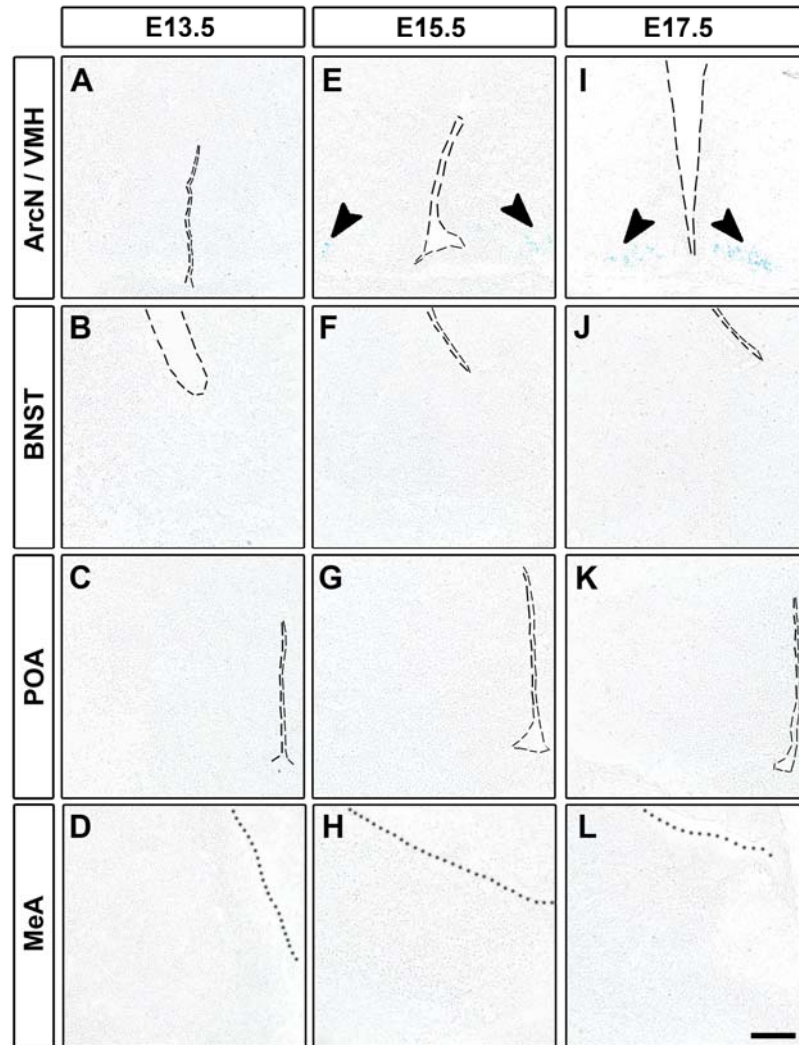


Figure S3

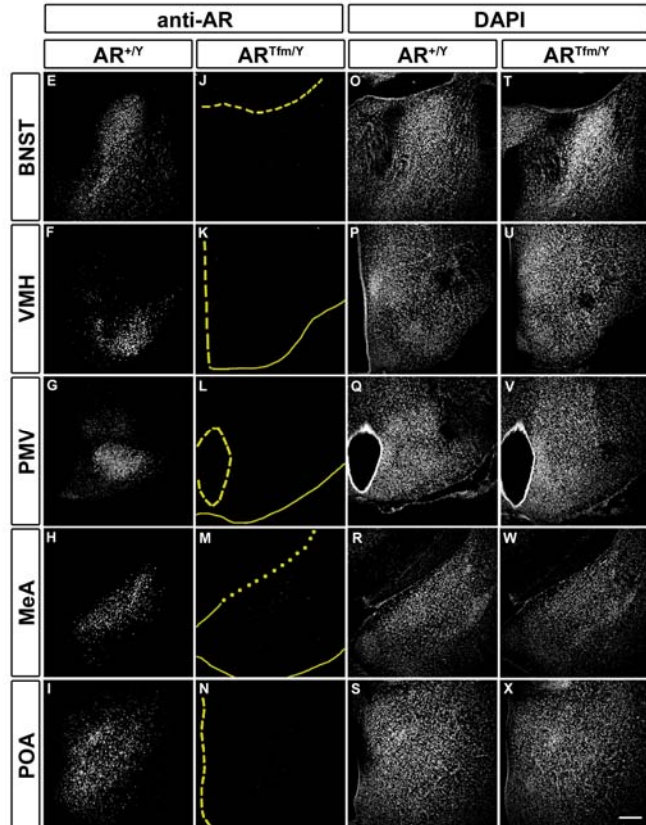
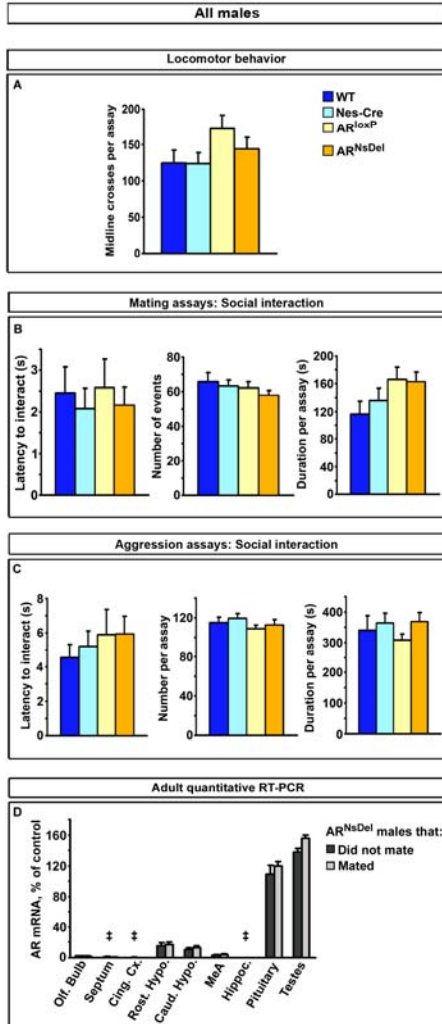
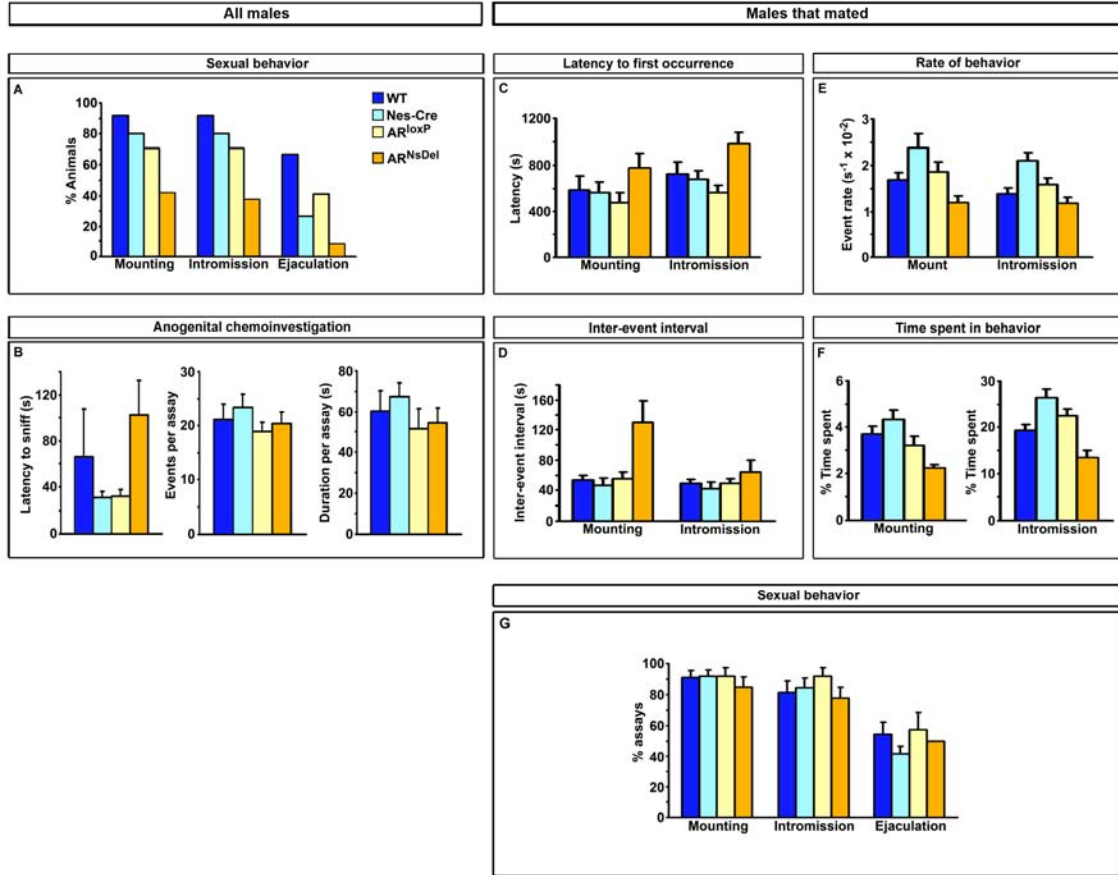


Figure S4



Conclusion

Sexually dimorphic displays are robust and innate and are thus likely to be genetically hardwired. However, these behaviors are complex and there are multiple subroutines that make up each behavior. Male mating, for example, consists of chemoinvestigation and identification of a female, courting of the female with ultrasonic vocalization, multiple mounts and intromissions, and finally ejaculation. Male aggressive behavior includes chemoinvestigation of the male intruder followed by aggressive behaviors directed at the intruder, which can include bites, tail rattles, wrestling, and chasing. Each of these components of male typical behavior contributes to successful copulation or territorial defense. Previous work has demonstrated that the gonadal steroid hormones testosterone and estrogen play integral roles in the display of these male typical behaviors (Matsumoto, et al., 2003; Ogawa, et al., 2000; Ogawa, et al., 1997; Sato, et al., 2004). Our data suggests that estrogen acts as a master regulator early in development to switch on the display of male aggressive behavior in adulthood (Chapter 2). While there is little estrogen in circulation in males at birth, the neonatal surge of testosterone in males is accompanied by an increase of estrogen in the male brain. Estrogen signaling (following conversion by aromatase of testosterone to estrogen) is also required acutely for activation of both these behaviors (Chapter 2). Acute testosterone signaling through the androgen receptor in the adult then modulates the intensity of both male specific aggressive behavior and male mating behavior (Chapter 3).

This model of testosterone signaling acting as a gain control on behaviors switched on by early estrogen elucidates how the two hormone signaling pathways are interacting to regulate male specific behaviors. However, these behaviors are not solely

under hormonal control. Appropriate display of sex specific behaviors also requires correct identification of conspecifics. Male typical mating is only elicited in the presence of a female intruder and male specific aggression is only directed towards male intruders. How information from sensory input and the hormonal milieu are integrated to regulate sex specific behaviors is not yet well understood. Animals lacking the ability to distinguish between male and female conspecifics display inappropriate sex-specific behavior or fail to display these behaviors at all (Leypold, et al., 2002; Mandiyan, et al., 2005; Stowers, et al., 2002). Our studies have demonstrated that female mice supplemented with estrogen at birth and testosterone in adulthood will display the full complement of male typical aggressive behaviors and sexual behaviors. It will be interesting to determine how hormonal and sensory control of sexually dimorphic behaviors interact to direct these displays.

The dual requirement of testosterone and estrogen to regulate male behaviors also indicates a critical role for aromatase, the enzyme that converts testosterone to estrogen. We have demonstrated that neonatal estrogen supplementation to females is sufficient to elicit male typical aggression. As there is little estrogen in the neonatal male circulation and aromatase is required for the conversion of testosterone to estrogen, this suggests that aromatase activity early in development plays a critical role in the masculinization of male behavior. Additionally, others have shown that the deficits in mating and aggression in aromatase knockout males can be rescued with neonatal estrogen supplementation (Toda, et al., 2001a; Toda, et al., 2001b). Our studies also show that aromatase is critical for acute activation of male behaviors. Testosterone supplementation to males mutant for the androgen receptor is sufficient to elicit male typical aggressive behavior (Chapter 2). Since testosterone cannot signal via the

androgen receptor in these males, aromatase must be converting the testosterone into estrogen to signal via the estrogen receptors to regulate male behavior. Aromatase activity is thus crucial for both the organization and activation of male typical aggression.

It will be interesting to determine whether the masculinization of aromatase expression serves only to increase aromatase activity in the male brain. At birth, most cells expressing aromatase in the male brain also express ER α . It is possible that aromatase is merely a marker of cells integral for male behavior and that increased survival of ER α positive neurons switches on the display of male typical aggression. It is unknown whether the estrogen that is synthesized in aromatase expressing neurons signals via ER α in the same cell or if this synthesized estrogen signals in a cell non-autonomous fashion. Alternately, these aromatase positive cells could be playing a crucial role in the circuitry underlying sexually dimorphic behaviors at the electrical level. Colocalization studies of aromatase and other neurotransmitters have yet to definitely determine whether aromatase expressing neurons are excitatory or inhibitory. A critical experiment for the future will be to determine the behavioral consequences of killing, silencing, or activating aromatase positive neurons

It is well documented that early estrogens also have effects on other species besides rodents. Prenatal estrogen treatment is sufficient to induce sex-reversal in toads (Villalpando and Merchant-Larios, 1990) and turtles (Crews, et al., 1991). Estrogen exposure also reverses sex in zebrafish (Orn, et al., 2003) and medaka (Yamamoto, 1958) as well as other fish species. This supports the compelling argument, especially combined with the data presented in this thesis, for improved regulation of environmental estrogens and increased research on the effects of these

agents on development and behavior (Hayes, et al., 2002; Suzawa and Ingraham, 2008).

The implications of the masculinizing effect of neonatal estrogen for human development and behavior are unclear. It appears that hormones may regulate male differentiation differently in humans as compared to rodents. Additionally, there is considerable influence of culture and social factors on human sexual development and behavior. The null mutation of the androgen receptor in humans is referred to as complete androgen insensitivity syndrome (CAIS). These XY patients appear physically female, are treated as females during childhood, and identify themselves as females in adulthood (Wisniewski, et al., 2000). Similar to female rodents given neonatal testosterone, XX females exposed to excess neonatal androgens (congenital adrenal hyperplasia, CAH) are physically virilized at birth (New and Levine, 1984). These females usually undergo sexual reassignment and are raised as females. Recently, however, it has been shown that many of these women with CAH are less satisfied with a female identity and heterosexual orientation (Hines, et al., 2004). Girls with CAH also show a preference for childhood play with boys' toys instead of girls' toys (Berenbaum and Hines, 1992). These studies indicate that androgens (whether or not they are aromatized to estrogen) are both necessary and sufficient for male typical behavior in humans.

There has only been a single reported case of a human with estrogen resistance (Smith, et al., 1994) and sex-typical behaviors have not yet been assessed. Additionally, the few humans presenting with aromatase deficiencies appear to be normal both in reproductive function and sexual identity and orientation (Rochira, et al., 2005). This is

in sharp contrast to rodent studies. It thus appears that hormone signaling in humans and rodents may not regulate sexual differentiation in the same way. It will be interesting to determine if this is due to the profound influence of culture on human sex typical behaviors or if pathways regulating sexually dimorphic behaviors are indeed different between humans and other mammals.

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