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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 ERα and ERβ, 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, 2000, 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

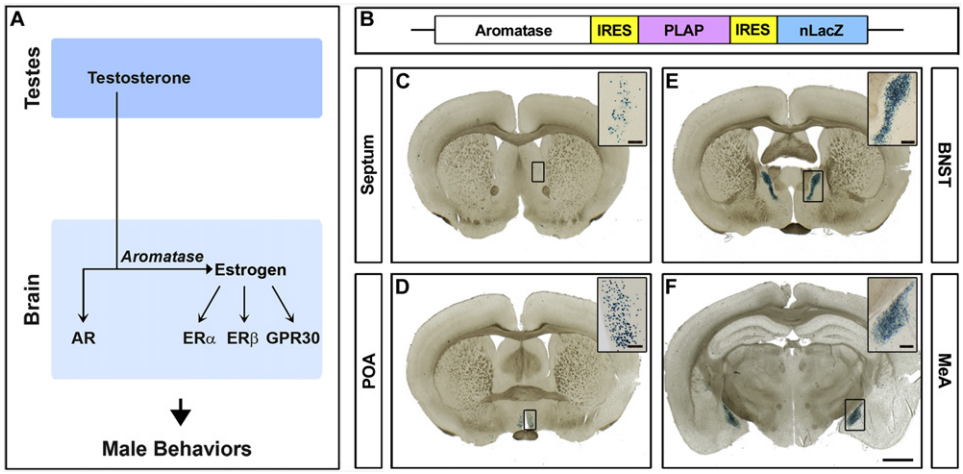

Figure 1. Visualizing Aromatase-Expressing Neurons in the Mouse Brain

(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 βgal 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 μm (C and D) and 50 μm (E and F).

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 coexpress 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 (βgal) 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 wild-type (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 (see Supplemental Text available online).

Analysis of βgal 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 βgal 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 βgal 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 βgal 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

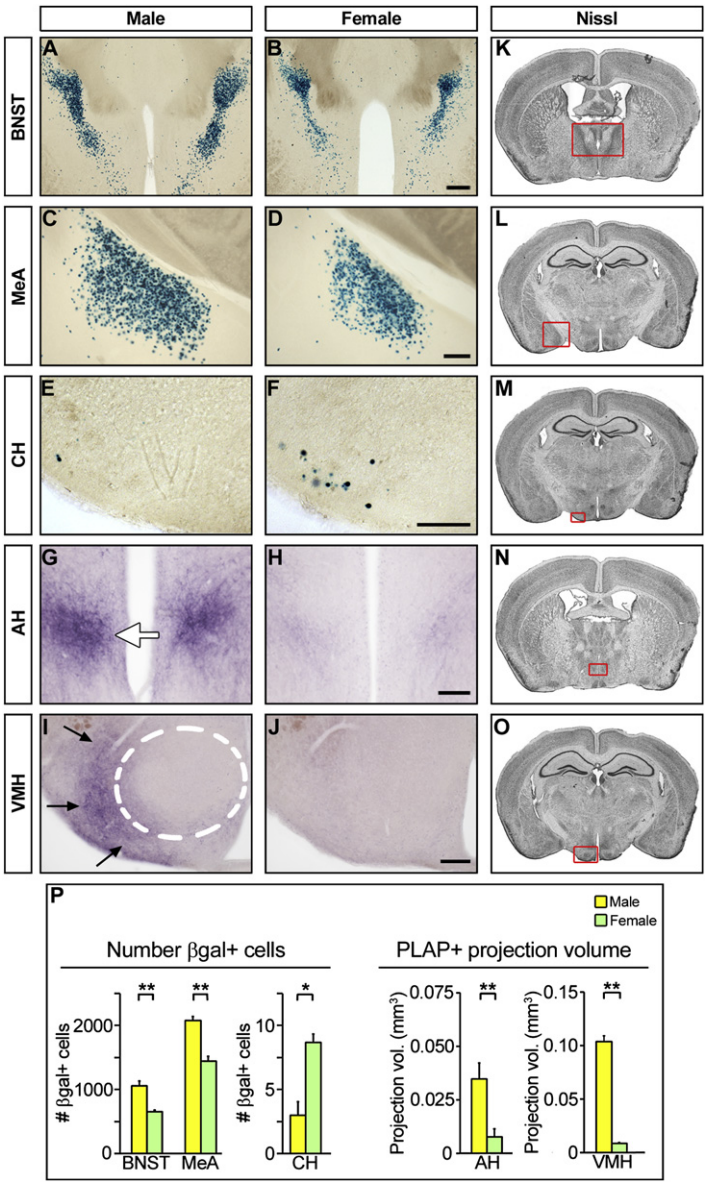

Figure 2. Extensive Sexual Dimorphism in Aromatase-Expressing Neural Pathways

(A–J) Coronal sections through the adult brain of male and female mice harboring the aromatase-IPIN allele stained for βgal (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 μm (A and B) and 250 μm (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 (βgal$^+$) and fiber tracts (PLAP$^+$) of neurons expressing aromatase. Mean and standard error of the mean (SEM) are shown; n ≥ 3; *p ≤ 0.015, **p ≤ 0.005.

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 βgal-positive cells (>95%; n ≥ 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 βgal 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, 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 βgal-positive cells in both sexes (Supplemental Text), indicating that the dimorphisms might 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 might 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,

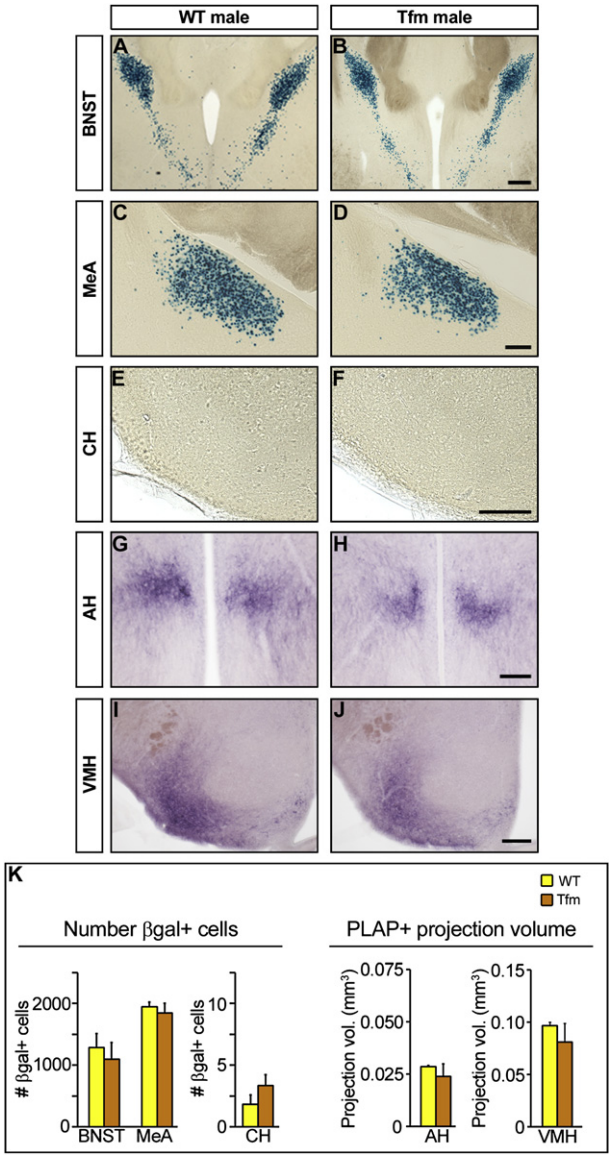

Figure 3. Masculinization of Aromatase-Expressing Neurons Is Independent of AR

(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 βgal (A-F) or PLAP activity (G–J). Scale bars equal 500 μm (A and B) and 250 μm (C–J). (K) There is no significant difference in the number of βgal+ cells or in the volume occupied by PLAP$^+$ fibers between WT and Tfm males. Mean ± SEM; $n \geq 3$; $p \geq 0.26$.

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 βgal-positive neurons in the BNST, MeA, and caudal hypothalamus is similar between Tfm and WT males (Figures 3A–3F and 3K). 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 βgal-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 time points (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

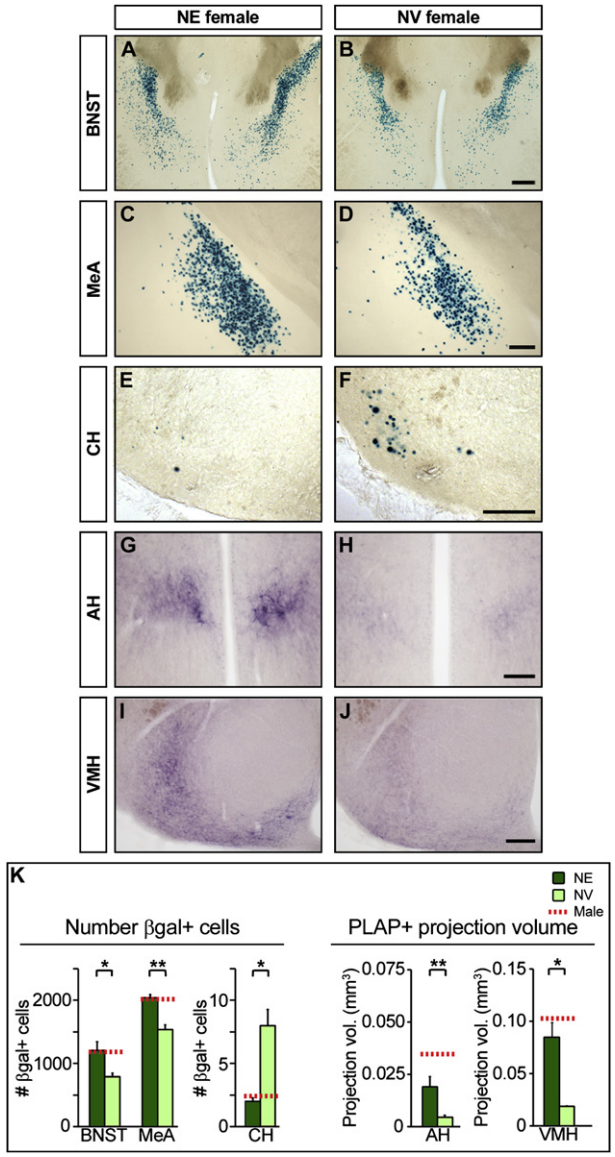

Figure 4. Estrogen Masculinizes Aromatase-Expressing Neural Pathways

(A–J) Coronal sections through the brain of adult NE and NV females bearing the aromatase-IPIN allele stained for βgal (A-F) or PLAP activity (G–J). Scale bars equal 500 μm (A and B) and 250 μm (C–J).

(K) Increase in βgal$^+$ 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 ± SEM; n ≥ 3; *p ≤ 0.028, **p ≤ 0.003; these p values were obtained for the comparisons between NE and NV females.

females compared with males (Figure 5J). Because the conditions for TUNEL preclude colabeling for βgal, we immunolabeled sections through the BNST and MeA for βgal 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 βgal in both sexes, with significantly more double-labeled cells present in females compared to males (% apoptotic cells expressing βgal in BNST: females, 50 ± 1; males, 26 ± 2, n = 3, $p < 0.001$; % apoptotic cells expressing βgal 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 with 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 toward 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)

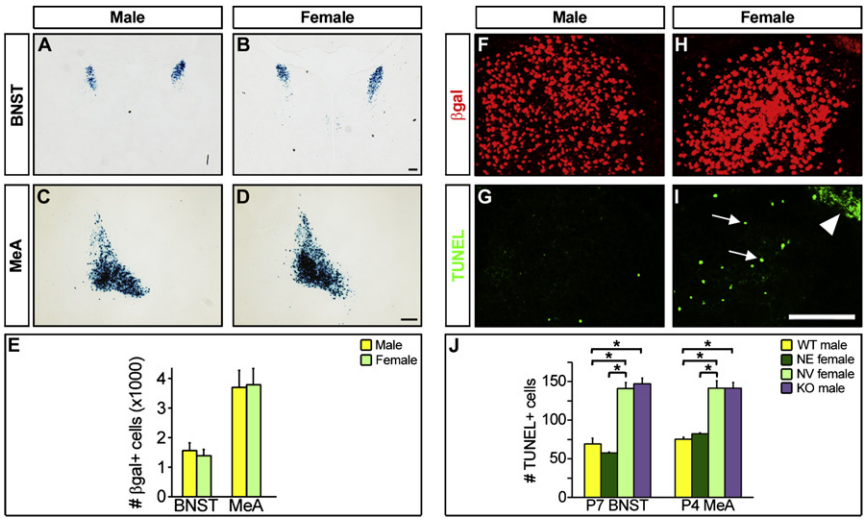

Figure 5. Estrogen Promotes Cell Survival in the Neonatal BNST and MeA

(A–D) Coronal sections through the brain of P1 male and female mice bearing the aromatase-IPIN allele stained for βgal activity. Scale bars equal 250 μm.

(E) No sex difference in the number of βgal^+ cells at P1. Mean ± 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 βgal or stained for apoptosis with TUNEL (arrows). Arrowhead (I) indicates autofluorescent material. Scale bars equal 100 μm.

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

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 masculizing 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 with 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 might 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

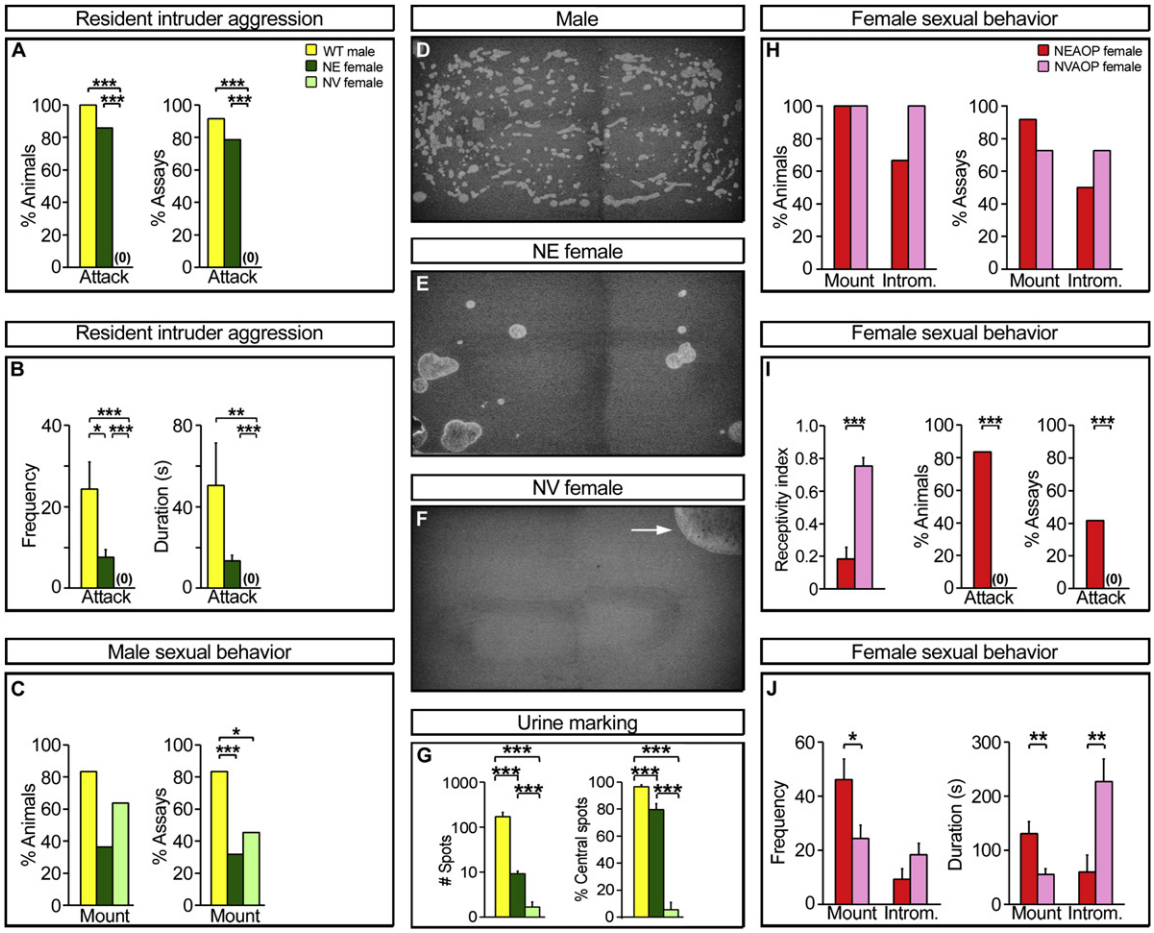

Figure 6. Estrogen Masculinizes Territorial but Not Sexual Behavior

(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 toward 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 ± SEM; $n \geq 6$; *$p \leq 0.037$, **$p \leq 0.022$, ***$p \leq 0.009$.

that NEAOT but not NVAOT females attack male intruders (Figure 7A; Movies S3 and 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 N.M.S., 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 with WT males, indicating that a male typical frequency of urine

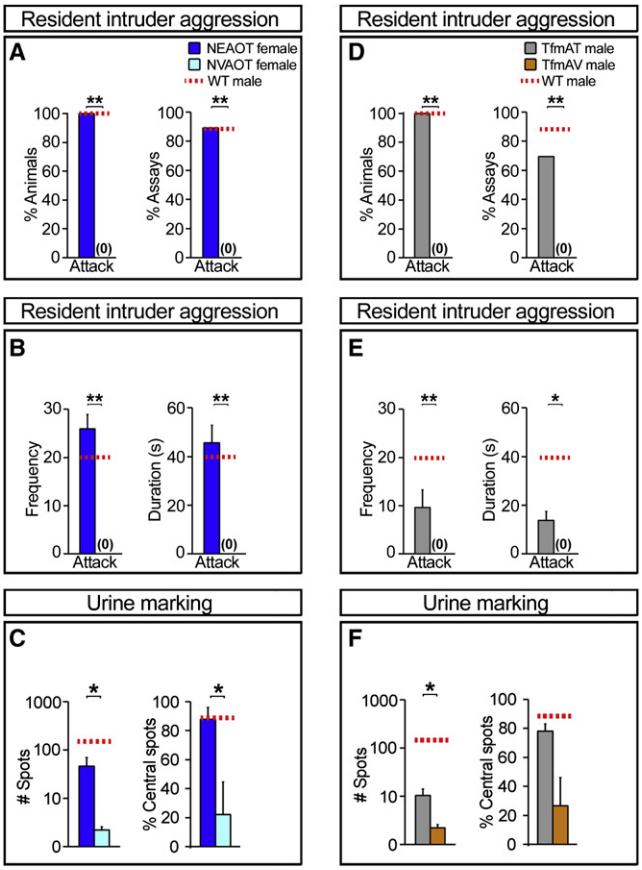

Figure 7. Neonatal Estrogen Masculinizes the Response to Adult Testosterone Administration

(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 ± 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.

marking may require additional contributions from AR signaling. Although TfmAT males deposited more urine spots in the cage center compared with 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% ± 0.5; n = 3) express AR, whereas only a subset of AR-positive neurons colabels with aromatase (35% ± 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 ERα 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 might 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 because the expression of βgal 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 ERα (Figure S4). Both regions also express ERβ (Figure S4), suggesting that estrogen might 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 because these mice direct their aggression, like WT males, exclusively toward 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 toward estrus intruders (5/5 NEAOT and 4/5 NVAOT females mated with estrous mice; $p = 0.29$, chi-square test). Such mating attempts were displayed in $\geq$ 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 prohormone 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 17β-estradiol benzoate (EB) (Sigma) or 100 μg testosterone propionate (TP) (Sigma) dissolved in 50 μl 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 3 weeks. We injected 100 μg 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 βgal activity was visualized in 80 μm (adult) or 12 μm (neonate) thick brain sections obtained from mice homozygous for the aromatase-IPIN allele. Immunolabeling was performed on 65 μm (adult) or 20 μm (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, ERα, and ERβ was localized by ISH as described in Supplemental Experimental Procedures. Sections of 16 μm 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- to 24-week-old singly housed mice in behavioral tests, which were done ≥ 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 1 hr, and then returned to their homecage. The marking pattern was visualized with UV transillumination. Residents were subsequently tested for aggression directed toward 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 nonparametric (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.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Text, Supplemental Experimental Procedures, six figures, and four movies and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00916-7.

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Supplemental Data

Estrogen Masculinizes Neural Pathways and Sex-Specific Behaviors

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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 ± 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 βgal in the two sexes in the BNST (males, 36.57 ± 1.3%; females, 28.2 ± 5.9%; n = 3; p = 0.17, Student's t test) as well as the MeA (males, 40.4 ± 2.3%; females, 41.4 ± 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 βgal 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 *Pml*I genomic clone containing the last two exons of the *aromatase* gene was used to design the targeting vector. An *Asc*I 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 *Asc*I 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 *Asc*I 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-IPIN 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 μg and 5 μg of EB followed by 50 μg of progesterone. The females were used for sexual behavior 4 - 6 hours after progesterone injection. Females were allowed to recover for ≥ 1 week between assays.

Immunostaining

The primary antisera used in this study are: rabbit anti-βgal (Cortex Biochem, 1:2000 or ICL, 1:5000), mouse anti-βgal (Promega, 1:2500), mouse anti-NeuN (Chemicon, 1:300), rabbit anti-ERα (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^{o}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^{o}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 μm, and adjacent sections were collected on alternating slides. One set of slides was immunolabeled for βgal 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 ERα and ERβ mRNA in the neonatal brain, and aromatase message in the adult brain. The probes used to detect ERα and ERβ 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 μm. These sections were treated with proteinase K (10 μg/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^{o}C. These sections were subsequently incubated for 14 - 18 hours at 65^{o}C in fresh hybridization buffer containing 0.5 μg/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^{o}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^{o}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 ERα 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 βgal activity (G-L). The expression of βgal mirrors that of aromatase message. There are no βgal positive cells in the AON and the thalamus, regions with no detectable aromatase message. There are small clusters of aromatase mRNA expressing and βgal positive cells in adjacent sections through the BNST, MeA, septum, and POA. **(S)** There is no sex difference in the number of βgal 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 μm.

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 βgal (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 βgal 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 μm (A, B) and 250 μm (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 μm.

Figure S4. ERα and ERβ 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 ERα or ERβ mRNA. There are ERα and ERβ 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 βgal (red) and ERα (green). The merged

images show that individual cells co-express (yellow) aromatase (βgal) and ERα in the BNST and MeA. The vast majority (>99%) of βgal positive cells co-express ERα. n ≥ 2. Scale bars equal 250 μm (A-H) and 100 μm (I-T).

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

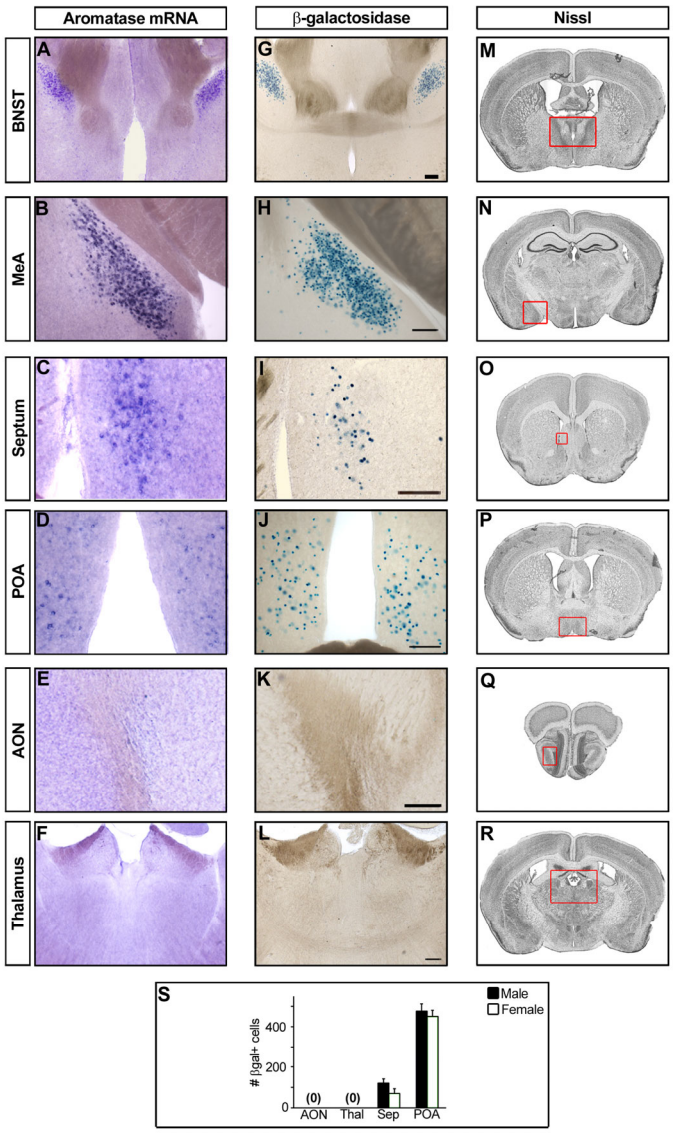
Representative coronal sections through the brain of adult females bearing the aromatase-IPIN allele treated with estrogen or vehicle as adults and stained for βgal (A-J) or PLAP activity (K-R). There appears to be no significant difference in aromatase positive neural pathways between estrogen treated females and their controls. Adult females were injected subcutaneously every other day with 5μg of estrogen or vehicle for three weeks. These animals were sacrificed and their brains processed for βgal 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 ≥ 2. Scale bars equal 250 μm.

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

(A) Adult females treated neonatally with testosterone (NT) have significantly more aromatase expressing (βgal+) 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 βgal+ 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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β-galactosidase
Nissl
BNST
MeA
Septum
POA
AON
Thalamus
A
B
C
D
E
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H
I
J
K
L
M
N
O
P
Q
R
S
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Female
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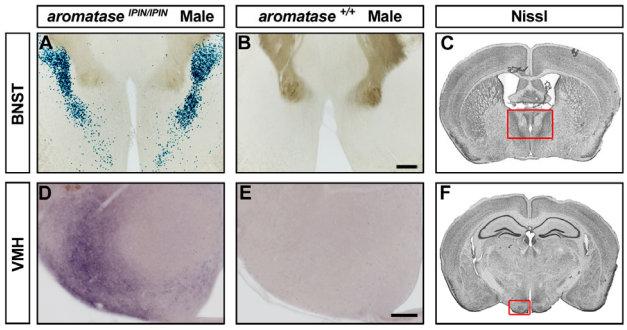
200
0
(0)
(0)
AON
Thal
Sep
POA

Figure S2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aromatase +/+ Male
Nissl
BNST
VMH
A
B
C
D
E
F

Figure S3

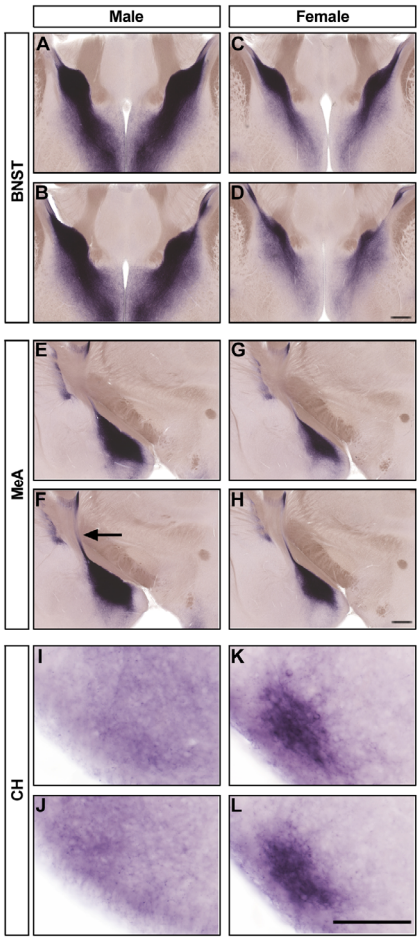


Figure S4

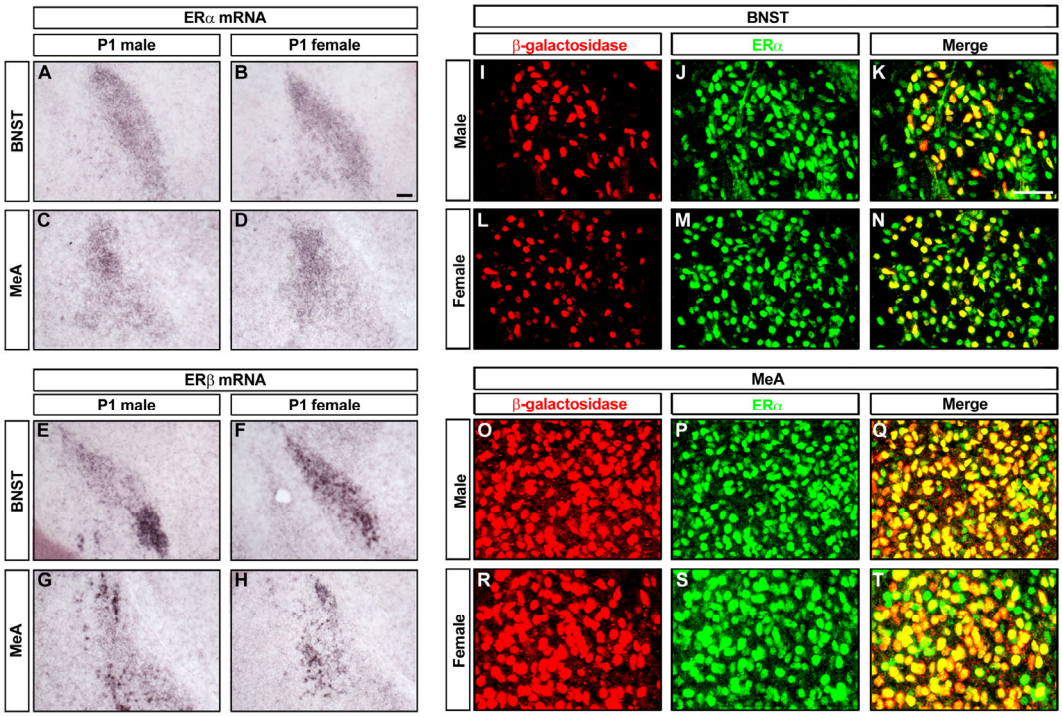
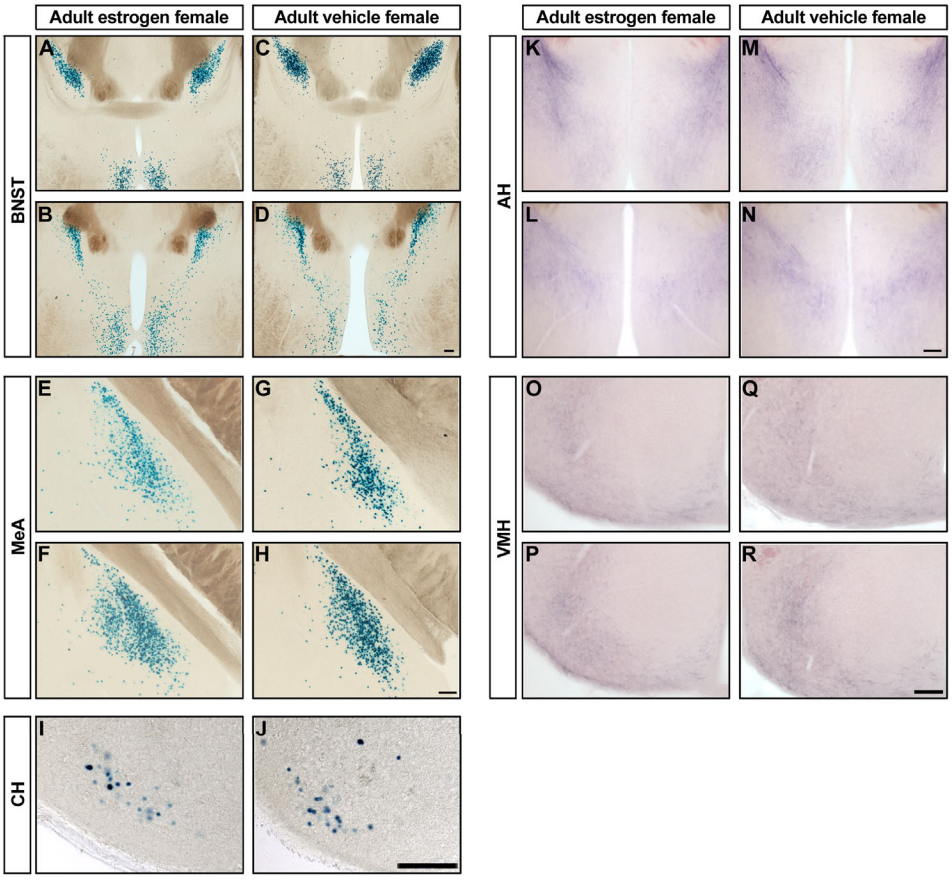

ERα mRNA
P1 male
P1 female
BNST
MeA
A
B
C
D
BNST
β-galactosidase
ERα
Merge
Male
Female
I
J
K
L
M
N
ERβ mRNA
P1 male
P1 female
BNST
MeA
E
F
G
H
MeA
β-galactosidase
ERα
Merge
Male
Female
O
P
Q
R
S
T

Figure S5


Adult estrogen female
Adult vehicle female
Adult estrogen female
Adult vehicle female
BNST
A
C
B
D
AH
K
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L
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G
F
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VMH
O
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CH
I
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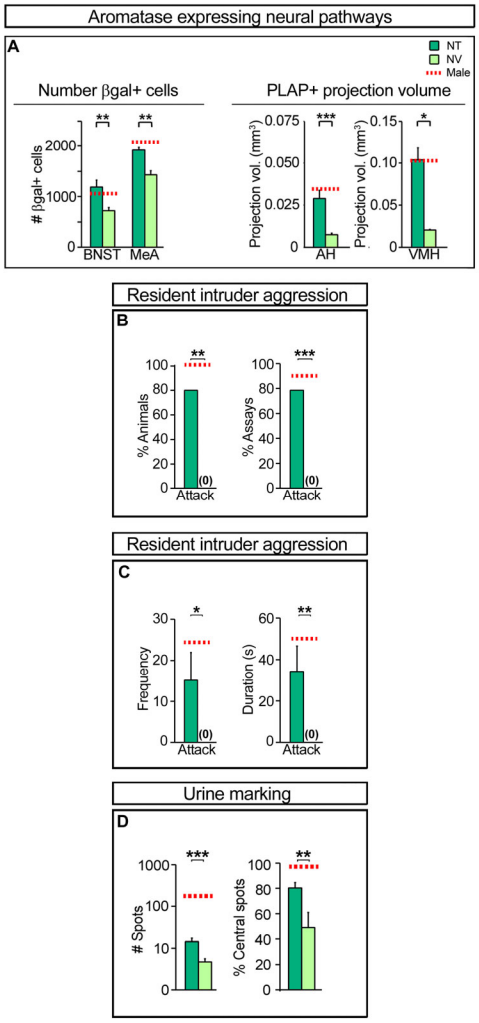
Figure S6


Aromatase expressing neural pathways
A
NT
NV
Male
Number βgal+ cells
PLAP+ projection volume
βgal+ cells
2000
1000
0
BNST
MeA
Projection vol. (mm³)
0.075
0.050
0.025
0
AH
0.15
0.10
0.05
VMH
Resident intruder aggression
B
% Animals
% Assays
100
80
60
40
20
Attack
(0)
Resident intruder aggression
C
Frequency
30
10
Duration (s)
Urine marking
D
Spots
1000
100
10
% Central spots