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

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SUMMARY

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

INTRODUCTION

All sexually reproducing animals exhibit gender dimorphisms in behaviors that are characteristic of the species. Such sex differences in behaviors can be observed in many displays, including in mating, territorial defense, and parental care. Gonadal steroid hormones play a critical role in the neural circuits that mediate sexually dimorphic behaviors: they organize the differentiation of these circuits in the developing animal, and activate these neural pathways to influence sex specific behaviors in the mature organism. Such an “organizational”

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effect is thought to lead to irreversible modifications in subsequent behavior, whereas the "activational" function of the hormones results in acute changes in the behavioral repertoire (Arnold et al., 2003; Goy and McEwen, 1980; Morris et al., 2004; Phoenix et al., 1959). Male typical patterns of behavior are controlled by both testosterone and estrogen in many vertebrates, including mammals. However, the relative contribution of these two hormone signaling pathways to the masculine differentiation of brain and behavior remains to be determined.

The requirement of estrogen for male behaviors appears counter-intuitive as this circulating ovarian hormone is essentially undetectable in the males of most species. Testosterone, or a related androgen, is an obligate precursor of estrogen, and circulating testosterone in males can be metabolized into estrogen in the brain by the enzyme aromatase (Balthazart and Ball, 1998; MacLusky and Naftolin, 1981; Naftolin and Ryan, 1975). It is this target derived estrogen that controls male behaviors, and male mice null for aromatase display profound deficits in male typical mating and aggression (Honda et al., 1998; Toda et al., 2001a; Toda et al., 2001b). The precursor-product relation between testosterone and estrogen raises the possibility that the sole function of testosterone in the neural control of male behaviors is to serve as a circulating prohormone for estrogen (Figure 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 often utilized dihydrotestosterone (DHT) to examine AR function, as this steroid is a non-aromatizable androgen. Recent evidence indicates, however, that 3βAdiol, a DHT metabolite found in vivo, is an estrogenic steroid capable of signaling via nuclear estrogen receptors (Ishikawa et al., 2006; Pak et al., 2005; Sikora et al., 2009; Wahlgren et al., 2008; Weihua et al., 2001). This makes it difficult to unambiguously define a role for AR in controlling male behaviors using DHT. The use of constitutive AR mutants is also not definitive in defining a role for AR in male behaviors as these animals have low, often undetectable, levels of circulating testosterone (Sato et al., 2004) (SAJ, unpublished observations) resulting from postnatal testicular atrophy. Consequently, the behavioral deficits in constitutive AR mutant males could result solely from inadequate estrogen synthesis and signaling in the brain due to the low levels of circulating testosterone.

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

specific patterning of gene expression and behavior, the extent to which this hormone signals through AR for masculinizing the brain and behavior remains unclear (Juntti et al., 2008).

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

RESULTS

Sparse expression of AR in the developing brain

There is a male specific transient spike in serum testosterone at birth (postnatal day 1, P1) followed by a sharp drop in circulating titer within 36 hours, and these low baseline levels of testosterone persist until puberty. By contrast, the ovaries are quiescent during the neonatal period, and there is little circulating estrogen (or testosterone) in female pups (McCarthy, 2008; Motelica-Heino et al., 1988). The male specific surge of testosterone and its subsequent conversion to estrogen is thought to be critical for the sexual differentiation of the neural circuits that control many dimorphic behaviors (Motelica-Heino et al., 1993; Peters et al., 1972; Phoenix et al., 1959; Whalen and Nadler, 1963; Wu et al., 2009). We wished to identify the neurons that respond to this critical testosterone surge via AR at birth. We analyzed AR expression in mice bearing a previously described knock-in AR allele (AR-IPIN allele) that permits co-expression of the sensitive, genetically encoded reporter, nuclear β-galactosidase (βgal), in all cells that express AR (Shah et al., 2004). Previous work has implicated AR expressing regions such as the medial amygdala (MeA), posterior medial component of the medial subdivision of the bed nucleus of the stria terminalis (BNST), and the preoptic hypothalamus (POA) as being critical for the display of male typical mating, aggression, and territorial marking (Commins and Yahr, 1984; Kondo et al., 1998; Liu et al., 1997; Meisel and Sachs, 1994). Surprisingly, we only detected occasional, faintly AR positive cells in these regions at P1 (Figures 2A–C and S1D). There were >15–90 fold fewer AR expressing cells in these regions at birth (BNST 12.1 ± 5.7; MeA 16.4 ± 10.3; POA 59.8 ± 20.3; n = 6) than in older animals (Figure 3) (Shah et al., 2004; Wu et al., 2009). Similar results were obtained by directly immunolabeling for AR (data not shown). We could detect more AR positive cells in the MeA, BNST, and POA at P4, a timepoint by which the testosterone surge has already subsided (Figures 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 (Figure S1A, B). The expression pattern of AR in the BNST, POA, and MeA resolved into widespread, intense labeling in these areas at P7, resembling the pattern observed in the adult brain (Figures 2G–L, S1, and data not shown).

The fetal testis produces testosterone from E13 (Crocoll et al., 1998) and we wondered if this hormone signaled via AR in the prenatal brain to masculinize neural pathways. We did not

observe AR in the brain at E13.5, using βgal expression to visualize AR positive cells in mice bearing the AR-IPIN allele (Figure S2A–D). By contrast, at E15.5 and E17.5, we could visualize AR expression in the neurons near the ArcN and VMH but not in the BNST, POA, or MeA (Figure S2E–L). The ontogeny of AR expression in the BNST, POA, and MeA makes it unlikely that this hormone receptor plays a major, cell-autonomous role in masculinizing these neural pathways for male typical behaviors prenatally or at the time of the neonatal testosterone surge.

Estrogen is necessary and sufficient for sexual differentiation of AR expression

Previous work demonstrates that adult AR expression is sexually dimorphic such that there are more AR positive neurons in the BNST, POA, and the basal forebrain in males compared to females (Shah et al., 2004). AR expression in the BNST and POA in the P7 male resembles that observed in the adult male (Figure 2G–L) whereas we did not observe AR positive cells in the basal forebrain at P7 (data not shown). We asked whether the adult pattern of sexual dimorphism in AR expression was also apparent at P7 in the BNST and POA. Immunolabeling for βgal at P7 revealed significantly more AR positive cells in the male BNST and POA than in these regions in the female (Figure 3), consistent with previous reports of sexual dimorphism in these regions (McAbee and DonCarlos, 1998; Shah et al., 2004).

The sexual dimorphism in AR expression is unlikely to arise from testosterone signaling via AR as this receptor is expressed in few cells in these regions at the time of the testosterone surge. Rather it is likely to result from the autonomous action of forebrain patterning genes or from estrogen signaling (Arnold et al., 2003; Hoch et al., 2009; Wu et al., 2009). Previous work indicates that both nuclear estrogen receptors (ERα, β) as well as aromatase are expressed in the early neonatal brain (Harada and Yamada, 1992; Wolfe et al., 2005; Wu et al., 2009). Indeed, we observed abundant expression of ERα, β and aromatase in the BNST and POA at P1 (Figure 4A–F), consistent with the notion that testosterone may masculinize AR expression after its conversion into estrogen.

If testosterone does masculinize AR expression in the BNST and POA subsequent to aromatization to estrogen, then either of these two hormones should be sufficient to drive male pattern differentiation of AR in these regions. Indeed, we find that administering testosterone or estrogen to P1 females masculinizes the number of AR positive cells in the P7 BNST and POA (Figure 4G–M), consistent with previous pharmacological studies in other vertebrates (Kim et al., 2004;McAbee and DonCarlos, 1999a, b). To determine whether the conversion of testosterone to estrogen is essential for the development of these sex differences in AR expression, we examined the BNST and POA of P7 aromatase$^{-/-}$ males (Honda et al., 1998). In these animals, the number of AR positive cells is indistinguishable from that observed in control females, and significantly lower than in control males (Figure 4G, N, O). Taken together, these results show that estrogen controls the sexual differentiation of AR expression in the BNST and POA in males. We note that the few AR positive cells in the P1 BNST and POA may also respond to the testosterone surge at birth by inducing sexual differentiation of AR in neighboring cells that do not express this receptor; in such a scenario, both estrogen and testosterone signal via their cognate receptors to regulate the sex difference in AR expression in a redundant manner. Nevertheless, our findings demonstrate that estrogen is necessary and sufficient to drive masculinization of AR expression in these brain regions.

Genetic deletion of AR in the nervous system

Our results suggest that testosterone serves primarily as a precursor for estrogen during the neonatal period of sexual differentiation of the brain. Consequently, testosterone signaling via AR is unlikely to be essential for the differentiation of the male typical repertoire of dimorphic behaviors. Constitutive deletion of AR in all tissues results in feminization of the external

genitalia, and eventual testicular atrophy, leading to a loss of circulating testosterone in adults (Lyon and Hawkes, 1970; Sato et al., 2004). To bypass the requirement for AR in the testes, we used a Cre-loxP strategy to engineer a deletion of AR specifically in the nervous system. We crossed mice bearing a previously described loxP-flanked allele of AR (AR^{loxP}) (De Gendt et al., 2004) to animals harboring the Nestin-Cre transgene (Nes-Cre) (Tronche et al., 1999) that drives Cre recombinase specifically in neural stem cells and glia (Figure 5A). This strategy should yield an early, nervous system restricted deletion of AR in males carrying the X-linked AR^{loxP} and the Nes-Cre transgenic alleles. Indeed, in contrast to the external phenotypes observed in constitutive AR mutant males, an examination of adult $AR^{loxP/Y}$; Nes-Cre (AR^{NsDel}) mice suggests normal AR function in non-neural tissues (Figure 5B–D, F): the genitalia of these mice are masculinized, and the testes and seminal vesicles, sensitive peripheral tissues responsive to AR signaling, are similar in weight to those of their control littermates (wildtype (WT), $AR^{loxP/Y}$, and Nes-Cre males). While there appears to be an elevation in circulating testosterone in AR^{NsDel} mice, this is not statistically different when compared to the titers of this hormone in males of the control genotypes ($p = 0.157$, Kruskal-Wallis test for multiple group comparisons) (Figure 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 (Figure 5I, J).

Inadvertent deletion of AR in non-neural tissues such as muscle could result in a failure to thrive, a generalized motor deficit or muscular weakness. However, AR^{NsDel} mice appear indistinguishable from their controls in body length and weight (Figure 5G). We also did not observe abnormal gait or gross motor deficits in our behavioral assays with these mutants (data not shown). In addition, AR^{NsDel} males were similar to their control littermates in general motor activity and social interactions such as grooming (Figure S3A–C). 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 as well as P7 in these mutants compared to 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 (Figure 5K–V). Taken together, our genetic strategy yields male mice that have intact peripheral masculinization and circulating testosterone and a deletion of AR that appears restricted to the nervous system. These mutants therefore afford the opportunity to assess the contribution of testosterone signaling via AR in the neural circuits that control male typical behaviors.

AR increases the frequency of male sexual behavior

In order to determine whether AR mediated signaling in the nervous system is essential for male sexual behavior, we examined the behavior of AR^{NsDel} mice in mating assays. In mice, mating consists of a series of stereotyped routines that include mounting, intromission or

penetration (as visualized by pelvic thrusting), and ejaculation. These behaviors can be reliably elicited in a 30 minute assay in which a WT estrous female is introduced into the cage of a singly housed WT male (Mandiyan et al., 2005; McGill, 1962). A statistically similar percent of AR^{NsDel} residents (42%; n = 24 mice) mounted at least once when tested in 2–3 assays for sexual behavior with different estrous females when compared to control residents (Figure S4A). When we analyzed the percent of all assays with mounts, we observed mating in fewer assays with AR^{NsDel} residents compared to males in the control cohort (Figure 6B). Notably, we find that some of the AR^{NsDel} mice never mate, whereas when they do exhibit sexual behavior, these mutants mate in most assays (85% ± 6), similar to control residents (Figure 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} animals (Figure 6C, D). Male mice do not always achieve ejaculation within a 30 minute assay (McGill, 1962), and we observed ejaculation at a similar, low frequency in males of all genotypes (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, F and S4C–F; Supplementary 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 wildtype males. This suggests that AR controls the probability of triggering male mating but not the pattern of this complex behavioral routine. Alternatively, the lowered likelihood of initiating mating behavior could simply reflect a large variability in the extent of AR deletion in the brain. In this scenario, AR^{NsDel} mice who do not mate may have little residual AR in the brain compared to the mutants who do initiate sexual behavior. Several lines of evidence favor the notion that AR regulates the probability of triggering male mating. First, qPCR analysis of AR deletion in individual AR^{NsDel} males reveals a consistent, strong diminution of AR message in all mutants, regardless of their performance in mating assays (Figure S3D). Second, when AR^{NsDel} mutants who did not mate in any of the three 30 minute mating assays were co-housed with females, they successfully sired litters (SAJ, preliminary observations). Finally, only a subset of constitutive AR mutant males attempted to mate when supplemented with testosterone (or estrogen) at doses that recapitulate wildtype circulating levels of this steroid hormone (Olsen, 1992; Sato et al., 2004) (MVW, unpublished observations). Taken together, these findings are consistent with the notion that AR functions in the brain to regulate the likelihood of initiating male sexual behavior.

Chemosensory cues emanating from females are 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 to their control male littermates (Figures S4B, 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 leads to gender discrimination, one consequence of which is female-directed ultrasonic vocalizations by the resident male (Nyby et al., 1977; Pankevich et al., 2004; Stowers et al., 2002). We observe that AR^{NsDel} mice vocalize to female, but not to male, intruders in their cage in a manner similar to their control counterparts, suggesting that sex discrimination is intact in these animals (Figure 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, B), the mutants spent significantly less time fighting compared to control residents, and this deficit persisted even when we restricted our analysis to the assays in which we observed aggressive interactions (Figure 7B, C). The reduction in total duration of attacks cannot be explained by alterations in chemoinvestigation, the latency to first attack, or by the total attack number, as these parameters were statistically similar in all resident males (Figures S5B–E). Rather, our analysis revealed a deficit in the pattern of aggression following the first fight initiated by AR^{NsDel} males (Figures 7D–F and S5F). Compared to the control residents, AR^{NsDel} mice spent less time fighting with the intruder and exhibited a longer interval between successive attacks (Figure 7D, F). 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 fight. Unlike the attack number metric, a measurement of attack rate eliminates from analysis the variable latency to the first attack in any particular assay, and as such represents a corrected, perhaps more sensitive, measure of the frequency of fighting. As part of territorial behavior, resident WT male mice mark their territory by depositing many urine spots across the cage floor, whereas females pool their urine in one or a few large spots in a corner of the cage (Desjardins et al., 1973; Kimura and Hagiwara, 1985). Thus, there is a dimorphism in the number as well as the pattern of urine marks deposited by male and female mice. The male pattern of urine marking appears independent of AR function in the nervous system as AR^{NsDel} residents also distribute their urine marks across the cage floor, similar to WT males (Figure 7H). By contrast, we find that AR^{NsDel} residents deposit fewer urine marks compared to 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 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 to our findings 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 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 inter-male aggression. We find that AR^{NsDel} mutants mark their territory in a male pattern, but they deposit far fewer urine marks, indicating a deficit in this component of male territorial display. Our studies also indicate that it is unlikely that the masculine behaviors observed in AR mutants result from a failure to delete AR prior to the early neonatal sexual differentiation of the brain. Indeed, we find that there is minimal AR expression in regions known to be critical for dimorphic behaviors during this period, when gonadal hormones orchestrate sexual differentiation of the brain (Meisel and Sachs, 1994; Morris et al., 2004; Motelica-Heino et al., 1993), and this sparse neonatal AR expression is largely eliminated in AR^{NsDel} males. Importantly, we demonstrate that sexual differentiation of AR expression itself is controlled by estrogen signaling. The sparse perinatal expression of AR in the brain suggests that the behavioral phenotype of AR^{NsDel} mice results from activational rather than organizational effects of testosterone acting on AR. We cannot exclude the possibility that AR also functions during the later postnatal period, including puberty, to influence the maturation of the neural circuits that drive male behaviors (Schulz et al., 2009). Regardless of the exact timepoint at which AR functions to control behaviors, our findings indicate that AR is not a master regulator for male behaviors, but rather, it serves as a gain control mechanism to regulate the extent of male sexual and territorial displays.

We find minimal AR expression in various brain regions of AR^{NsDel} males, demonstrating that most neurons do not need to signal via this receptor to drive male sexual and territorial behaviors. As we have deleted AR in the developing and adult animal, we cannot exclude the possibility that masculine differentiation of behaviors in these mutants reflects compensatory mechanisms that are activated in the absence of AR signaling. Mice 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 wildtype levels (Olsen, 1992; Sato et al., 2004; Scordalakes and Rissman, 2004). Such studies therefore suggest that AR in the brain may also control the expression of other genes that modulate the levels of male behavioral displays. Irrespective of the exact molecular mechanisms, our data demonstrate that testosterone signaling via AR is essential for wildtype male behavior.

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

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

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

generates the male typical levels of these behaviors. It will be interesting in future studies to identify the molecular and circuit level mechanisms that are controlled by these hormones.

EXPERIMENTAL PROCEDURES

Animals

Mice were housed in a rodent barrier facility at UCSF with a 12:12 hour light:dark cycle. All studies with animals were done in accordance with UCSF IACUC protocols. The AR-IPIN knock-in 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 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 hemizygous Nes-Cre males to generate males bearing both alleles ($AR^{loxP/Y}$; Nes-Cre) as well as 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 in 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 hour in a fresh cage, and then returned to the homecage. Males were subsequently tested for male mating for 30 min with an estrous intruder female. Following mating tests, mice were tested for aggression for 15 min in the resident-intruder paradigm, using an adult male intruder who was group-housed with other intruders between testing sessions. The males were subsequently tested for ultrasonic vocalizations for 3 min in response to an intruder in their cage. Each resident was tested for vocalization separately with a male and a female intruder. All animals were tested 2–3 times each in assays of 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 $\geq$ 2 days.

Histology

We used age matched mice for histological experiments. We visualized βgal activity in 20 μm thick sections using brightfield optics. Fluorescent immunolabeled sections (20 μm thick, P7; 65 μm 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-βgal (1:2500, Promega). The anti-AR antibody appears specific to AR as we did not observe AR+ cells in various brain regions in constitutive AR mutant males (Tfm) (Figure S3). Staining for βgal activity and fluorescent immunolabeling were performed as described previously (Shah et al., 2004; Wu et al., 2009). Quantitation of cell numbers was performed using stereology and other experimental approaches.

Brain regions were identified based on landmarks as defined in standard atlases of the mouse brain (Paxinos and Franklin, 2001; Paxinos et al., 2007). At P1 and P7, βgal 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 data with Lillefors' goodness-of-fit test of normality. Datasets not violating this test were analyzed with parametric tests (Student's t-test for 2 groups or one-way ANOVA), otherwise we used non-parametric analyses (Kolmogorov-Smirnov (KS) test for 2 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 control cohorts (WT, $AR^{loxP/Y}$, and Nes-Cre).

qPCR

At each age, we collected tissue from AR^{NsDel} mice and each control group to quantitate AR mRNA levels. 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. As AR expression was similar across all control genotypes but was significantly different from AR^{NsDel} mice in all brain 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 5(I, J) and S3D as the percent of AR mRNA in AR^{NsDel} males in various tissues compared to the control cohort.

Hormones

Serum testosterone and estradiol titers were determined with kits from DRG International and Cayman Chemicals, respectively. We induced estrus in adult ovariectomized 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 μL subcutaneous injection of hormone or vehicle. We injected either 100 μg testosterone propionate (Sigma) or 5 μg of estradiol benzoate dissolved in sesame oil.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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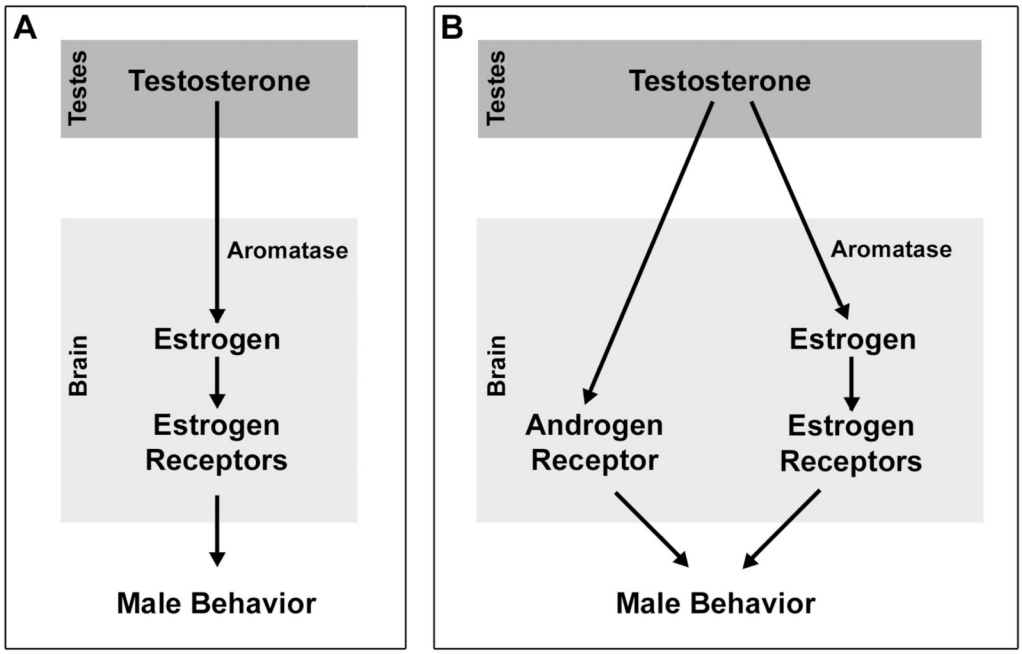

Figure 1. Models for the role of testosterone in masculinizing the brain and behavior

Schematics illustrating possible mechanisms whereby testosterone controls male typical behaviors.

(A) Testosterone 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, androgen receptor (AR), to influence directly the neural circuits that control male behaviors.

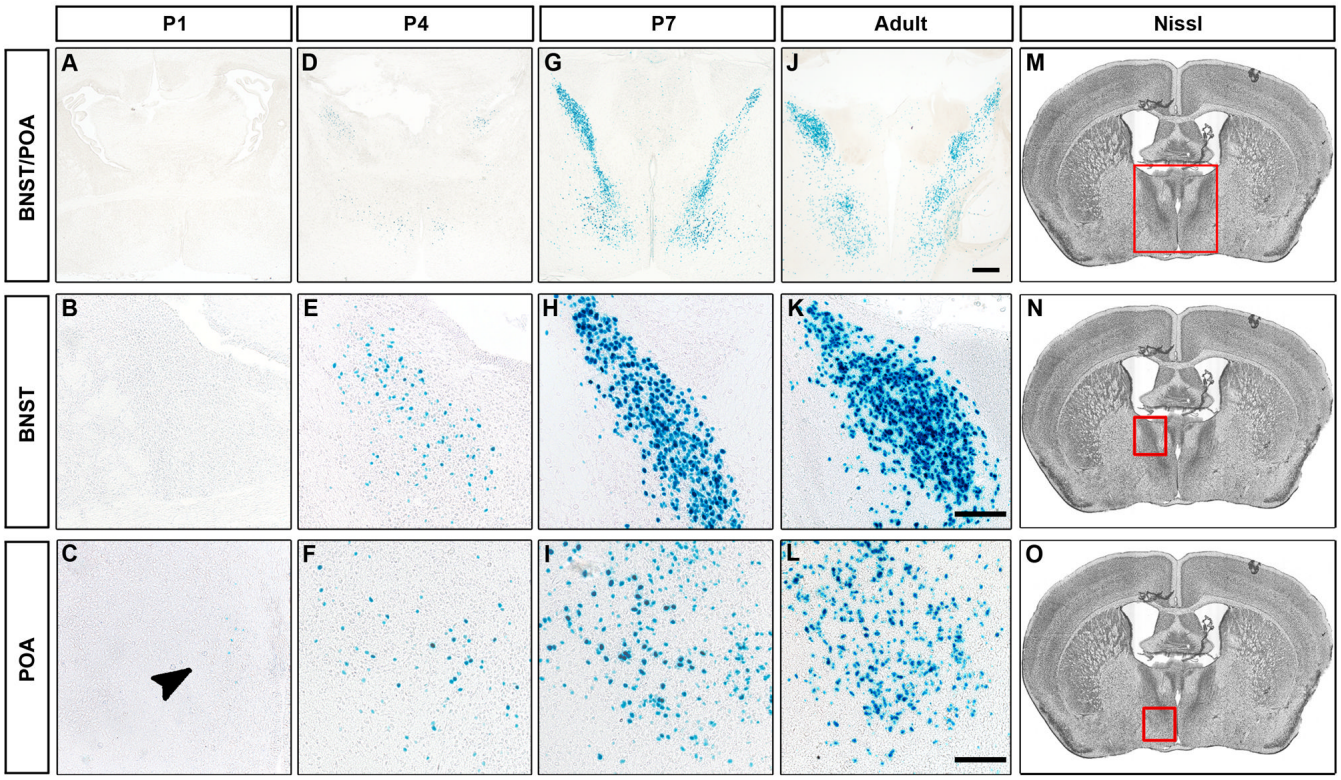

Figure 2. Limited expression of AR in the newborn brain

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

(M–O) Nissl stained sections highlighting (red box) the BNST (M, N) and POA (M, O). $n \geq 3$ at P1, P4, P7.

See also Figures S1, S2.

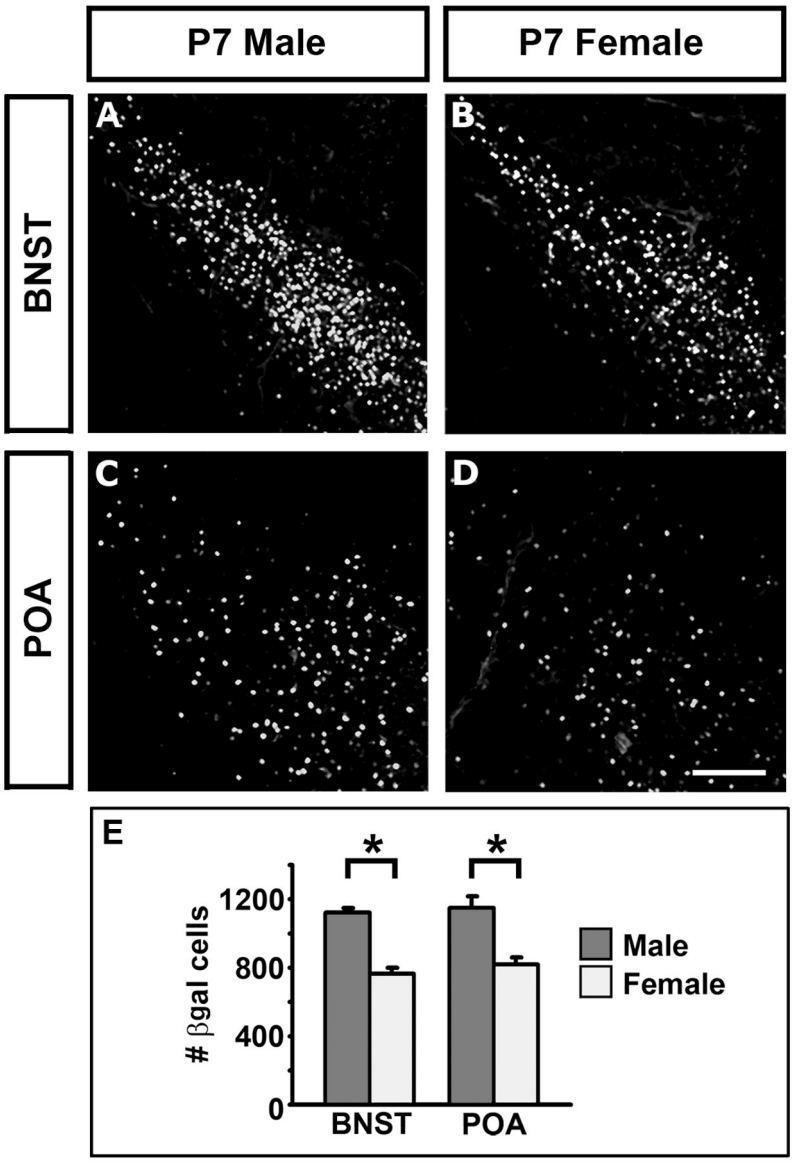

Figure 3. Sexual dimorphism in AR expression

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

(E) There are more βgal+ cells in the BNST and POA of $AR^{IPIN/Y}$ males than in $AR^{IPIN/IPIN}$ females.

Mean ± SEM; n = 4 for each genotype, * $p \leq 0.005$. Scale bar equals 100 μm.

See also Figure S2.

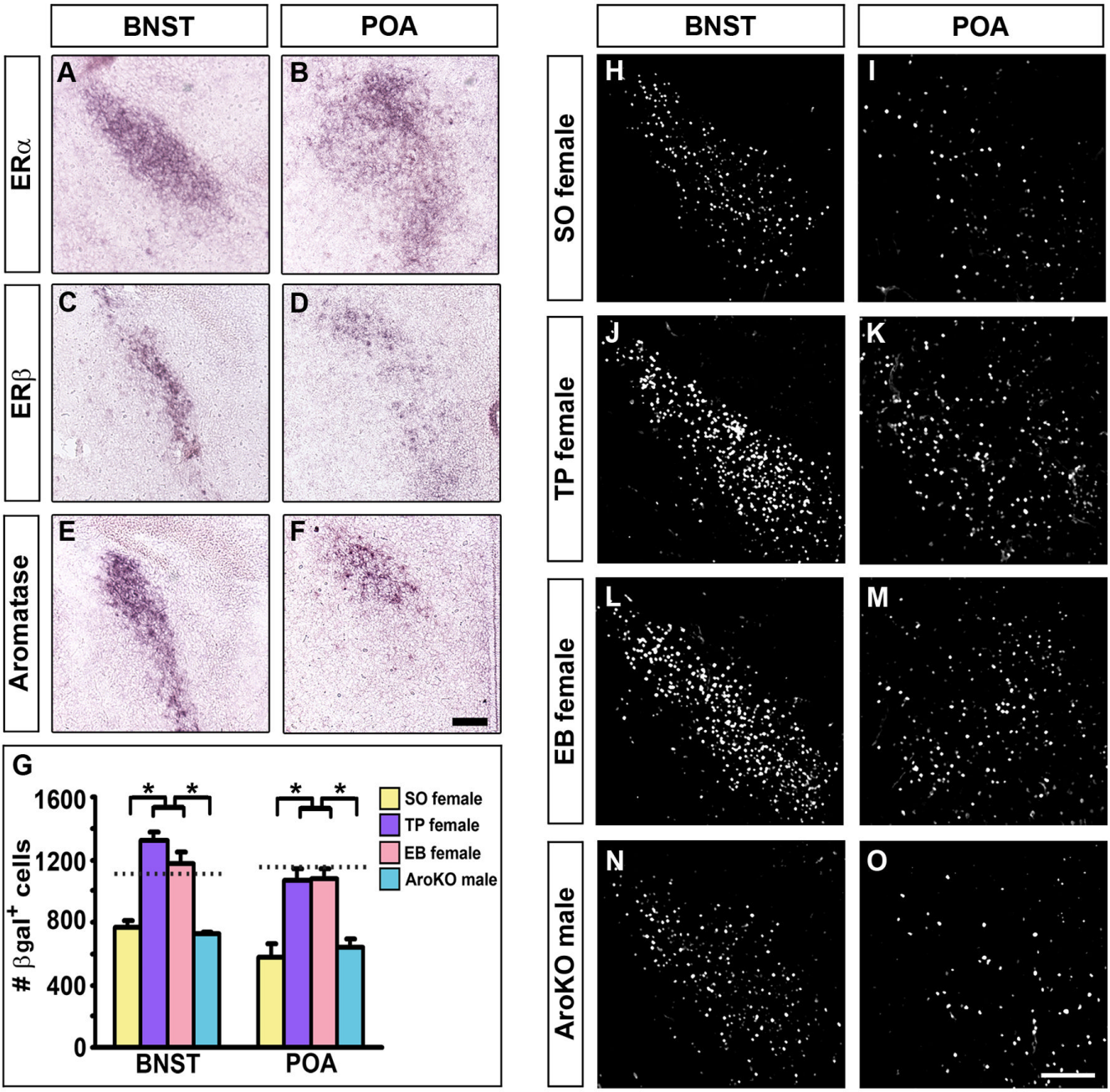

Figure 4. Estrogen masculinizes AR expression

(A–F) Coronal sections through the BNST and POA of P1 males labeled for ERα, ERβ, or aromatase mRNA.

(G–O) There are more βgal^{+} cells in the P7 BNST and POA of $AR^{IPIN/IPIN}$ females treated at P1 with testosterone (TP female) or estrogen (EB female) compared to vehicle (SO female) or to $aromatase^{-/-}$; $AR^{IPIN/Y}$ (AroKO) males. Horizontal dashed lines represent the mean value in WT males shown in Figure 3. Mean ± 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 μm (A–F) and 100 μm (H–O).

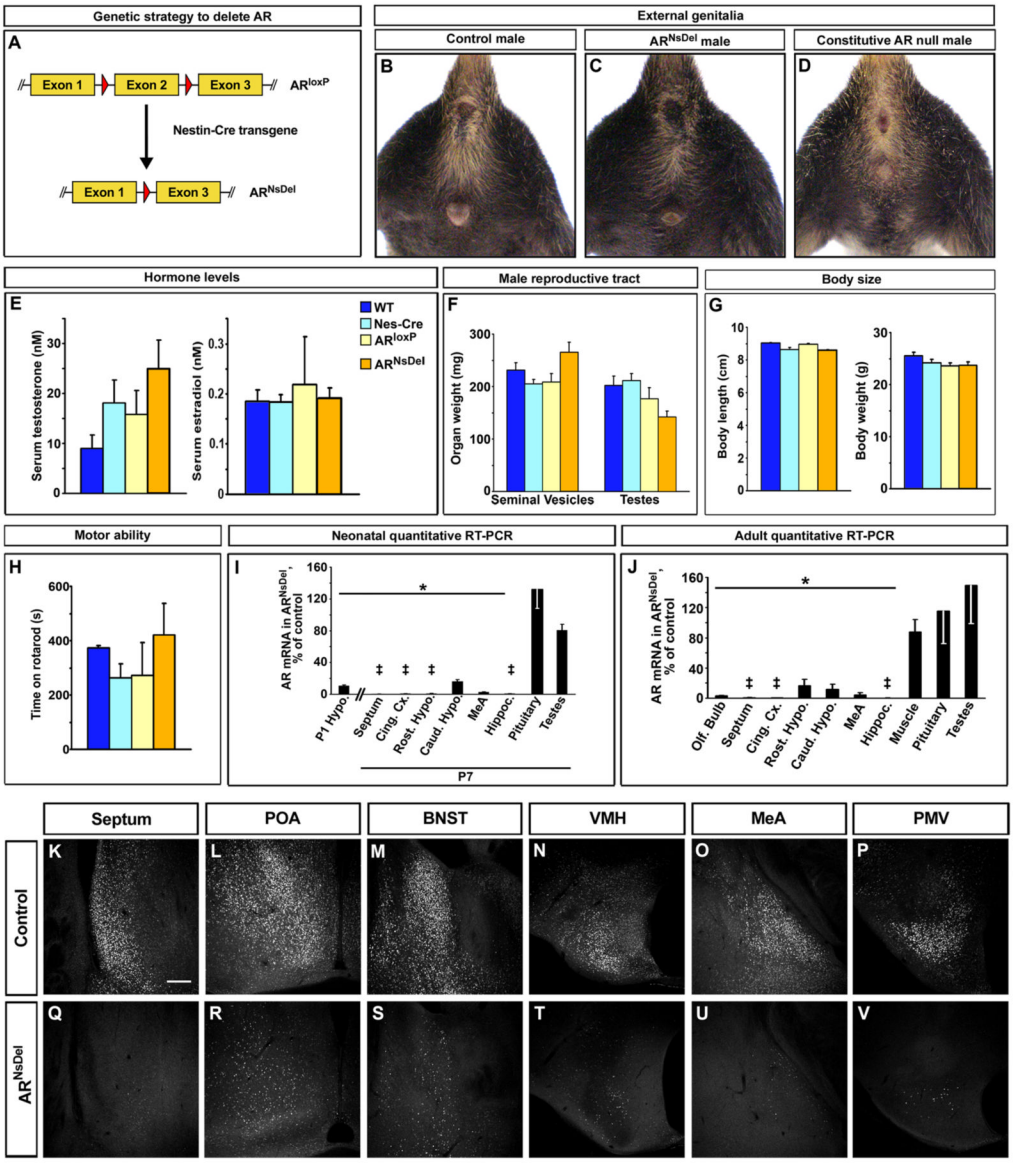

Figure 5. Targeted deletion of AR in the nervous system

(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 in all males ($n \geq 12$/genotype).

(F) Similar weight of testes and seminal vesicles in all males ($n \geq 7$/genotype).

(G) Similar body length (snout to base of tail, $n \geq 4$/genotype) and weight ($n \geq 12$/genotype) in all males.

(H) No difference in time to fall from rotarod between control and AR^{NsDel} males ($n \geq 4$/genotype).

(I, J) Reduction in normalized AR mRNA in the brain of P1 (I), P7 (I), and adult (J) AR^{NsDel} males shown as percent of AR mRNA levels in controls (Hypo., hypothalamus; Cing. Cx., cingulate cortex; Rost. Hypo., rostral hypothalamus; Caud. Hypo., caudal hypothalamus; Hippoc., hippocampus). Similar AR mRNA levels between AR^{NsDel} and control males in other tissues. Mean ± SEM; ‡ mRNA < 0.5% of control, * $p < 5\times10^{-4}$, $n = 4$ 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 compared to control males. Scale bar equals 100 μm.
See also Figure S3.

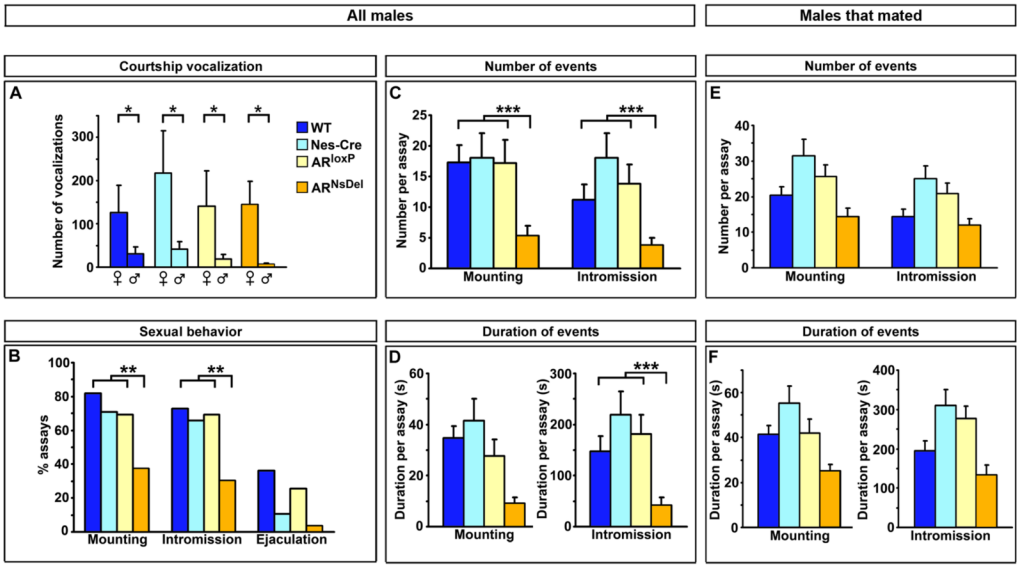

Figure 6. AR increases the frequency of male mating

(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) No statistical difference between AR^{NsDel} and control males in time spent mounting, but as a group, AR^{NsDel} mice intromit for a shorter duration than controls.

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

Mean ± 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, S2.

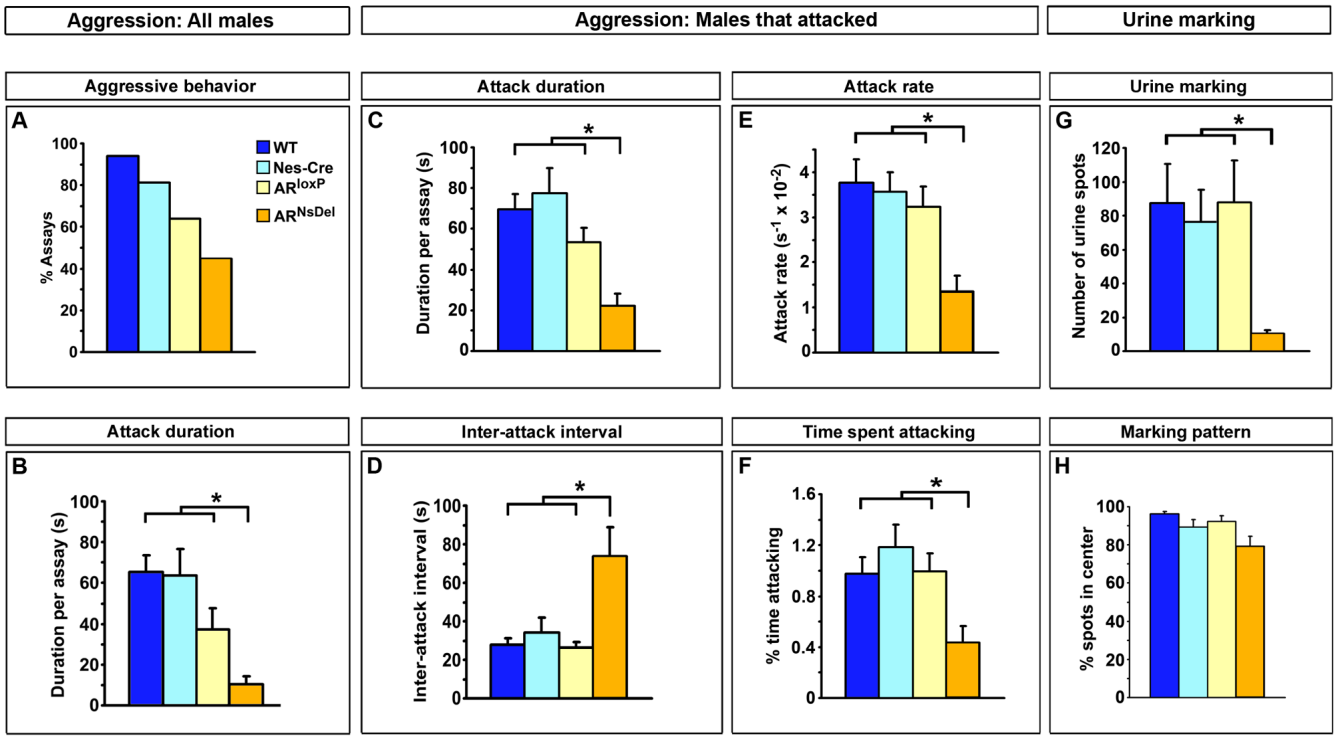

Figure 7. AR increases the levels of male territorial displays

(A) No statistical difference between AR^{NsDel} and control males in percent assays containing aggression.

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

(C) When AR^{NsDel} residents fight, they do so for a shorter duration compared to 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 for a smaller percent of the duration of the assay (F) compared to controls.

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

(H) No difference between AR^{NsDel} and control males in the percent of urine marks away from cage perimeter.

Mean ± SEM; * $p < 0.05$, Tukey's test following Kruskal-Wallis comparison, $n \geq 12$/genotype. See also Figure S5.