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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" 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 counterintuitive because this circulating ovarian hormone is essentially undetectable in the males of most species. Testosterone, or a related androgen, is an obligate precursor of estrogen, and circulating testosterone in males can be metabolized into estrogen in the brain by the enzyme aromatase (Balthazart and Ball, 1998; MacLusky and Naftolin, 1981; Naftolin and Ryan, 1975). It is this target-derived estrogen that controls male behaviors, and male mice null for aromatase display profound deficits in male-typical mating and aggression (Honda et al., 1998; Toda et al., 2001a, 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, because this steroid is a nonaromatizable androgen. Recent evidence indicates, however, that 3βAdiol, a DHT metabolite found in vivo, is an estrogenic steroid capable of signaling via nuclear estrogen receptors

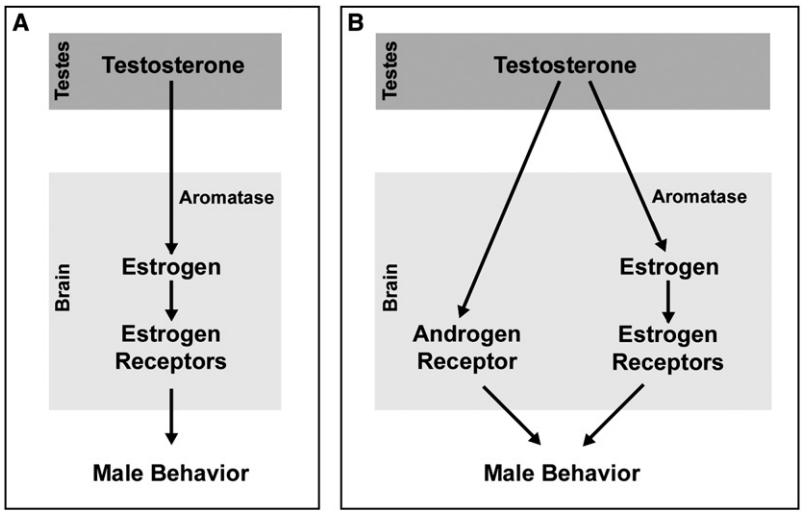

Figure 1. Models for the Role of Testosterone in Masculinizing the Brain and Behavior

Schematics illustrating possible mechanisms whereby testosterone controls male-typical behaviors are shown.

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

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

(Ishikawa et al., 2006; Pak et al., 2005; Sikora et al., 2009; Wahlgren et al., 2008; Weihua et al., 2001). This makes it difficult to unambiguously define a role for AR in controlling male behaviors using DHT. The use of constitutive AR mutants is also not definitive in defining a role for AR in male behaviors because these animals have low, often undetectable, levels of circulating testosterone (Sato et al., 2004) (S.A.J., unpublished data) 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 and ERβ 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., 1997, 2000; Ohno et al., 1974; Sato et al., 2004; Wersinger et al., 1997) (M.V.W., unpublished data). Both testosterone and estrogen, however, appear critical during development and adult life in males for the display of dimorphic behaviors such as intermale aggression (Bakker et al., 2006; Finney and Erpino, 1976; Motelica-Heino et al., 1993; Wu et al., 2009). Consistent with the apparent nonredundant 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 (Pérez et al., 2003; Shah et al., 2004; Simerly et al., 1990). Thus, AR and ERα and ERβ are widely expressed in interconnected limbic regions such as the medial amygdala (MeA), the posteromedial component of the medial subdivision of the bed nucleus of the stria terminalis (BNST), and the preoptic hypothalamus (POA). Despite numerous studies documenting a role for testosterone in male-specific patterning of gene expression and behavior, the extent to which this hormone signals through AR for masculinizing the brain and behavior remains unclear (Juntti et al., 2008).

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

RESULTS

Sparse Expression of AR in the Developing Brain

There is a male-specific transient spike in serum testosterone at birth (postnatal day 1, P1) followed by a sharp drop in circulating titer within 36 hr, and these low baseline levels of testosterone persist until puberty. By contrast, the ovaries are quiescent during the neonatal period, and there is little circulating estrogen (or testosterone) in female pups (McCarthy, 2008; Motelica-Heino et al., 1988). The male-specific surge of testosterone and its subsequent conversion to estrogen is thought to be critical for the sexual differentiation of the neural circuits that control many dimorphic behaviors (Motelica-Heino et al., 1993; Peters et al., 1972; Phoenix et al., 1959; Whalen and Nadler, 1963; Wu et al., 2009). We wished to identify the neurons that respond to this critical testosterone surge via AR at birth. We analyzed AR expression in mice bearing a previously described knockin AR allele (*AR-IPIN* allele) that permits coexpression 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 MeA, the BNST, and the POA as being critical for the display of

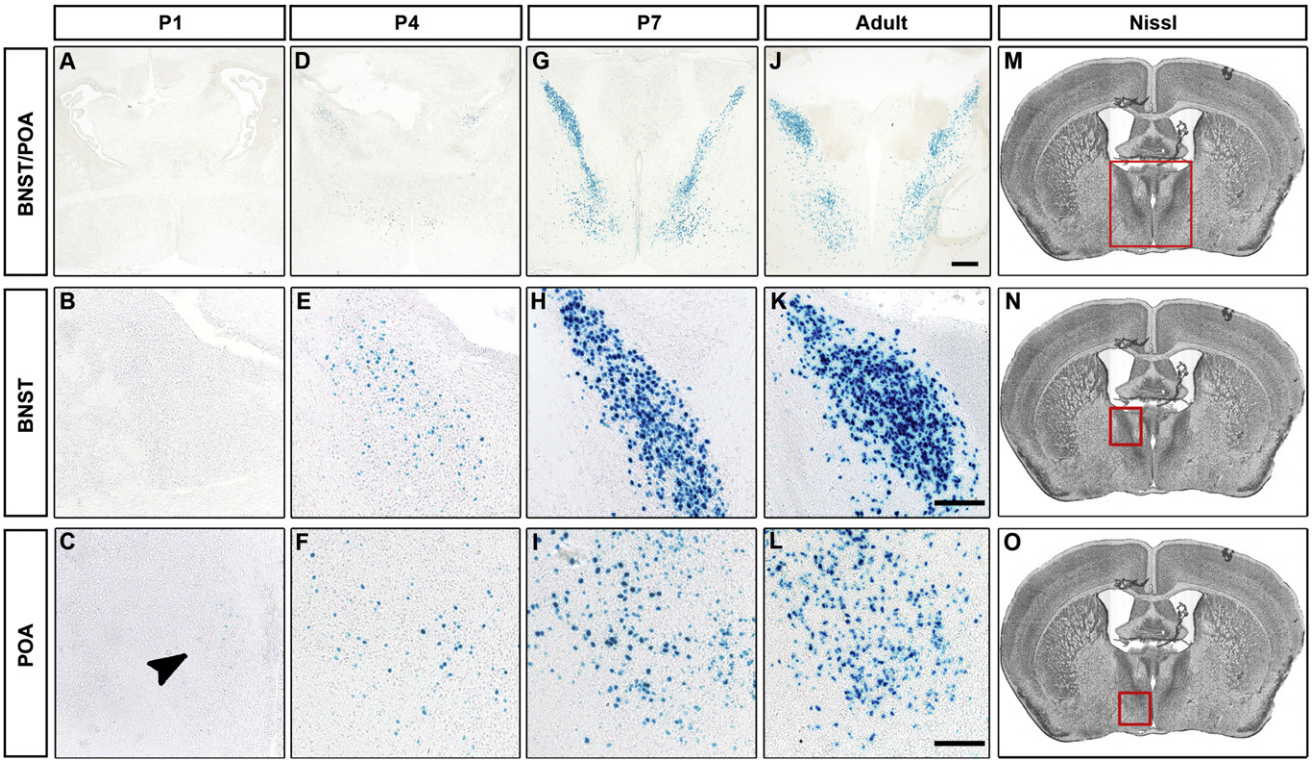

Figure 2. Limited Expression of AR in the Newborn Brain

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

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

See also Figures S1 and S2.

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–2C and S1D, available online). There were >15- to 90-fold fewer AR-expressing cells in these regions at birth (BNST 12.1 ± 5.7; MeA 16.4 ± 10.3; POA 59.8 ± 20.3; n = 6) than in older animals (Figure 3) (Shah et al., 2004; Wu et al., 2009). Similar results were obtained by directly immunolabeling for AR (data not shown). We could detect more AR-positive cells in the MeA, BNST, and POA at P4, a time point by which the testosterone surge has already subsided (Figures 2D–2F and S1E), but even at this age there appeared to be significantly fewer AR-expressing cells than observed in adults. In contrast to this sparse and faint AR labeling in the BNST, POA, and MeA, AR expression could be reliably detected in a small pool of neurons in the vicinity of the arcuate (ArcN) and ventromedial (VMH) nuclei of the hypothalamus at P1 and P4 (Figures S1A and S1B). The expression pattern of AR in the BNST, POA, and MeA resolved into widespread, intense labeling in these areas at P7, resembling the pattern observed in the adult brain (Figures 2G–2L, 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 (Figures S2A–S2D). By contrast, at E15.5 and E17.5, we could visualize AR expression in the neurons near the ArcN and VMH, but not in the BNST, POA, or MeA (Figures S2E–S2L). The ontogeny of AR expression in the BNST, POA, and MeA makes it unlikely that this hormone receptor plays a major, cell-autonomous role in masculinizing these neural pathways for male-typical behaviors 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 with females (Shah et al., 2004). AR expression in the BNST and POA in the P7 male resembles that observed in the adult male (Figures 2G–2L), whereas we did not observe AR-positive cells in the basal forebrain at P7 (data not shown). We asked whether the adult pattern of sexual dimorphism in AR expression was also apparent at P7 in the BNST and POA. Immunolabeling for βgal at P7 revealed significantly more AR-positive cells in the male BNST and POA than in these regions in the female

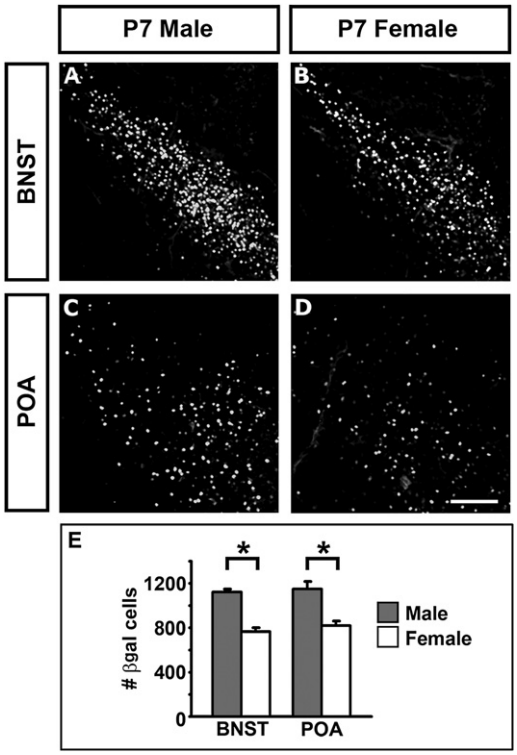

Figure 3. Sexual Dimorphism in AR Expression

(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-positive 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 ≤ 0.005. Scale bar equals 100 μm. See also Figure S2.

(Figure 3), consistent with previous reports of sexual dimorphism in these regions (McAbee and DonCarlos, 1998; Shah et al., 2004).

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

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

Genetic Deletion of AR in the Nervous System

Our results suggest that testosterone 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 nonneural tissues (Figures 5B–5D and 5F): the genitalia of these mice are masculinized, and the testes and seminal vesicles, sensitive peripheral tissues responsive to AR signaling, are similar in weight to those of their control littermates (wild-type [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 from the titers of this hormone in males of the control genotypes ($p = 0.157$, Kruskal-Wallis test for multiple group comparisons) (Figure 5E). The titers of circulating estrogen in these mutants are also comparable to those of the control males (Figure 5E). These findings suggest that testicular function is likely unimpaired in the absence of AR in the nervous system. Indeed, we find that AR^{NsDel} mutants can sire litters when cohoused with WT females (data not shown), and that AR transcript levels in the testis as well as the pituitary are comparable between AR^{NsDel} mice and their controls (Figures 5I and 5J).

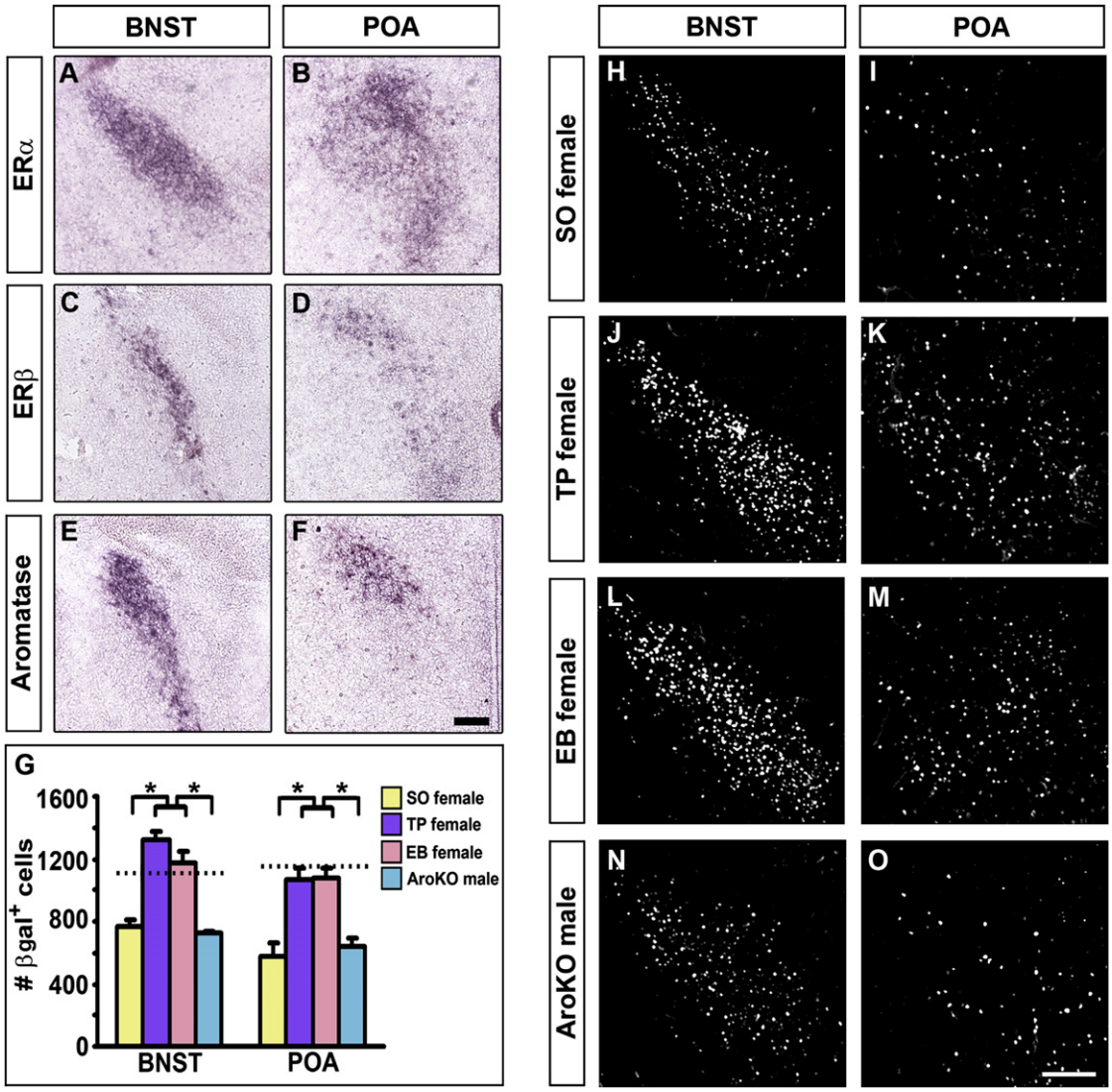

Figure 4. Estrogen Masculinizes AR Expression

(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-positive cells in the P7 BNST and POA of $AR^{IPIN/IPIN}$ females treated at P1 with testosterone (TP female) or estrogen (EB female) than those in females treated with vehicle (SO female) or $aromatase^{-/-}$; $AR^{IPIN/Y}$ (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 ≤ 0.01 by Tukey's post hoc test following one-way ANOVA. Scale bar equals 200 μm (A–F) and 100 μm (H–O).

Inadvertent deletion of *AR* in nonneural tissues such as muscle could result in a failure to thrive, a generalized motor deficit, or muscular weakness. However, AR^{NsDel} mice appear indistinguishable from their controls in body length and weight (Figure 5G). We also did not observe abnormal gait or gross motor deficits in our behavioral assays with these mutants (data not shown). In addition, AR^{NsDel} males were similar to their control littermates in general motor activity and social interactions such as grooming (Figures S3A–S3C). When assayed for motor performance on the rotarod, AR^{NsDel} mice performed equivalently to control males (Figure 5H). In accord with these findings, quantitative RT-PCR (qPCR) also reveals comparable levels of AR mRNA in skeletal muscle obtained from AR^{NsDel} and control males (Figure 5J).

In contrast to these findings in nonneural 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

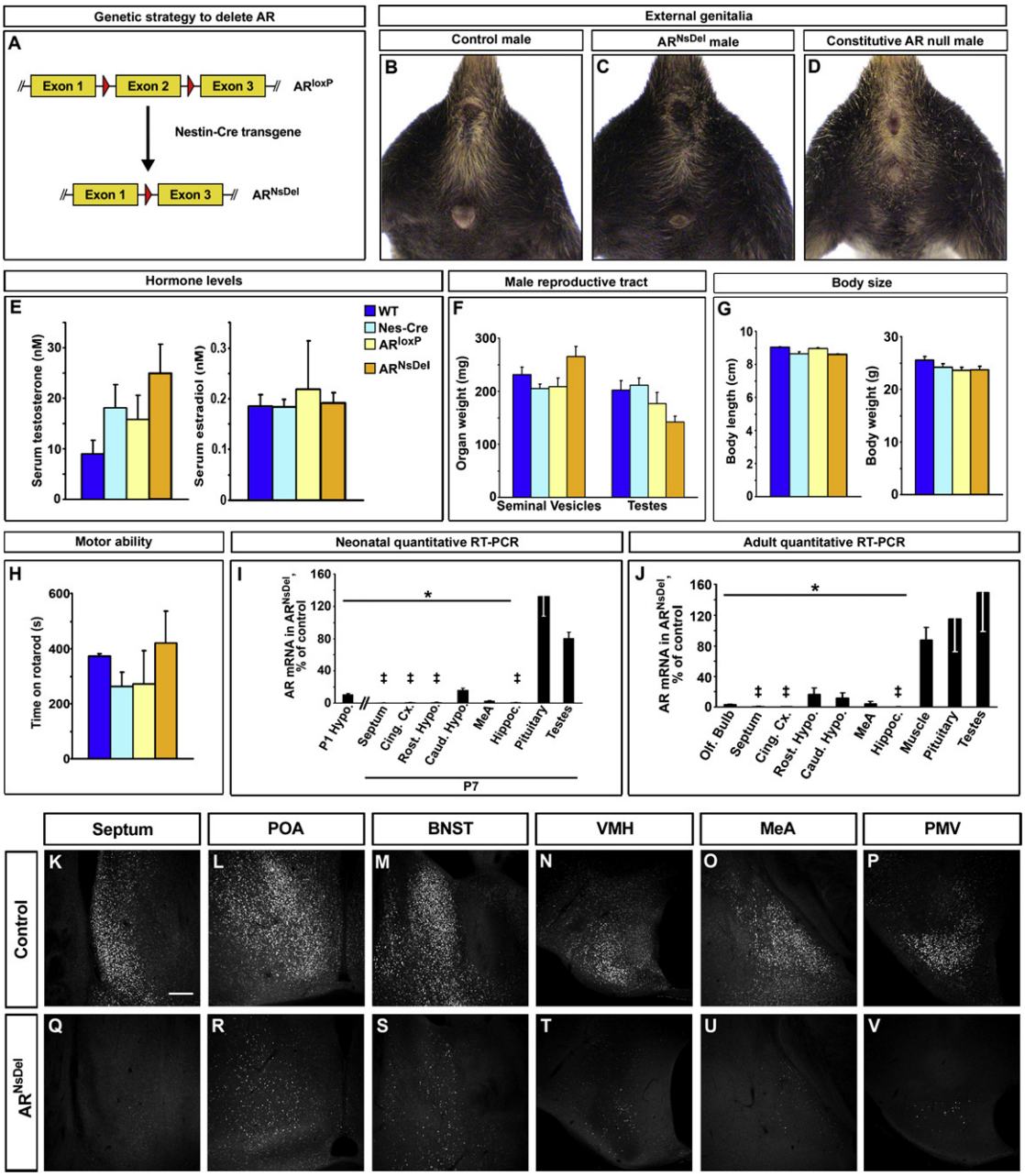

Figure 5. Targeted Deletion of AR in the Nervous System

(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 and J) Reduction in normalized AR mRNA in the brain of P1 (I), P7 (I), and adult (J) AR^{NsDel} males shown as a percent of AR mRNA levels in controls (Hypo., hypothalamus; Cing. Cx., cingulate cortex; Rost. Hypo., rostral hypothalamus; Caud. Hypo., caudal hypothalamus; Hippoc., hippocampus). There are similar AR mRNA levels between AR^{NsDel} and control males in other tissues. Mean ± 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 than those in control males. Scale bar equals 100 μm.

See also Figure S3.

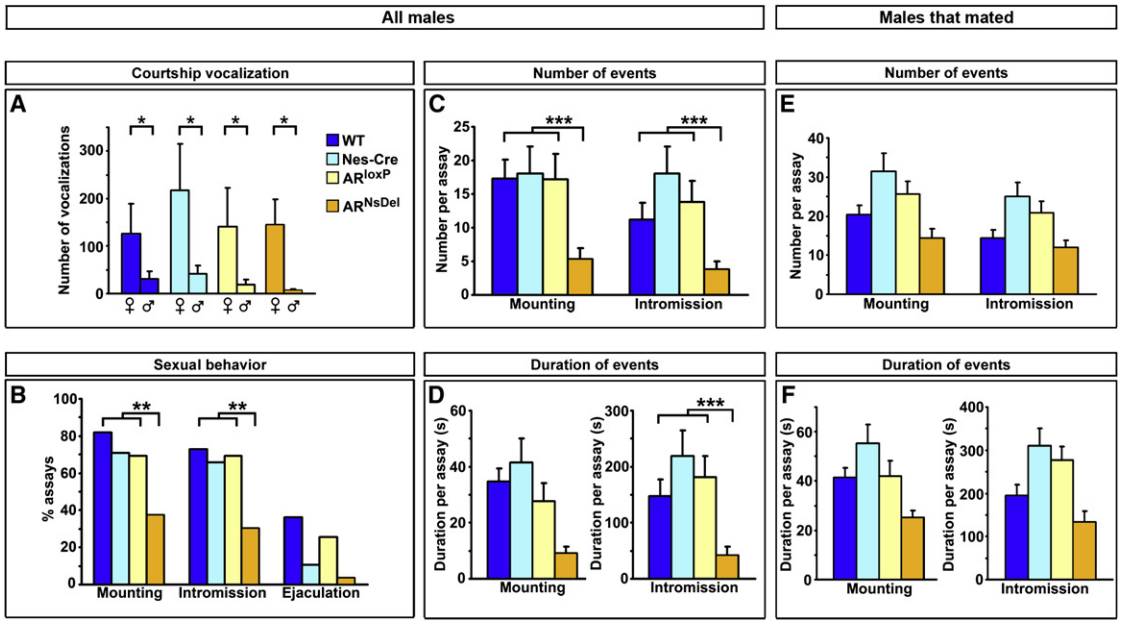

Figure 6. AR Increases the Frequency of Male Mating

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

Mean ± SEM; *$p < 0.033$, $n \geq 3$/genotype; **$p < 0.05$, post hoc Bonferroni's correction for Fisher's exact test, $n \geq 12$/genotype; ***$p < 0.05$, Tukey's test following Kruskal-Wallis comparison, $n \geq 12$/genotype. See also Figure S4 and Movies S1 and S2.

expression at P1 and P7 in these mutants compared to that of their control male littermates (Figure 5I). In agreement with these results, immunolabeling reveals very few AR-positive cells in the forebrain of adult AR^{NsDel} males compared with controls (Figures 5K–5V). Taken together, our genetic approach yields male mice that have intact peripheral masculinization and circulating testosterone and a deletion of AR that appears restricted to the nervous system. These mutants therefore afford the opportunity to assess the contribution of testosterone signaling via AR in the neural circuits that control male-typical behaviors.

AR Increases the Frequency of Male Sexual Behavior

In order to determine whether AR-mediated signaling in the nervous system is essential for male sexual behavior, we examined the behavior of AR^{NsDel} mice in mating assays. In mice, mating consists of a series of stereotyped routines that include mounting, intromission or penetration (as visualized by pelvic thrusting), and ejaculation. These behaviors can be reliably elicited in a 30 min assay in which a WT estrous female is introduced into the cage of a singly housed WT male (Mandiyan et al., 2005; McGill, 1962). Forty-two percent of AR^{NsDel} residents (n = 24 mice), a percentage statistically similar to that of control residents, mounted at least once with different estrous females when tested in two or three assays for sexual behavior (Figure S4A). When we analyzed the percent of all assays with mounts, we observed mating in fewer assays with AR^{NsDel} residents than in those with males in the control cohort (Figure 6B). Notably, we find that some of the AR^{NsDel} mice never mate, though when they do exhibit sexual behavior, these mutants mate in most assays (85% ± 6%) similarly to control residents (Figure S4G).

The lowered probability of initiating mating across all assays was also reflected in the diminution of the number of mounts and intromissions, as well as in the duration of intromissions, exhibited by AR^{NsDel} animals (Figures 6C and 6D). Male mice do not always achieve ejaculation within a 30 min assay (McGill, 1962), and we observed ejaculation at a similar, low frequency in males of all genotypes (Figures 6B and S4A). The deficits in some parameters of sexual behavior in AR^{NsDel} mice reflected an analysis of all assays, including those in which the resident did not initiate mating. We also examined these behavioral parameters by restricting our analysis to include only the assays in which males mated. Strikingly, this analysis revealed that once AR^{NsDel} mice initiate sexual behavior, they mate in a manner similar to their controls (Figures 6E, 6F, and S4C–S4F; and Movies S1 and S2, available online).

Thus, while AR^{NsDel} mutants are less likely to mate, once sexual behavior is initiated, its display appears similar to that of WT males. This suggests that AR controls the probability of triggering male mating, but not the pattern of this complex behavioral routine. Alternatively, the lowered likelihood of initiating mating behavior could simply reflect a large variability in

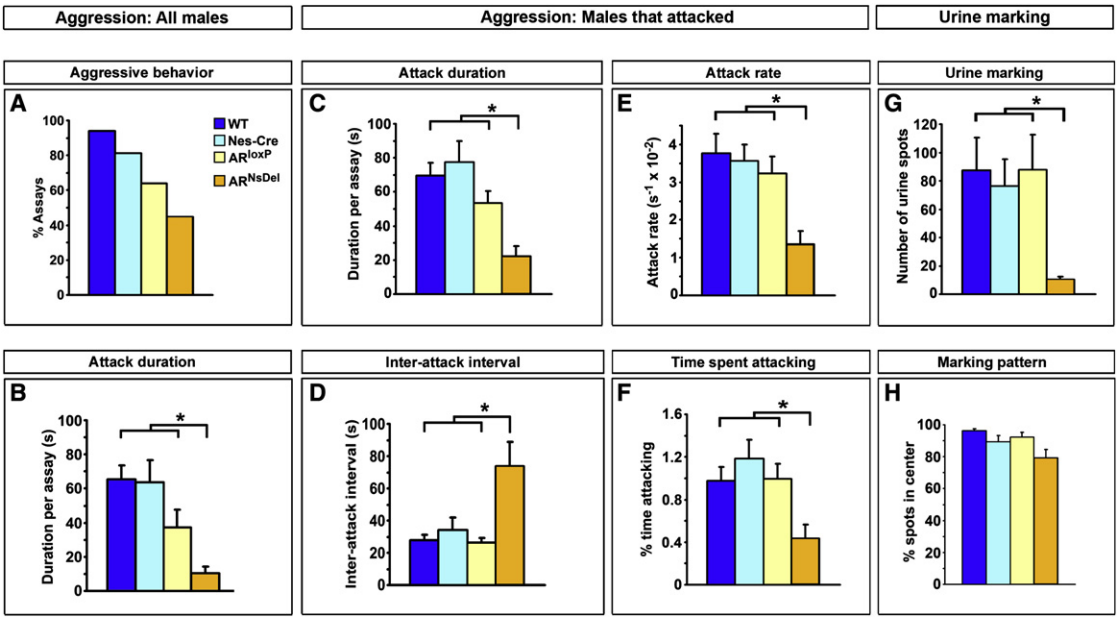

Figure 7. AR Increases the Levels of Male Territorial Displays

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

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

(C) When AR^{NsDel} residents fight, they do so for a shorter duration than controls.

(D–F) In assays with fighting, AR^{NsDel} residents exhibit an increase in time between fights (D), attack at a slower rate (E), and attack for a smaller percent of the duration of the assay (F) compared with controls.

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

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

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

the extent of AR deletion in the brain. In this scenario, AR^{NsDel} mice who do not mate may have little residual AR in the brain compared with the mutants who do 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 min mating assays were cohoused with females, they successfully sired litters (S.A.J., unpublished data). Finally, only a subset of constitutive AR mutant males attempted to mate when supplemented with testosterone (or estrogen) at doses that recapitulate WT circulating levels of this steroid hormone (Olsen, 1992; Sato et al., 2004) (M.V.W., unpublished data). Taken together, these findings are consistent with the notion that AR functions in the brain to regulate the likelihood of initiating male sexual behavior.

Chemosensory cues emanating from females are critical for triggering male sexual behavior, and WT males engage in extensive anogenital chemoinvestigation of females prior to initiating sexual behavior (Keverne, 2004; Mandiyan et al., 2005; Yoon et al., 2005). The reduced frequency of male sexual behavior we observe with AR^{NsDel} mice may be a consequence of deficits in such chemoinvestigation. However, we find that AR^{NsDel} animals chemoinvestigate conspecifics in a manner comparable with that of their control male littermates (Figures S4B and S5C). Previous work shows that, unlike WT males, constitutive AR mutants have a preference for male rather than female odors (Bodo and Rissman, 2007). Chemosensory cues from the two sexes lead to gender discrimination, one consequence of which is female-directed ultrasonic vocalizations by the resident male (Nyby et al., 1977; Pankevich et al., 2004; Stowers et al., 2002). We observe that AR^{NsDel} mice vocalize to female, but not to male, intruders in their cage in a manner similar to their control counterparts, suggesting that sex discrimination is intact in these animals (Figure 6A). Taken together, these results suggest that the reduced frequency of male sexual behavior of AR^{NsDel} mice is not a consequence of reduced chemoinvestigation of females or an inability to distinguish the sexes.

AR Controls the Degree of Male Territorial Behaviors

We next tested AR^{NsDel} animals in assays of male territorial behaviors. Singly housed WT male, but not female, resident mice attack male intruders in their homecage (resident-intruder aggression test) (Miczek et al., 2001). AR^{NsDel} males were less aggressive than control residents by several measures. Although there was no statistical difference in the number of attacks or in the percent of tests with aggression (Figures 7A, S5A, and S5B), the mutants spent significantly less time fighting compared with control residents, and this deficit persisted even when we restricted our analysis to the assays in which we observed

aggressive interactions (Figures 7B and 7C). The reduction in total duration of attacks cannot be explained by alterations in chemoinvestigation, the latency to first attack, or by the total attack number, as these parameters were statistically similar in all resident males (Figures S5B–S5E). Rather, our analysis revealed a deficit in the pattern of aggression following the first fight initiated by AR^{NsDel} males (Figures 7D–7F and S5F). Compared with the control residents, AR^{NsDel} mice spent less time fighting with the intruder and exhibited a longer interval between successive attacks (Figures 7D and 7F). While AR^{NsDel} residents and their controls attacked a similar number of times in an assay (Figure S5E), the mutants exhibited a lower attack rate (Figure 7E), initiating fewer attacks per unit time following the first 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 because AR^{NsDel} residents also distribute their urine marks across the cage floor, similar to WT males (Figure 7H). By contrast, we find that AR^{NsDel} residents deposit fewer urine marks compared with control resident males (Figure 7G). Importantly, AR^{NsDel} males deposit more urine marks (10.2 ± 2.1 spots) than WT females who pool urine (1.7 ± 0.5 spots) (Wu et al., 2009), suggesting that AR is not required to masculinize this parameter of urine marking, but rather AR enhances the display of this behavior. Taken together, these findings show that AR functions in the nervous system to control specific parameters of male-typical urine marking and fighting.

DISCUSSION

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

Our genetic strategy permits us to define a functional contribution of AR signaling in the neural circuits that mediate male behaviors. Using an approach identical to ours, a recent study also showed deficits in mating and fighting in males lacking AR in the nervous system (Raskin et al., 2009). Differences in the phenotypes reported in that study compared with those in our study likely arise from variations in the experimental design or strain differences. Here, we have significantly refined the analysis of AR^{NsDel} mutants to provide new mechanistic insight into the role of AR in masculinizing the brain and behavior. We have compared AR^{NsDel} males to each of the control genotypes (WT, Nes-Cre, $AR^{loxP/Y}$) to assess the contributions of these distinct genetic backgrounds to all mutant phenotypes. We have also developed analytical tools to examine in an extensive manner the behavioral deficits in sexual and territorial behaviors of AR^{NsDel} males. In the absence of AR function in the nervous system, males discriminate between the sexes and initiate appropriate behavioral responses, mating with females and fighting with males. Our analysis of male mating suggests that AR in the brain regulates the probability of triggering sexual behavior, but not the pattern of various components of male mating. Our analysis of territorial behaviors reveals a previously unreported role of AR in the brain in controlling the duration and pattern of intermale aggression. We find that AR^{NsDel} mutants mark their territory in a male pattern, but they deposit far fewer urine marks, indicating a deficit in this component of male territorial display. Our studies also indicate that it is unlikely that the masculine behaviors observed in AR mutants result from a failure to delete AR prior to the early neonatal sexual differentiation of the brain. Indeed, we find that there is minimal AR expression in regions known to be critical for dimorphic behaviors during this period, when gonadal hormones orchestrate sexual differentiation of the brain (Meisel and Sachs, 1994; Morris et al., 2004; Motelica-Heino et al., 1993), and this sparse neonatal AR expression is largely eliminated in AR^{NsDel} males. Importantly, we demonstrate that sexual differentiation of AR expression itself is controlled by estrogen signaling. The sparse perinatal expression of AR in the brain suggests that the behavioral phenotype of AR^{NsDel} mice results from activational rather than organizational effects of testosterone acting on AR. We cannot exclude the possibility that AR also functions during the later postnatal period, including puberty, to influence the maturation of the neural circuits that drive male behaviors (Schulz et al., 2009). Regardless of the exact time point at which AR functions to control behaviors, our findings indicate that AR is not a master regulator for male behaviors, but rather, it serves as a gain control mechanism to regulate the extent of male sexual and territorial displays.

We find minimal AR expression in various brain regions of AR^{NsDel} males, demonstrating that most neurons do not need to signal via this receptor to drive male sexual and territorial behaviors. Because we have deleted AR in the developing and adult animal, we cannot exclude the possibility that 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, 2008b). Nevertheless, our study indicates that AR functions in the nervous system to control various parameters of male sexual and territorial behaviors, but it is not essential to masculinize this behavioral repertoire in mice.

A Model for Hormonal Control of Male Sexual and Territorial Behaviors

Testosterone is essential for male behaviors. We set out to distinguish two competing models of testosterone's function in male behaviors: in one scenario, testosterone simply serves as a prohormone for estrogen in the brain, and it is estrogen signaling via

its cognate receptors that masculinizes the brain and behavior (Figure 1A). Alternatively, testosterone may serve not only as a precursor for estrogen, but it may also signal via AR to control male behaviors (Figure 1B). We find that male mice mutant for AR in the nervous system do not exhibit male-typical levels of mating and territorial behaviors. AR can regulate the activity and expression of aromatase (Roselli et al., 2009), and therefore might serve to amplify male behaviors by regulating the levels of local estrogen synthesis. However, supplementation of constitutive AR mutants with estrogen does not restore mating and territorial displays to WT levels (Olsen, 1992; Sato et al., 2004; Scordalakes and Rissman, 2004). Such studies therefore suggest that AR in the brain may also control the expression of other genes that modulate the levels of male behavioral displays. Irrespective of the exact molecular mechanisms, our data demonstrate that testosterone signaling via AR is essential for WT male behavior.

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

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

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

EXPERIMENTAL PROCEDURES

Animals

Mice were housed in a rodent barrier facility at UCSF with a 12:12 hr light:dark cycle. All studies with animals were done in accordance with UCSF IACUC protocols. The AR-IPIN knockin and aromatase knockout mice have been described previously (Honda et al., 1998; Shah et al., 2004). Animals bearing the AR^{loxP} allele (De Gendt et al., 2004) or *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 hr 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 two or three times each in assays of mating and aggression, and once each for urine marking and ultrasonic vocalization. Experimental animals were always exposed to intruder mice they had not encountered previously, and each assay was separated by $\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 because we did not observe AR-positive 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. Data sets not violating this test were analyzed with parametric tests (Student's t test for two groups or one-way ANOVA); otherwise, we used nonparametric analyses (Kolmogorov-Smirnov [KS] test for two groups or Kruskal-Wallis test). We used Tukey's post hoc test following one-way ANOVA and Kruskal-Wallis tests to determine which groups differed significantly. For all experiments, we deemed an effect of the *AR$^{loxP/Y}$; Nes-Cre* (AR^{NsDel}) genotype to be statistically significant only if this genotype differed from each of the 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. Because 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 with those of AR^{NsDel} males. For visualization purposes, this data is presented in Figures 5I, 5J, and S3D as the percent of AR mRNA in AR^{NsDel} males in various tissues compared with that of the control cohort.

Hormones

Serum testosterone and estradiol titers were determined with kits from DRG International and Cayman Chemicals, respectively. We induced estrus in adult ovariectomized mice with injections of estrogen and progesterone as described previously (Wu et al., 2009). For hormonal manipulation of neonates, females were treated on the day of birth (P1) with a single 50 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.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes Supplemental Experimental Procedures and five figures and can be found with this article online at doi:10.1016/j.neuron.2010.03.024.

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Supplemental Information

The Androgen Receptor Governs Execution, but Not Programming, of Male Sexual and Territorial Behaviors

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Genotyping animals

Animals were genotyped for the AR^{loxP} allele (5' primer: AATGCATCACATTAAGTTGATACC; 3' primer: TCAGAATTCTACGGTCTTCTGAG) and for the Nes-Cre transgene (5' primer: CGCTTCCGCTGGGTCACTGTCG; 3' primer: TCGTTGCATCGACCGGTAATGCAGGC).

Behavioral assays

We used 9-16 week old male mice in behavioral assays, which were initiated ≥ 1 hour after the onset of the dark cycle. After being singly housed for 2-7 days, mice were first tested for urine marking, in which the animal was allowed to explore a fresh cage lined with Whatman filter paper for 1 hour, and then returned to its homecage. The urine marks were imaged with UV trans-illumination, and the number of spots was enumerated. The proportion of urine marks not abutting a cage wall was also determined (% center spots). The males were subsequently tested for male sexual behavior in their homecage as described previously (Wu et al., 2009). Briefly, a female primed to be in estrus was introduced into the male's cage and animals were allowed to interact for 30 minutes. Following the mating assay, mice were tested for aggression in the

resident-intruder test, using group housed adult 129/SvEv (Taconic Farms) males as intruders. A single intruder was inserted into the resident's cage and the males were allowed to interact for 15 minutes. An aggressive interaction was defined as containing one or more instances of biting, tumbling, wrestling, and chasing. In tests of mating or aggression, a behavioral interaction was scored as "social interaction" when the resident initiated contact with the intruder; such interactions included grooming as well as non-anogenital chemoinvestigation. Various parameters of mating or fighting were scored as such rather than as general "social interaction". Following the resident-intruder test, the mice were tested for ultrasonic vocalizations produced in response to an intruder in their cage. A bat detector tuned to detect sound at 60-80 kHz (Mini-3, Noldus) was placed above the animal's cage and an intruder was introduced into the cage for 3 minutes. Each resident was tested for vocalization separately with a male and a female intruder. All animals were tested 2-3 times each in assays of sexual behavior and aggression, and once each for urine marking and ultrasonic vocalization. The experimental animals were always exposed to intruder mice they had not encountered previously, and each assay was separated by $\geq$ 2 days. Mating and aggression tests were recorded using an infrared sensitive video camera. All tests were scored by an experimenter blind to the genotype of the resident with a software package we have developed in Matlab. The mice were sacrificed 3-7 days following their last behavioral assay, their serum was processed to determine hormone titers, and their brains were analyzed for AR expression using immunolabeling or qPCR.

Histology

Brains were dissected from paraformaldehyde-perfused postnatal animals, and fixed for an additional 2 hours (adult) or 45 minutes (P1, P4, P7) at 4^{o}C. Freshly dissected heads of E13.5,

E15.5 and E17.5 embryos were immediately rinsed in cold phosphate-buffered saline and fixed in 4% paraformaldehyde for 45 minutes at 4°C. Embryonic and postnatal brains were cryoprotected overnight in 20% sucrose at 4°C and embedded in 1:1 TissueTek OCT (Sakura) and Aquamount (VWR). Sections were obtained from these animals at 20 μm thickness, and serially adjacent sections from various brain regions were collected on sets of three slides. These slides were either stored frozen at -80°C for subsequent histological analyses or immediately processed for staining for βgal activity or immunolabeling as described previously (Shah et al., 2004; Wu et al., 2009). To examine residual AR expression in the adult brain of AR^{NsDel} mice, the brains of these males and their controls were fixed as described above, embedded in 3% bacto-agar, and sectioned at 65 μm thickness, using a Leica vibrating microtome; these slices were processed as described previously (Shah et al., 2004; Wu et al., 2009).

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

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

To quantitate AR expressing cells at P1, we enumerated cells labeled for βgal activity in every third section through the entire BNST, POA, and MeA using brightfield optics and a 20X objective lens. To estimate the size of the sex difference in AR positive cells within the BNST and POA at P7, we imaged every third histological section (20 μm thickness) immunolabeled for βgal through the entire extent of the left BNST and POA. This strategy yielded 5-6 imaged sections per animal. Each of these histological sections was imaged with a 10X objective lens using a Nikon C1si confocal microscope, and image stacks were generated with five 4μm thick optical slices through the section. The images were subjected to despeckling and background noise subtraction using ImageJ (NIH) as described previously (Shah et al., 2004; Wu et al., 2009). The βgal is targeted to the nucleus and we enumerated all labeled nuclei in the central optical slice of each histological section, and these counts are reported in Figures 3 and 4. We therefore effectively enumerated βgal positive nuclear figures in optical slices separated by 56 μm. The diameter of βgal labeled nuclei is significantly smaller than 56 μm and does not exhibit a sex difference (POA: male, 6.1 ± 0.04 μm and female, POA 6.1 ± 0.03 μm; BNST: male, 6.4 ± 0.03 μm and female, 6.2 ± 0.05 μm; n = 3 animals with 30 labeled nuclei measured in each

region per animal, $p > 0.32$), indicating that our estimate of the size of the sexual dimorphism in the BNST and POA is likely to be unbiased. Indeed, we observed a similar sexual dimorphism in the BNST and POA (JT, unpublished observations), using unbiased stereology with an Optical Fractionator probe whose parameters were set per the manufacturer's instructions (StereoInvestigator, MicroBrightField) and published protocols (Keuker et al., 2001).

qPCR

We collected testes, pituitary, the gastrocnemius muscle, and brain from AR^{NsDel} mice and each of the control groups to quantitate the levels of AR mRNA. Adult and P7 brains were dissected to obtain tissue from the olfactory bulb, septum, rostral hypothalamus (including the bed nucleus of the stria terminalis), caudal hypothalamus, cingulate cortex, hippocampus, and medial amygdala, using anatomical landmarks and stereo-coordinates of the mouse brain (Paxinos and Franklin, 2001; Paxinos et al., 2007). We defined the demarcation between the rostral and caudal hypothalamus as the coronal plane 0.6 mm caudal to bregma (adult), or 4.5 mm caudal to the anterior tip of the olfactory bulb (P7). P1 brains were dissected to obtain tissue from the hypothalamus. Each tissue and brain region for individual animals (P1, P7 or adult) was processed separately for RNA extraction (Qiagen RNeasy kit), oligo-dT primed cDNA synthesis (SuperScript III, Invitrogen), and qPCR. We used separate qPCR reactions to detect AR (5' primer in exon 1: GTGAAATGGGACCTTGGATG; 3' primer in exon 2: AGGTCTTCTGGGGTGGAAAG) as well as the ubiquitous ribosomal protein Rpl32 (5' primer: CGGTTATGGGAGCAACAAGAAAAC; 3' primer: GGACACATTGTGAGCAATCTCAGC) that was used for normalization of AR expression.

Estrus induction

We induced estrus in adult, ovariectomized 8-24 week old mice with sequential daily injections of 10 μg and 5 μg of 17β-estradiol benzoate (Sigma), and 50 μg of progesterone (Sigma) dissolved in 50-100 μL sesame oil (Sigma) (Beach, 1976). The females were used for sexual behavior 4-6 hours after progesterone injection. Females were allowed to recover for ≥ 1 week between assays.

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SUPPLEMENTARY FIGURE LEGENDS

Figure S1: Limited expression of AR in the newborn brain

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

Figure S2: Sparse expression of AR in the prenatal brain

(A-L) Representative coronal sections through the POA, BNST, MeA, and ArcN/VMH of E13.5, E15.5, E17.5 male mice bearing the AR-IPIN allele labeled for βgal activity. We did not observe βgal+ cells in these regions at E13.5; note that at this age, panel B shows the brain region containing the presumptive BNST. At E15.5 and E17.5, βgal+ cells (arrowheads, E, I) appear limited to the ArcN/VMH region. The area circumscribed by the dashed line delineates the lateral (B, F, J) and third (A, C, E, G, I, K) ventricles. The dotted line (D, H, L) marks the optic tract. Dorsal is at the top. The midline is in the center (A, E, I) or to the right (B-D, F-H, J-L). $n \geq 2$ at each age. Scale bar equals 200 μm.

Figure S3: AR is not required in the nervous system for locomotor behavior and social interactions

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

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

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

(D) Normalized AR transcript levels in adult AR^{NsDel} males as a percentage of the levels in control male mice. There is no difference in the residual AR mRNA in various brain regions between AR^{NsDel} males that mated and AR^{NsDel} males that did not. We re-analyzed the results in the main Figure 5J with additional AR^{NsDel} mice to generate the comparison shown in this panel. ‡ mRNA < 0.5% of control. $n \geq 3$ per group. Mean ± SEM. (A-C) One-way ANOVA or Kruskal-Wallis test: $p > 0.05$ for all parameters. (D) KS test: $p \geq 0.15$ for each pairwise comparison.

(E-X) Coronal sections through the brain of adult WT ($AR^{+/Y}$) and Tfm ($AR^{Tfm/Y}$) male mice immunolabeled with a rabbit monoclonal anti-AR antibody reveal AR+ cells in WT but not in mutant males. Cells expressing AR are easily visualized in the BNST, VMH, PMV, MeA and POA of the WT brain (E-I). By contrast, there are essentially no AR labeled cells in these regions in the Tfm animal (J-N). The immunolabeled sections in the WT and Tfm animals have also been co-stained with DAPI (O-S, T-X, respectively) to enable visualization of all cells in these regions. Solid yellow lines indicate the ventral surface of the brain, dashed yellow lines indicate the edge of the lateral (J) or the third (K, L, N) ventricle, and the dotted yellow line indicates the ventrolateral edge of the optic tract. Similar results were observed in 3 animals of each genotype. Scale bar equals 200 μm.

Figure S4: AR is not essential in the nervous system for many parameters of male sexual behavior

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

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

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

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

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

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

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

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

Figure S5: AR is essential in the nervous system for experiential changes in aggression

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

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

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

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

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

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

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

Figure S1

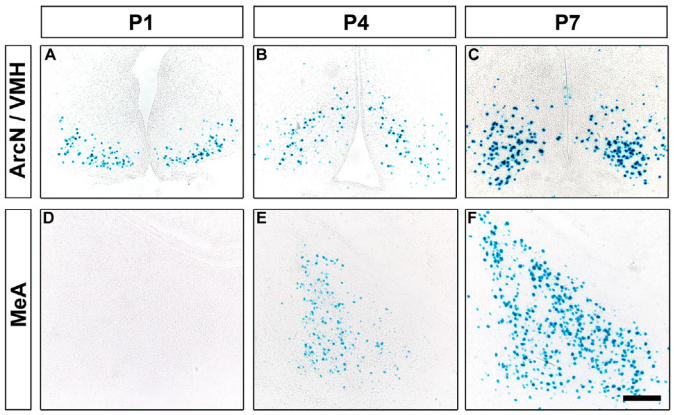

Figure S2

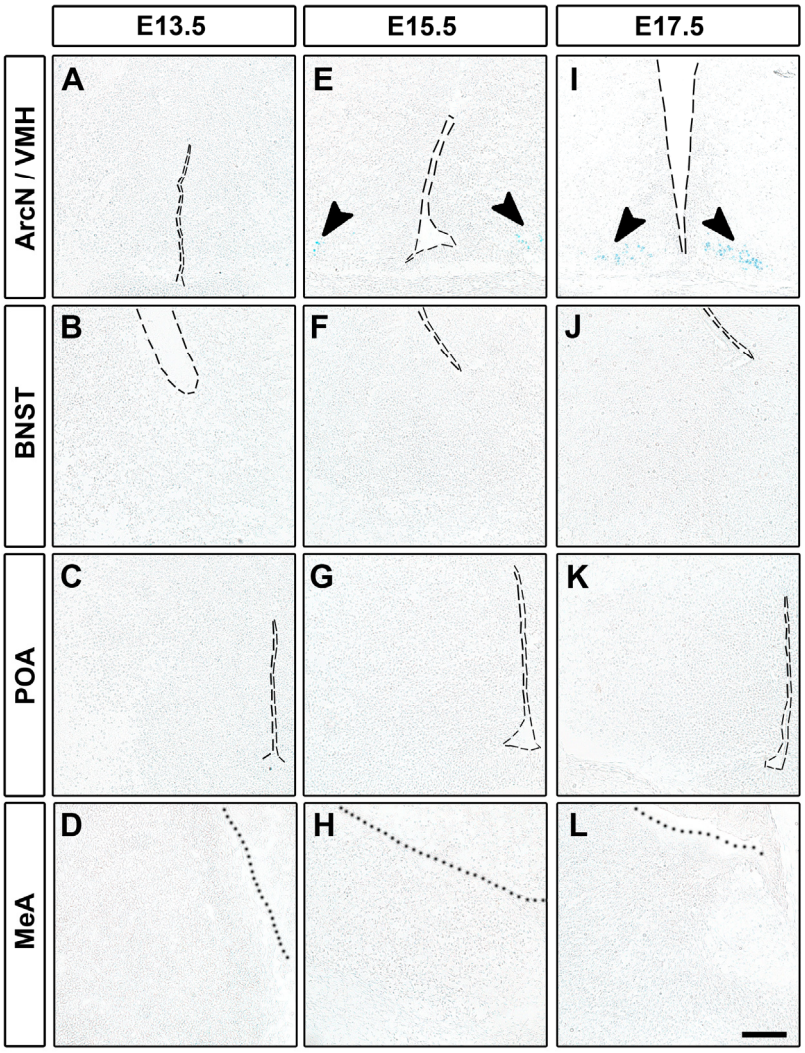

Figure S3

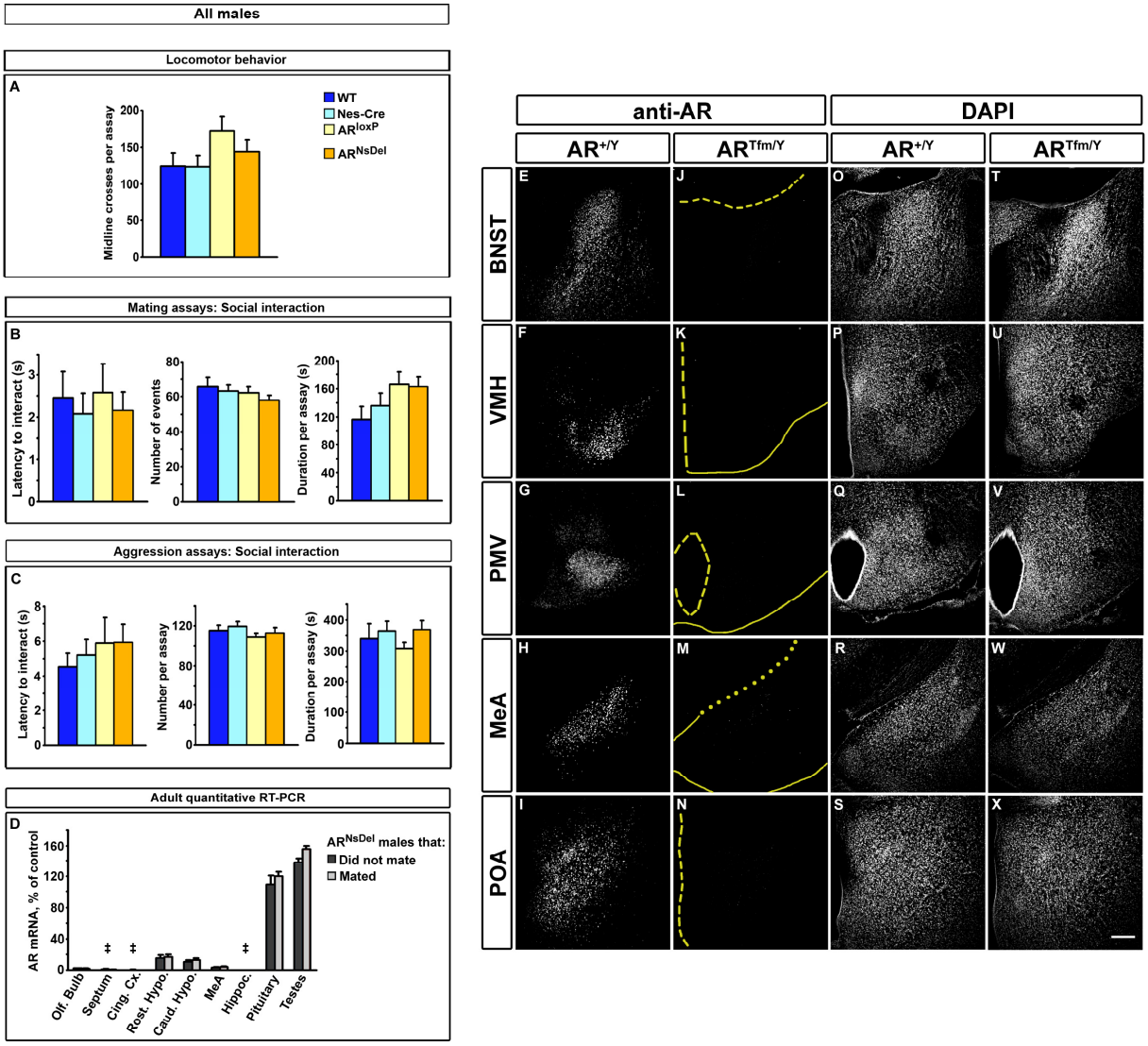
All males
Locomotor behavior
A
Midline crosses per assay
WT
Nes-Cre
AR^loxP
AR^NsDel
Mating assays: Social interaction
B
Latency to interact (s)
Number of events
Duration per assay (s)
Aggression assays: Social interaction
C
Latency to interact (s)
Number per assay
Duration per assay (s)
Adult quantitative RT-PCR
D
AR mRNA, % of control
AR^NsDel males that:
Did not mate
Mated
Olf. Bulb
Septum
Cing. Cx.
Rost. Hypo.
Caud. Hypo.
MeA
Hippoc.
Pituitary
Testes
anti-AR
DAPI
AR+/Y
AR^Tfm/Y
AR+/Y
AR^Tfm/Y
BNST
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Figure S4

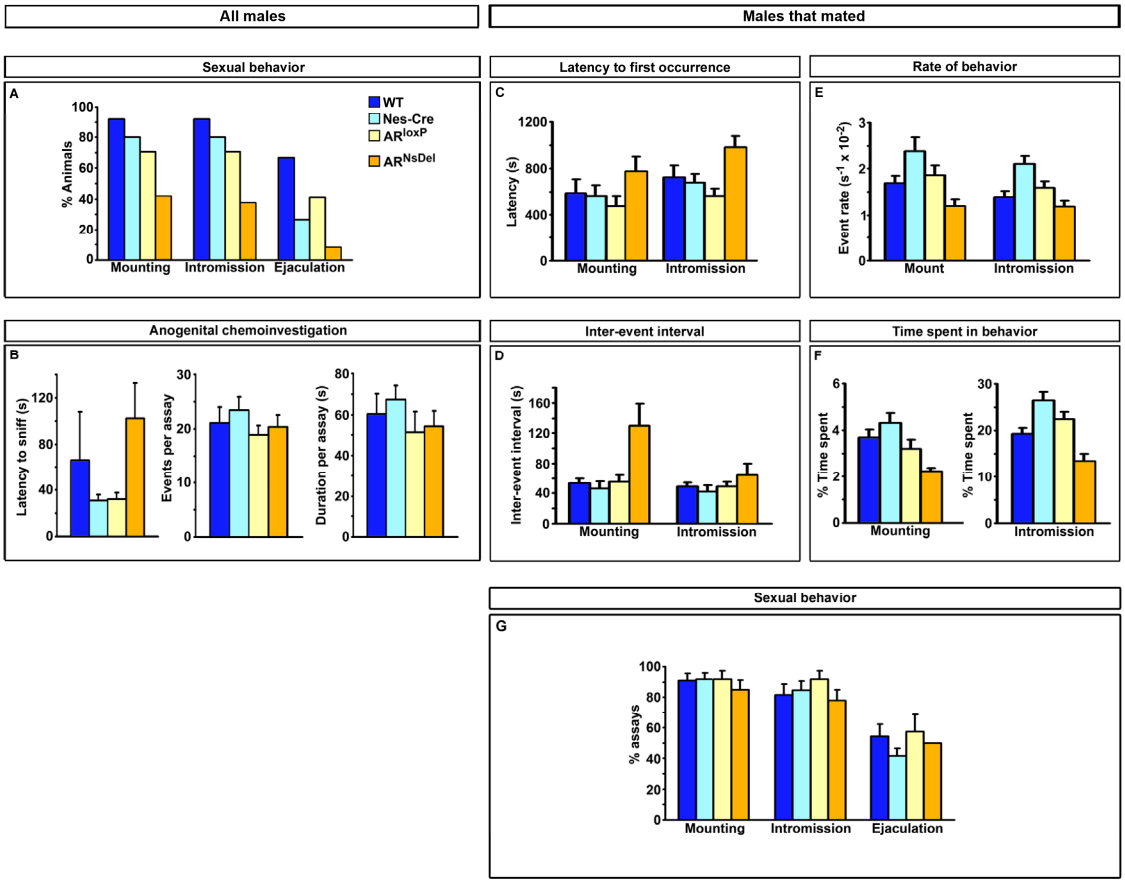
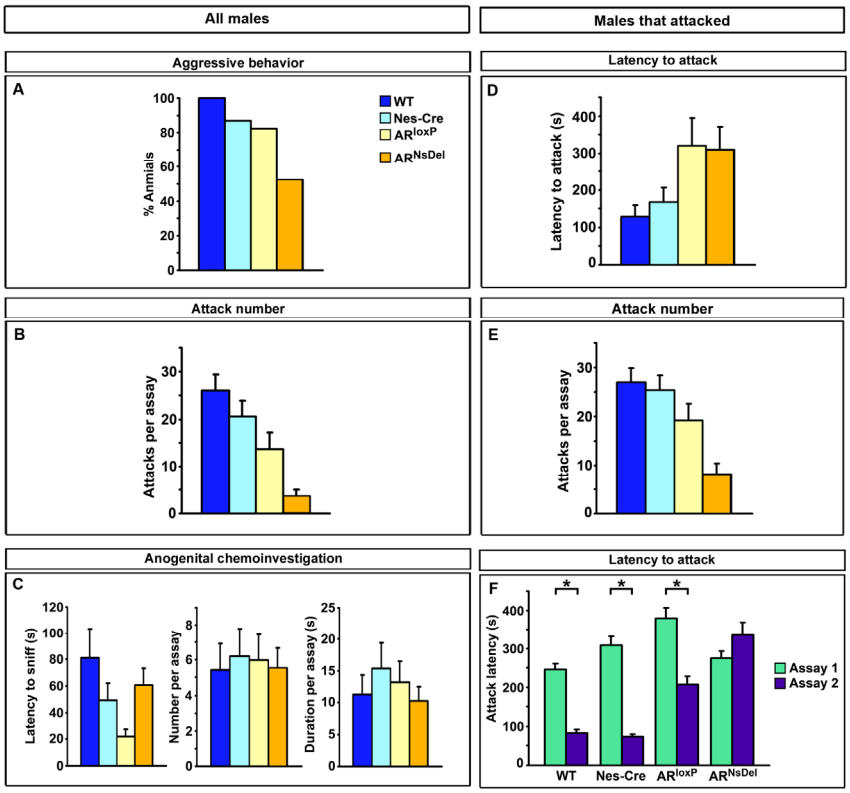

All males
Males that mated
Sexual behavior
A
% Animals
WT
Nes-Cre
AR loxP
AR NsDel
Mounting
Intromission
Ejaculation
Latency to first occurrence
C
Latency (s)
Mounting
Intromission
Rate of behavior
E
Event rate (s-1 x 10-2)
Mount
Intromission
Anogenital chemoinvestigation
B
Latency to sniff (s)
Events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

Figure S5


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