

Visualizing Sexual Dimorphism in the Brain

Report

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Summary

Sexually dimorphic behaviors are likely to involve neural pathways that express the androgen receptor (AR). We have genetically modified the *AR* locus to visualize dimorphisms in neuronal populations that express AR. Analysis of AR-positive neurons reveals both known dimorphisms in the preoptic area of the hypothalamus and the bed nucleus of the stria terminalis as well as novel dimorphic islands in the basal forebrain with a clarity unencumbered by the vast population of AR-negative neurons. This genetic approach allows the visualization of dimorphic subpopulations of AR-positive neurons along with their projections and may ultimately permit an association between neural circuits and specific dimorphic behaviors.

Introduction

All animals have evolved a repertoire of innate behaviors that results in stereotyped social and sexual responses to the environment. These innate behaviors can be observed in naive animals without prior learning or experience, suggesting that the neural circuits that mediate these behaviors must be developmentally programmed (Tinbergen, 1951). The development of neural pathways that govern behavioral repertoires and the activation of these circuits are controlled by internal regulators such as hormones and external cues that are recognized by sensory systems. Sexually dimorphic behaviors represent a robust set of innate responses that includes mating, nursing, aggression, and territorial marking. Male behaviors including mating and aggression require androgens during a critical period in development as well as during adulthood, presumably to establish and maintain the neural circuits responsible for sexually dimorphic behaviors (Edwards, 1969; reviewed in Goy and McEwen, 1980).

Despite dramatic behavioral differences between the sexes, surprisingly few anatomic and molecular distinctions differentiate the male and female brain (reviewed in Cooke et al., 1998). Quantitative differences in cell number as well as the density of projections have been observed in the preoptic nuclei of the hypothalamus (Raisman and Field, 1971), the bed nucleus of the stria

terminalis (a relay between amygdala and hypothalamus) (Gu et al., 2003; Hines et al., 1985; Hutton et al., 1998; Stefanova and Ovtcharoff, 2000; Zhou et al., 1995), and the nucleus of the bulbocavernosus (a spinal motor nucleus controlling erection) (Breedlove and Arnold, 1980). In the rat, the sexually dimorphic nucleus of the preoptic area (SDN-POA) is 3- to 4-fold larger in males (Gorski et al., 1980), and an analogous region of the human hypothalamus is larger in men (Allen et al., 1989; Byne et al., 2000; LeVay, 1991).

Dimorphic behaviors may derive from circuits that differ not only anatomically but biochemically. However, analysis of mRNA populations fails to reveal genes that are expressed solely in male or female neurons. The only exceptions are the genes on the Y chromosome and *Xist* RNA, which controls dosage compensation in all cells in the female (Dewing et al., 2003; Eriksson et al., 1999; Lahr et al., 1995; Xu et al., 2002). Since females expressing transgenic *SRY* can be masculinized in the absence of a Y chromosome, it is unlikely that additional genes on the Y chromosome play an essential role in generating sexually dimorphic behaviors (Carruth et al., 2002; De Vries et al., 2002; Koopman et al., 1991). An increasingly large number of genes with more subtle quantitative differences in expression between the sexes has been identified (reviewed in De Vries, 1990; Simerly, 2002; Stefanova and Ovtcharoff, 2000). Thus, despite numerous behavioral dimorphisms, there are only rare examples of robust molecular or anatomic differences between the sexes.

The identification of dimorphic cell populations has been hindered by the confounding complexity of the mammalian brain. The mouse brain, for example, is thought to have 10^8 neurons that are interconnected by 10^{11} synapses, a complexity that renders it difficult to identify dimorphic circuits responsible for specific behaviors. We have developed a genetic approach that allows us to visualize a subpopulation of neurons in the mouse brain that is likely to participate in sexually dimorphic behaviors. Testosterone and its receptor are required for the elaboration of male-specific behavioral repertoires (Goy and McEwen, 1980; Ohno et al., 1974). We reasoned that these dimorphic behaviors are likely to be mediated by anatomically dimorphic neuronal subpopulations that express the androgen receptor (AR). We therefore used gene targeting to modify the *AR* gene in mice such that cells that express AR co-express two reporter molecules, nuclear targeted lacZ (nLacZ) and placental alkaline phosphatase (PLAP). Nuclear β -galactosidase (β -gal) labels neuronal nuclei, whereas PLAP robustly stains neuronal processes, allowing the tracing of projections from AR-positive cells. Moreover, since AR is expressed in less than 10% of the neurons in the mouse brain, visualizing only AR-positive cells allows a cellular resolution unobscured by the vast majority of CNS neurons.

Analysis of AR-positive neurons in the brains of these mice reveals previously described dimorphisms in the preoptic area (POA) of the hypothalamus and the bed nucleus of the stria terminalis (BNST). Moreover, we

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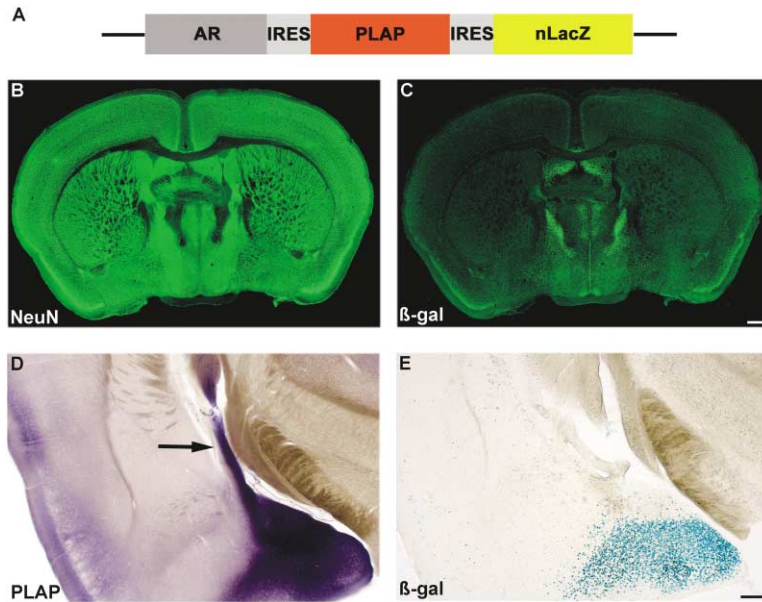


Figure 1. Visualizing AR-Positive Neurons in the Brain

(A) Schematic of genetic modification at the *AR* locus. Using gene targeting in ES cells, an IRES was placed in the 3' UTR of the *AR* gene, directing expression of PLAP. A second IRES was inserted 3' of the *PLAP* gene to drive translation of nLacZ.

(B and C) Coronal section through adult brain of *AR*-IRES-PLAP-IRES-nLacZ male stained with anti-NeuN (B) and anti-β-gal (C) antibodies. Only a small fraction of neurons in the brain labels with AR.

(D and E) Adjacent coronal sections through amygdala of adult male bearing the modified *AR* gene stained for PLAP (D) and β-gal (E) activity. Dorsal is to the top and midline is to the right. Note that PLAP not only labels the amygdala stained by β-gal but also marks the stria terminalis (arrow), the fiber tract that courses dorsally from the amygdala. Scale bars equal 500 μm (C) and 200 μm (E).

identify sexually dimorphic islands of neurons in the basal forebrain that have not been described previously. We observe a significant induction of Fos expression in the BNST and the POA following male mating, consistent with the notion that these dimorphic regions may be involved in sex-specific behaviors. Our genetic approach allows us to visualize dimorphic subpopulations of AR-positive neurons along with their projections and may ultimately permit an association between neural circuits and specific dimorphic behaviors.

Results

Visualizing AR-Positive Neurons in the Brain

We have used gene targeting in ES cells to modify the *AR* locus such that all AR-positive cells also express a membrane-associated alkaline phosphatase (PLAP) (Deprimo et al., 1996; Fields-Berry et al., 1992; Leighton et al., 2001) and nuclear β-gal (Figure 1A). An internal ribosome entry site (IRES) was introduced into the 3' untranslated region of the *AR* gene, directing the expression of PLAP (Mombaerts et al., 1996). A second IRES was placed 3' of the *PLAP* gene to direct the translation of nLacZ. This construct was linked to a selection cassette containing the *neomycin^R* gene and introduced into ES cells to obtain homologous recombinants at the *AR* locus (see Experimental Procedures). Males and females carrying the targeted *AR*-IRES-PLAP-IRES-nLacZ allele were viable, fertile, and displayed no apparent anatomic or behavioral abnormalities. Serum testosterone concentration was similar between targeted males and wild-type littermates (mean ± SEM; targeted males, 6.9 ± 3.5 nM; wild-type males, 7.6 ± 3.8 nM; n = 7; p = 0.565, Mann-Whitney U test; EIA kit from DRG International).

Analysis of AR-positive neurons allows the visualization of individual clusters with single-cell resolution and also permits us to observe the projections of AR-expressing cells. Sections stained for the ubiquitous neuronal

marker, NeuN, and for nuclear β-gal allow clear visualization of the small subpopulations of AR-positive neurons with less than 10% of the neurons evident upon nLacZ staining (Figures 1B and 1C). Staining for β-gal in sections through the amygdala reveals intense labeling of the nuclei of AR-positive neurons, whereas PLAP activity reveals the soma as well as the fiber tract of the AR-positive neurons that constitute the stria terminalis (Figures 1D and 1E).

We have compared the expression of the unmodified *AR* allele with that of the *AR*-IRES-PLAP-IRES-nLacZ allele to ask whether this reporter gene reproduces the pattern of endogenous AR expression. The *AR* locus is X-linked and undergoes random inactivation in females. Thus, in females heterozygous for the unmodified *AR* allele, half the cells will express AR from the wild-type allele and half will express the modified *AR* allele. Double labeling with antibodies to β-gal and AR demonstrates that brain regions that reveal cells expressing the wild-type *AR* allele (AR-positive, β-gal-negative) also contain cells that express the modified allele (AR-positive, β-gal-positive). All AR-expressing populations we have examined, including the basal forebrain clusters (see below), the sexually dimorphic BNST (see below), the POA, the medial amygdala, and the septal nuclei, also contain β-gal-positive cells (not shown). Moreover, in regions such as the caudate-putamen and the dentate gyrus that contain few or no AR-positive cells, we do not detect staining with the β-gal antibody (data not shown). Thus, the modified *AR* allele appears to mimic the pattern of endogenous AR expression. Moreover, the pattern of expression of β-gal is in accord with that observed for AR in sensitive radioactive in situ hybridization experiments (Simerly et al., 1990).

Sexual Dimorphisms in the Brain

Analysis of sections through male and female brains from mice bearing a targeted *AR* allele reveals clear sexual dimorphisms in three regions of the forebrain:

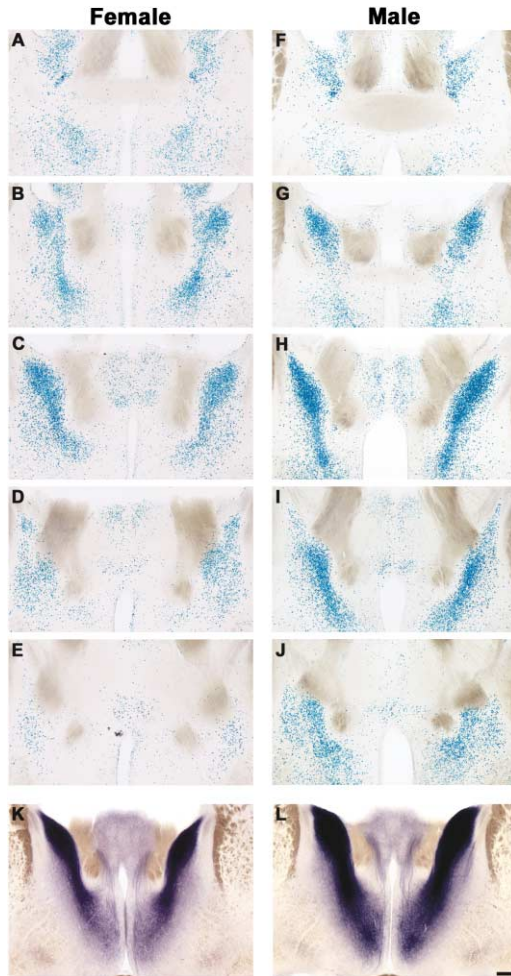


Figure 2. Sexual Dimorphism in the Bed Nucleus of the Stria Terminalis

(A–J) Serially adjacent coronal sections through the bed nucleus of the stria terminalis (BNST) stained for β -gal activity from adult female (A–E) and male (F–J) mice harboring the *AR-IRES-PLAP-IRES-nLacZ* allele. Top panels in each case are the most rostral sections and each successive lower panel is further caudal. These sections reveal a greater number of AR-positive cells in the male BNST.

(K and L) Coronal sections through the BNST stained for PLAP activity from adult female (K) and male (L) brains bearing the targeted *AR* allele. The fiber tract that emanates from the BNST and projects ventromedially toward the hypothalamus is larger and more intense in males. Scale bar equals 200 μ m.

the bed nucleus of the stria terminalis (BNST), the preoptic area of the hypothalamus (POA), and the basal forebrain. The BNST is a collection of neurons within the stria terminalis that is reciprocally connected with the amygdala, the hypothalamus, the cortex, and the brainstem (Dong et al., 2001; Simerly, 2002). Sections through the BNST stained for β -gal reveal a dimorphic cluster of neurons that begins dorsal to the anterior commissure where it crosses the midline and extends caudally for about 350 μ m (Figures 2A–2J). This cluster also extends ventromedially toward the third ventricle and lies immediately lateral to the stria medullares and the fornix. These boundaries suggest that this neuronal cluster corresponds to the posterior medial component of the me-

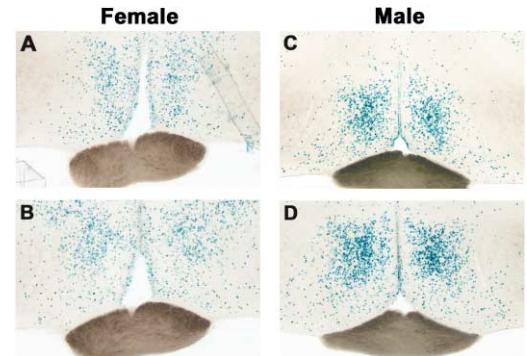


Figure 3. Sexual Dimorphism in the Preoptic Hypothalamus
Serially adjacent coronal sections through the preoptic area of the hypothalamus of adult female (A and B) and male (C and D) mice harboring an *AR-IRES-PLAP-IRES-nLacZ* allele stained for β -gal activity. (A) and (C) are rostral to (B) and (D). The β -gal-positive cells in the POA are more abundant in the male.

dial subdivision of the BNST (Paxinos and Franklin, 2001). Quantitation of β -gal-stained BNST neurons reveals 1.8 times as many AR-expressing neurons in the male BNST (females, 2380 ± 323 ; males, 4208 ± 207 ; $n = 4$; $p = 0.021$, Mann-Whitney U test). The majority of NeuN-labeled neurons in the BNST of both males and females express AR (females, $79.6\% \pm 1.1\%$; males, $89.2\% \pm 3.7\%$; $n = 3$), and only a small fraction of AR-positive cells in the BNST (<5% in females and males) is NeuN negative. Thus, the difference in the number of AR-positive cells between the sexes reflects an absolute increase in neuronal number in the BNST rather than an increase in the proportion of cells expressing AR.

Sections through the BNST stained for PLAP activity reveal that the increase in cell number is associated with a richer fiber tract in the male brain that emanates from the BNST and projects ventromedially toward the hypothalamus (Figures 2K and 2L). Analysis of serial sections suggests that these projections terminate in the anterior preoptic area of the hypothalamus. However, fibers from the BNST intermingle with fibers of the stria terminalis that directly connect the medial amygdala and the hypothalamus (Dong et al., 2001; Simerly, 2002). Both the medial amygdala and the hypothalamus are also rich in AR-positive neurons (Figures 1D, 1E, and 3; Simerly et al., 1990), making it difficult to distinguish the termini of BNST projections. It is nonetheless clear that both the number of cells in the posterior medial BNST and their projections are significantly greater in the male brain.

A second cluster of sexually dimorphic neurons resides in the POA of the hypothalamus (Figure 3). AR-expressing neurons are scattered throughout the preoptic hypothalamus in both males and females. An increase in cell number in the male is evident in a cluster of AR-positive neurons that appears as the anterior commissure crosses the midline and may correspond to the SDN-POA in the rat (Gorski et al., 1980). The continuous expression of AR rostrocaudally in subsets of cells beyond this nucleus makes it difficult to quantitate the difference in cell number, but the dimorphism is reproducible in four independent experiments. The extensive

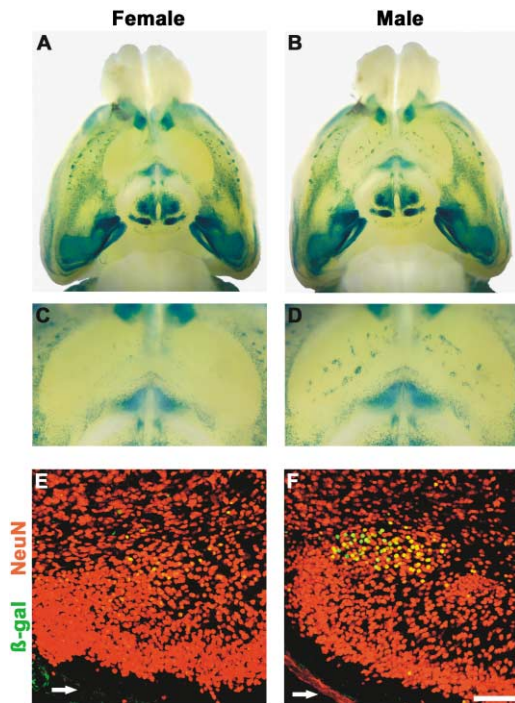


Figure 4. Sexual Dimorphism in the Basal Forebrain
(A–D) Whole-mount preparations revealing the ventral surface of brains from adult female (A and C) and male (B and D) mice bearing the AR-IRES-PLAP-IRES-nLacZ allele stained for β -gal. (C) and (D) are higher magnifications of (A) and (B). AR-positive clusters are more numerous in the male.
(E and F) Coronal sections through the basal forebrain region of adult female (E) and male (F) mice bearing the modified AR gene stained with anti-NeuN antibody (red fluorescence) and anti- β -gal antibody (green fluorescence). A large cluster of β -gal-positive neurons is visible in the male with far fewer β -gal-labeled neurons in the female. Arrows point to the ventral cortical surface. Scale bar for (E) and (F) equals 200 μ m.

reciprocal connectivity of the POA with collections of AR-expressing neurons in the BNST, the medial amygdala, and the ventromedial hypothalamus (reviewed in Simerly, 2002) is revealed in the dense PLAP staining observed in the POA (not shown). However, this richness in PLAP-stained neurites makes it difficult to visualize a distinct fiber tract emanating from the POA.

A novel dimorphism was identified in the basal forebrain. We observe several clusters of AR-expressing neurons in the basal forebrain that are more numerous in males. This dimorphic collection of neurons lies close to the ventral surface of the forebrain and has a rostro-caudal extent of about 1.25 mm. These clusters are observed caudally as far as the midline crossing of the anterior commissure. This dimorphism is evident in whole-mount preparations of the brain that reveal numerous intensely stained AR-positive clusters in the male basal forebrain and fewer clusters in the corresponding ventral region in the female (Figures 4A–4D). In coronal sections, these AR-positive clusters do not appear to lie within the boundaries of well-defined neuronal populations such as the olfactory tubercle, the ventral pallidum, or the islands of Calleja. Rather, they appear to be interspersed with AR-negative neurons in

these regions (Figures 4E and 4F). Quantitation reveals a 2-fold increase in the number of clusters in the male brain (females, 53 ± 9.4 ; males, 105 ± 7.3 ; $p = 0.009$, Mann-Whitney U Test; $n = 5$). The extent of the dimorphism in AR expression is likely to be even greater since each cluster in the male basal forebrain has on average 1.5-fold more neurons than female clusters (females, 11 ± 0.6 ; males, 17 ± 1.5 ; $n = 4$; $p = 0.021$, Mann-Whitney U test). We cannot distinguish whether the increase of AR-expressing neurons in this region in the male results from the induction of AR in pre-existing neurons or reflects an absolute increase in AR-positive neurons.

Thus, genetic modifications that label AR-expressing neurons reveal two previously identified sexually dimorphic nuclei, the POA and the BNST, with a cellular resolution greater than is possible with standard histological reagents. Moreover, we identify novel, dimorphic islands of neurons within the basal forebrain.

Sexually Dimorphic Nuclei and Dimorphic Behaviors

We have performed experiments to ask whether the sexually dimorphic AR-positive cells are activated by mating in mice. Activation of the immediate early gene, *c-fos*, can result from elevations in intracellular calcium such that the expression of this gene is often a marker of neural activity (Morgan and Curran, 1991). We have examined Fos expression in the amygdala, BNST, and hypothalamus, as well as in the dimorphic clusters in the basal forebrain after mating. In mice, mating is thought to be initiated by pheromones that activate both the main and vomeronasal olfactory systems. One possible circuit involves the flow of sensory information from the olfactory bulbs to the amygdala (Scalia and Winans, 1975). The amygdala in turn projects via the stria terminalis to the BNST and ultimately to the hypothalamus (Dong et al., 2001). Sexually experienced males expressing the modified AR allele were presented with an estrus female for 60 min, whereas control males (with equivalent prior sexual experience) did not receive a female. All experimental males exhibited robust mating behavior. After 60 min, the males were examined for Fos and nuclear β -gal staining. We observe a ~ 30 -fold increase in the number of cells expressing Fos in the BNST of mated males compared to control males (mated male, 237 ± 58 ; not mated male, 7 ± 3 ; $n = 3$; $p < 0.05$, Mann-Whitney U test). Only about 10% of the cells in the BNST express elevated levels of Fos (Figure 5). $78\% \pm 7\%$ of the Fos-positive neurons in the dimorphic, postero-medial component of the medial subdivision of the BNST also express AR. Fos-expressing neurons are dispersed within this region, suggesting that if this component of BNST mediates different behavioral functions, this is not apparent in a simple anatomic partitioning of the nucleus.

We also observe a significant increase in the number of cells exhibiting Fos expression in a dispersed population of neurons in the preoptic area of the hypothalamus and in the medial amygdala (not shown). We did not observe an increase in Fos in the sexually dimorphic AR-expressing clusters in the forebrain (not shown). Our data are consistent with previous experiments that have

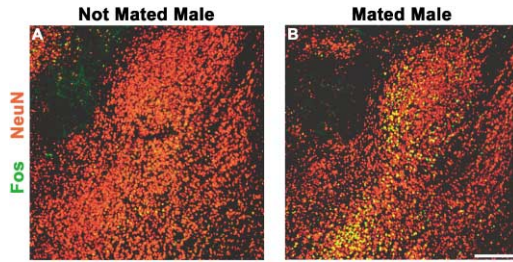


Figure 5. Mating Elicits Fos Expression in the BNST
Representative coronal sections through the BNST of adult males either mated (B) or not mated (A) stained with anti-NeuN antibody (red fluorescence) and anti-Fos antibody (green fluorescence). There is a 30-fold increase in the number of Fos-positive neurons in the mated male compared to the control male. However, only ~10% of the neurons in the BNST express Fos. Scale bar equals 200 μ m.

analyzed Fos expression following male mating (Greco et al., 1998a, 1998b; Joppa et al., 1995; Kollack-Walker and Newman, 1995; reviewed in Pfau and Heeb, 1997; Veening and Coolen, 1998; Wang et al., 1997). Despite the diversity of stimuli that can lead to Fos upregulation and the inability to discern at present what aspect of the behaviors elevates Fos expression, these observations are in accord with previous data implicating the sexually dimorphic BNST and the preoptic area in mating behavior.

Discussion

Visualizing Anatomic Dimorphisms

Innate behaviors result from the activation of genetically programmed neural circuits. Only in rare instances has it been possible to identify defined neural circuits and associate these circuits with specific behaviors. These experiments, successful largely in invertebrate nervous systems, have provided important insight into how specific sensory input elicits motor output and how these circuits change with learning and experience (Kandel, 2001). The anatomic complexity of the mammalian brain, however, confounds the identification of circuits responsible for specific behaviors. The development of genetic approaches to visualize neurons along with their projections can significantly reduce the “complexity” problem if genes are chosen that mark only small subpopulations of cells in the brain. We have genetically modified the AR gene to visualize only the subset of neurons in the mouse brain that expresses AR.

AR is expressed in less than 10% of the cells in the mouse brain and we can therefore visualize neurons and their processes unencumbered by the vast population of AR-negative cells. It is likely that neural circuits responsible for sexually dimorphic behaviors will include AR-positive neurons, since dimorphic behaviors in part result from differences in androgen levels between the two sexes. In mice bearing a modified AR gene, the previously described dimorphisms in the BNST and the POA of the hypothalamus are revealed with clarity (Allen et al., 1989; Byne et al., 2000; Gorski et al., 1980; Gu et al., 2003; Hines et al., 1985; Hutton et al., 1998; LeVay, 1991; Raisman and Field, 1971; Stefanova and Ovtcharoff, 2000; Zhou et al., 1995). Moreover, our experimental

design reveals previously undetected islands of neurons in the basal forebrain that are more abundant in the male.

Anatomic Dimorphisms and Dimorphic Behavior

Sexual dimorphisms in neural circuits are thought to be responsible for dimorphic behavior. For example, in songbirds such as canaries and zebra finches, only males sing, and this dimorphic behavior is paralleled by anatomic dimorphisms in several nuclei in the song circuit (Nottebohm and Arnold, 1976; reviewed in Konishi and Gurney, 1982, and in Cooke et al., 1998). Similarly, in the African clawed frog, only males emit courtship calls, and the motoneurons controlling the laryngeal muscles involved in these vocalizations are sexually dimorphic (reviewed in Kelley, 1986).

In mammals, it has been difficult to associate specific behaviors with defined neural circuits, and therefore the functional significance of anatomic dimorphisms remains elusive. In mice, mating is thought to be initiated by pheromones that activate sensory neurons that project to the olfactory bulb. This information is relayed to the amygdala and ultimately to the hypothalamus via the BNST (Dong et al., 2001; Scalia and Winans, 1975; Segovia and Guillaumon, 1993). Regions of the BNST and hypothalamus are sexually dimorphic and it is possible that these anatomic distinctions underlie the distinct sexual behaviors exhibited by males and females. Indeed, lesions to either the BNST or POA impair male mating behavior (Claro et al., 1995; Emery and Sachs, 1976; Liu et al., 1997). Our data, along with previous studies, reveal a striking increase in the expression of Fos, a reporter of neural activity, in the amygdala, BNST, and POA of the hypothalamus in males after mating (reviewed in Pfau and Heeb, 1997). Elevations in Fos in the BNST, for example, are restricted to perhaps 10% of the cells in this region. The frequency of AR-positive neurons is 80% in both the Fos-positive and -negative neurons in the BNST. These observations are consistent with the participation of two anatomically dimorphic brain loci, the BNST and the POA, in circuits governing sexually dimorphic mating behaviors. One interesting possibility is that AR-expressing cells in the medial amygdala, the BNST, and the POA are interconnected in a circuit that influences mating behavior.

It remains unclear whether androgens act solely through AR to elicit male-specific differences in brain anatomy and behaviors or whether the conversion of testosterone to estradiol could contribute to these dimorphisms. An interesting “paradox” emerges from experiments that indicate that estrogen administration neonatally can masculinize the female brain (MacLusky et al., 1987). In addition, male mice with mutations in the two estrogen receptors, ER α and ER β , fail to exhibit sex-specific aggressive and mating behaviors (Rissman et al., 1997; Ogawa et al., 2000). These data suggest that testosterone may exert its masculinizing effects on behavior via estradiol (Simpson and Davis, 2000). Moreover, null mutations in aromatase, the enzyme responsible for converting testosterone to estradiol, result in dramatic diminutions in aggression and mating behavior in male mice (Honda et al., 1998; Toda et al., 2001). Thus, locally synthesized estradiol in concert with circulating androgen may be required for the dimor-

phisms we observe in AR-positive neurons and the dimorphic behaviors of the two sexes.

In mice, sexual dimorphisms are evident in mating, nursing, aggression, territoriality, and exploratory behaviors. However, relatively few anatomic differences in neural organization distinguish the two sexes (Segovia and Guillamón, 1993). Despite the overt differences in behaviors, the vast majority of brain functions are shared by males and females. The circuits responsible for these common CNS functions will also be shared and the anatomic complexity of common neural pathways will obscure identification of the rarer dimorphic circuits. Identification of more subtle or "hidden" sex differences will require novel approaches to visualize distinct subpopulations of neurons unencumbered by the vast majority of shared circuits. The genetic marking of AR-positive cells provides one such approach and indeed reveals a novel dimorphic locus in the forebrain. This approach not only allows us to visualize dimorphic subpopulations of AR-positive neurons along with their projections but may ultimately permit an association between these neural loci and specific dimorphic behaviors.

Experimental Procedures

Generation of Mice Bearing a Modified AR Gene

A genomic clone containing the last exon of the AR gene was used to design the targeting vector for electroporation into mouse ES cells. The reporter cassette, IRES-PLAP-IRES-nLacZ, and a neomycin selection fragment were introduced after the stop codon of the AR gene in the targeting vector. Homologous recombinant ES clones were used in blastocyst injections to obtain chimeric males. Chimeric males that transmitted the modified AR allele were crossed to C57Bl/6J females to obtain the mice used in our analyses.

Staining and Quantitation

Age-matched adult (>3 months of age) homozygous (female) and hemizygous (male) mice were used for all comparative analyses. Except in the mating assay, mice were sexually naive and group-housed according to sex after weaning. To visualize β -gal or PLAP activity in the brains of mice bearing the modified AR allele, sections were collected at 75 μ m and processed as described previously (DePrimo et al., 1996; Fields-Berry et al., 1992; Leighton et al., 2001; Mombaerts et al., 1996).

Brain sections were collected at 65 μ m for immunostaining. For quantitation of β -gal-positive neurons in the BNST, sections through this region were stained with anti- β -gal and anti-NeuN and imaged using a confocal microscope. Image files were collected as Z-stacks, and β -gal-positive cells were enumerated using a combination of NIH ImageJ and Neurolucida (MicroBrightField). For quantitation of the AR-positive basal forebrain clusters, ≥ 5 β -gal-positive cells surrounded by β -gal-negative cells in this region were defined as a single cluster. To quantitate the number of AR-positive cells within each cluster, 17–20 clusters per animal were analyzed.

A more detailed description of the experimental techniques and reagents used in this study is available in the Supplemental Data posted online (<http://www.neuron.org/cgi/content/full/43/3/313/DC1>).

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Supplemental Data for:Shah et al., *Neuron* 43, pp. 313–319**Supplemental Experimental Procedures****Generation of Mice Bearing a Modified AR Gene**

Genomic clones containing AR exons were obtained by screening a 129/SvJ lambda phage library (Stratagene). A 8.7 kb *EcoRV* genomic clone containing the last exon of the *AR* gene was isolated after restriction and sequence analysis. Site-directed mutagenesis (Stratagene) was used to introduce an *AscI* restriction site 3 bp after the stop codon of the *AR* gene in this 8.7 kb *EcoRV* fragment. This results in a targeting vector with 1.7 kb and 7 kb of homology 5' and 3' of the *AscI* site, respectively. The reporter cassette IRES-PLAP-IRES-nLacZ was generated from separate plasmids containing IRES-PLAP (obtained from Lukas Sommer and David J. Anderson) and IRES-nLacZ (obtained from Silvia Arber and Thomas M. Jessell). The self-excising neomycin selection plasmid (see below), ACN, was introduced 3' to the nLacZ. The IRES-PLAP-IRES-nLacZ-ACN cassette was inserted in to the targeting vector as an *AscI* fragment. The AR-IRES-PLAP-IRES-nlacZ-ACN construct was electroporated into a 129/SvEv mouse ES cell line followed by G418 selection. This resulted in a targeting frequency of 40% for homologous recombinants as detected by PCR for the 5' arm of the targeting vector using an external primer (5'-AGGGAGAACACAGCTCCTGGACATTCC) and a primer (5'-GAATTCGGCGCGCCTCTTCACTGTGTG) unique to the modified allele. A subset of the positive ES clones was also subjected to PCR for the 3' arm using an external primer (5'-CAAATAGCCCACCAGCTGTGTGACACATG) and a primer (5'-GACCGCTTCCTCGTGCTTTACGGTATCG) from ACN. Chimeric males that transmitted the modified allele were obtained from one positive clone. As described previously, ACN contains a *neo^R* gene that is self-excised upon passage through the male germline (Bunting et al., 1999). Consistent with this, F1 progeny obtained by crossing the chimeric males to C57Bl/6J or 129/SvEv females had deleted ACN as determined by PCR. The transgenic line bearing the modified *AR* gene was maintained by crossing heterozygous females to hemizygous males.

Nuclear b-Gal and PLAP Staining

Brains were dissected from paraformaldehyde (PFA) perfused animals and fixed at 4°C for an additional 1–1.5 hr (b-gal staining) or for 2.5–3 hr (PLAP staining). The brains were embedded in 3% agar and sectioned at 75 µm on a vibrating microtome. b-gal activity was visualized using staining reagents as described previously (Mombaerts et al., 1996). The staining reaction was stopped after incubating the sections at 37°C for 16–18 hr protected from light. Sections were washed and postfixed in 4% PFA. The sections were transferred to glass slides, allowed to air dry briefly, and then mounted in Aquamount (Polysciences). Fixation and staining conditions were identical for visualizing b-gal in whole-mount brain preparations. These whole-mount brains were viewed under a dissecting microscope after dehydration and clearing in benzyl alcohol and benzyl benzoate (1:1).

Sections to be stained for PLAP were incubated at 72°C for 60–90 min to inactivate endogenous alkaline phosphatases. The staining reaction was performed in Tris buffer (0.1 M Tris [pH 9.5], 0.1 M NaCl, 0.05 M MgCl₂) containing BCIP and NBT (0.2 mg/ml and 1 mg/ml, respectively) at room temperature for 2–3 hr and protected from light. The sections were washed, postfixed briefly in 4% PFA, and destained with a series of dehydration and rehydration steps in ethanol. Sections were mounted as described above for b-gal staining.

Immunostaining

Brains were perfused and fixed as described above for b-gal staining. Sections of 65 µm thickness were collected to ensure adequate penetration of antibodies. Primary antisera used in this study are: rabbit anti-AR (Oncogene Science, 0.5 µg/ml), rabbit anti-b-gal (Cortex Biochemicals, 1:2000), goat anti-b-gal (Arnel, 1:800), mouse anti-NeuN (Chemicon, 1:400), and rabbit anti-Fos (Ab2 from Oncogene Science, 1:100). Sections were stained with primary antibodies overnight at 4°C. The fluorophore-conjugated secondary antisera used are: Cy3 donkey anti-rabbit (Jackson ImmunoResearch, 1:800), Alexa 488 donkey anti-goat (Molecular Probes, 1:300) and Alexa 488 donkey anti-mouse (Molecular Probes, 1:300). Sections were exposed to secondary antibodies for 2 hr at room temperature. Staining was performed on free-floating sections in D-PBS containing 0.1% Triton X100 and either 10% donkey serum (initial blocking step) or 1% donkey serum (all subsequent steps). Sections were transferred to glass slides and mounted in Vectashield (Vector Labs).

Quantitation of b-Gal-Positive Neurons in the BNST

Sections were stained with rabbit anti-b-gal and mouse anti-NeuN antibodies as described above. Images of each histological section of the BNST were collected as Z-stacks (2.5 μm steps) using a 10 \times objective lens (Nikon E800) on a BioRad MRC 1024 confocal microscope. Each histological section resulted in 2 Z-stacks of the BNST (left and right) and typically there were 6 sections per animal. NIH ImageJ was used to despeckle and to subtract background pixel intensities (as determined from sections obtained from wild-type animals and processed in parallel with the experimental sections) from these Z-stacks and finally to obtain a flat projection of each Z-stack. A contour of the BNST was traced in NeuroLucida (Confocal Module, MicroBrightField) for each of the flat projection files generated by NIH ImageJ. The despeckled and background-subtracted Z-stack was imported into NeuroLucida and only b-gal stained cells lying within the BNST contour were analyzed. Cells observed in the central optical slice (at $\sim 30 \pm 5 \mu\text{m}$ of a 65 μm section) of each Z-stack were marked and enumerated. Our confocal Z stacks afforded us the opportunity to count only intact nuclear profiles. We feel, therefore, that is highly unlikely that our cell counts are significantly distorted by counting split cells that appeared in consecutive histological sections. The cell counts of all Z-stacks obtained from an animal were then combined (and divided by 2) to yield a measure of the number of AR-positive cells in each BNST (left and right). Both b-gal and NeuN are localized to the nucleus, making it unlikely that our counts were skewed by any differences in cell soma size between males and females.

To obtain the fraction of neurons that co-expresses b-gal and NeuN, BNST sections were imaged on the confocal microscope using a 60 \times oil-immersion objective lens. Cells were enumerated using a combination of NIH ImageJ and NeuroLucida as described above with only minor modifications. The BNST is densely packed with cells and most easily delineated from neighboring regions in the middle two sections in its rostrocaudal extent. We therefore imaged subregions within these sections of the BNST and analyzed at least 150 cells for colabeling per animal.

Quantitation of b-Gal-Positive Neurons in Forebrain Clusters

For quantitation of the sexual dimorphism in the basal forebrain, ³⁵S b-gal-positive cells surrounded by b-gal-negative cells in this region were defined as a single cluster. Either X-Gal stained or anti-b-gal stained sections were used to count the number of clusters using a 10 \times objective lens mounted on an upright microscope. The total number of such clusters was obtained by analyzing all forebrain sections. To quantitate the number of AR-positive cells within each cluster 17–20 clusters per animal were analyzed along the entire AP axis at high magnification (40 \times) on an upright microscope with epifluorescence optics (Nikon).

Mating Assay

Sexually naive adult males bearing the modified *AR* gene were housed individually and given mating experience. Briefly, an adult female was introduced in to the cage and the pair was allowed to cohabit for 6–7 days. Following this, the female was removed from the cage and the male was singly housed for up to a week. The male obtained sexual experience with at least three adult females using this procedure. During the day of the test (performed during the light cycle for mice), estrus females were selected from a cohort of adult C57Bl/6J females based on genital examination. The female was introduced into the sexually experienced male's cage and the pair was allowed to interact for 60 min. Males achieving ejaculation with the female were then processed for Fos and b-gal staining as described above.

Supplemental References

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