SUMMARY

Sex hormones such as estrogen and testosterone are essential for sexually dimorphic behaviors in vertebrates. However, the hormone-activated molecular mechanisms that control the development and function of the underlying neural circuits remain poorly defined. We have identified numerous sexually dimorphic gene expression patterns in the adult mouse hypothalamus and amygdala. We find that adult sex hormones regulate these expression patterns in a sex-specific, regionally restricted manner, suggesting that these genes regulate sex typical behaviors. Indeed, we find that mice with targeted disruptions of each of four of these genes (Brs3, Cckar, Irs4, Sytl4) exhibit extremely specific deficits in sex specific behaviors, with single genes controlling the pattern or extent of male sexual behavior, male aggression, maternal behavior, or female sexual behavior. Taken together, our findings demonstrate that various components of sexually dimorphic behaviors are governed by separable genetic programs.

INTRODUCTION

Sexually reproducing animals display sex-specific social behaviors. Recent genetic studies have elucidated some of the rules that control such behaviors in mice. These studies show that estrogen sets up the repertoire of sexual and territorial behaviors, and testosterone controls the extent of these displays in males (Wu et al., 2009; Ogawa et al., 2000; Raskin et al., 2009; Juntti et al., 2010; Kudwa and Rissman, 2003; Kudwa et al., 2006; Wu and Shah, 2011). However, the molecular pathways employed by these overarching hormonal mechanisms to influence neural circuits underlying sex-typical behaviors are poorly understood.

Sex steroids can be regarded as master regulators of sex-specific behaviors (Morris et al., 2004; Baum, 2003). The developmental influence (organizational role) of sex hormones can lead to enduring effects on brain and behavior. By contrast, in adults sex steroids elicit reversible changes (activational role) in neural circuits and behavior. Gonadal hormones bind to distinct nuclear hormone receptors that are essential for sex-typical displays (Scordalakes and Rissman, 2003; Raskin et al., 2009; Kudwa and Rissman, 2003; Wersinger et al., 1997; Juntti et al., 2010; Ogawa et al., 2000; Lydon et al., 1995). These receptors directly regulate gene expression by binding DNA (Mangelsdorf et al., 1995), and they can initiate nontranscriptional signaling via mechanisms such as interactions with intracellular kinases and transmembrane receptors (Foradori et al., 2008; Lishko et al., 2011; Micevych and Dominguez, 2009; Revankar et al., 2005; Vasudevan and Pfaff, 2008; McDevitt et al., 2008). Sex hormones or their metabolites can also bind to neurotransmitter receptors to gate their activity (Henderson, 2007). Such nontranscriptional signaling can control neural function at time scales that allow real time modulation of behavior.

Prior work has identified genes downstream of sex hormones that regulate sexually dimorphic behaviors (Kayasuga et al., 2007; Wersinger et al., 2002; Nelson et al., 1995; Winslow and Insel, 2002). The relative paucity of such genes is in contrast to the diversity of these behaviors, and suggests that the underlying neural circuits may be regulated largely by nontranscriptional hormone signaling. However, such genes may also be difficult to identify because they are expressed at low levels or in a few neurons. We used an unbiased approach to identify genes that are downstream of sex hormones and that control dimorphic behaviors. We reasoned that such genes are expressed dimorphically; using microarrays, we therefore sought to identify sex differences in gene expression in the hypothalamus since this region is essential for dimorphic behaviors and contains sex hormone receptor expressing neurons. We identified 16 genes with sexually dimorphic expression in the hypothalamus and medial amygdala (MeA). Adult sex hormones control the expression of most of these genes, suggesting that they regulate dimorphic behaviors. Indeed, we find that mice singly mutant for four of these genes exhibit deficits in specific components of male mating, intermale aggression, maternal behavior, or female sexual receptivity. Thus, our results show that dimorphic behaviors are modular in the sense that components of these displays are genetically separable.
RESULTS

Identification of Sex Differences in Gene Expression in the Hypothalamus

We compared gene expression between adult male or female hypothalamus and whole brain using dual color microarrays (Figure 1A) (Verdugo and Medrano, 2006). Our gene profiling and subsequent analysis were devised to identify dimorphic hypothalamus-enriched mRNAs. We disregarded Y-specific genes, X-linked genes with Y paralogs, and genes involved in X-inactivation, because they were not hypothalamus-enriched. Our analysis identified 84 sexually dimorphic candidate transcripts (Table S1, available online).

We screened all 84 genes for sexually dimorphic expression by in situ hybridization (ISH) through the adult forebrain (Figures 1A–1C). Putative dimorphisms were validated by ISH on ≥ 2 more pairs of males and females. These studies revealed 16 dimorphically expressed genes (Figure 1D). These encode a neuropeptide (CART), GPCRs (Cckar, Brs3, Gpr165), neurotransmitter-gated ion channels (Gabrg1, Glra3), intracellular signaling proteins (Dgkk, Irs4, Pak3, Rps6ka6, Syt4), a transcription factor (Er2a), a protease (Ecel1), and those with poorly understood function (Chodl, Greb1, Nnat). Although the neural function of most of these genes is unknown, they largely encode signaling proteins that could regulate neuronal function and behavior acutely. Many of these genes (Dgkk, Gabrg1, Greb1, Pak3, Rps6ka6) are implicated in various human disorders that occur with sex-skewed ratios (Morrow et al., 2008; Ghosh et al., 2000; Enoch et al., 2009; Yntema et al., 1999; van der Zanden et al., 2011). Seven of these 16 genes are X-linked (Figure 1D), a distribution unlikely to occur by chance ($p < 1 \times 10^{-4}$). The X-linked genes are not simply female-upregulated because they escape X-inactivation. Rather, with the exception of Brs3, whose expression is upregulated only in females, the expression of other X-linked genes is upregulated only in males or in distinct regions in both sexes (Figure 2, Figure 3, Figure 4, and Figure S1). Thus, our screen has yielded many dimorphic transcripts with unexpected features and whose functions in dimorphic behaviors are unknown.
Complex Patterns of Sexually Dimorphic Gene Expression

We find dimorphic gene expression in the hypothalamus, the bed nucleus of the stria terminalis (BNST), and MeA (Figure 2, Figure 3, Figure 4, and Figure S1). The BNST was included in our tissue dissection for gene profiling since it is intermingled with hypothalamic areas, expresses sex hormone receptors, and regulates dimorphic behaviors (Emery and Sachs, 1976; Simerly et al., 1990; Gammie and Nelson, 2001). By contrast, the MeA was not included in our dissection and it is surprising that many of these genes are dimorphic in the MeA. The MeA receives pheromonal input essential for social behaviors and it provides afferents to the BNST and most hypothalamic centers with dimorphic gene expression (Figure 4A) (Canteras et al., 1995; Dulac and Wagner, 2006). Thus, sex differences within the MeA could influence pheromonal information relayed to the BNST and hypothalamus. The sex differences in gene expression within these regions are restricted to specific neuronal pools that are thought to control dimorphic behaviors (Cooke et al., 1998; Simerly, 2002; Blaustein, 2008). These include the BNSTmpm (posteromedial area of the medial BNST), BNSTmpl (posterolateral area of the medial BNST), MeApv (posteroventral MeA), MeApd (posterodorsal MeA), and the POA (ventral premammillary nucleus), VMHvl (ventrolateral area of the ventromedial nucleus), PMV (ventral premammillary nucleus), and perIV (rostral periventricular region) in the hypothalamus (Figure 2, Figure 3, Figure 4, and Figure S1).

Of these 16 genes, ten are male-upregulated, two are female-upregulated, and four exhibit a compound dimorphism such that each is upregulated in the female VMHvl and in 1 male brain region (Figure 2, Figure 3, Figure 4, and Figure S1). All 16 transcripts were also expressed in a nondimorphic pattern in other discrete brain regions (Figure S2B). Microarray studies cannot reveal such complexity in expression patterns, validating the utility of ISH. A microarray study previously identified Sytl4 as being male-upregulated in the brain (Yang et al., 2006), although its dimorphic expression was not confirmed or localized histologically. We find Sytl4 to be upregulated in the male BNSTmpm (Figures 1B–1C and Figures 2A and 2B). Male-upregulated POA expression of Gabrg1 has been described in the rat (Nett et al., 1999), and our data extend these findings to the mouse. Some but not other studies have reported sexually dimorphic ERα expression in rodents (Lauber et al., 1991; Simerly et al., 1990; Shughre et al., 1992; Koch and Ehret, 1989). Our results show unequivocal sex differences in ERα expression (Figure 4A, Figures S1S–S1L, and Figure S2A).

These 16 genes are not expressed in white matter, and they label cells that appear to bear a neuronal morphology. We find genes that are upregulated in the female BNSTmpm and MeApd and in the male BNSTmpl even though these regions contain
more neurons in the other sex (Figure 2, Figure 4A, and Figure S1) (Guillamón et al., 1988; Holmes et al., 2009; Morris et al., 2008a; Shah et al., 2004; Wu et al., 2009). Most sex differences in gene expression also withstand normalization to neuronal number in RT-qPCR studies (Figure S2A). Thus, the sex difference in gene expression cannot be accounted for solely by dimorphic neuronal numbers.

**Testicular Hormones Control Sex Differences in Gene Expression**

Castration of adult males abrogates sex-typical behaviors (McGill and Tucker, 1964; Beeman, 1947). We tested if these deficits are accompanied by altered dimorphic gene expression by performing ISH in adult male castrates and controls. The expression of most male-upregulated genes is downregulated in castrates and appears feminized (Figure 2, Figure 3, and Figure 4B). Brs3 and ERα, which are normally female-upregulated, were upregulated in male castrates (Figure 2 and Figure 4B). Thus, testicular hormones enhance, inhibit, or leave unaffected gene expression in a region-specific manner, suggesting that they utilize distinct molecular mechanisms to drive male-typical behaviors. The expression profile in castrate males is plastic, and testosterone provision restores expression of most genes to levels observed in intact males (Figure S3). Thus, testosterone can masculinize expression of most genes we have identified.
Restricted Control of Sexual Dimorphisms in Gene Expression by Ovarian Hormones

Castration rapidly eliminates estrous cycling and female sexual behavior (Allen and Doisy, 1923; Wiesner and Mirskaia, 1930; Ring, 1944), and we compared gene expression between adult castrate females and controls. In contrast to the wholesale changes in gene expression in male castrates, we observed highly circumscribed changes in castrate females. Castration reduced expression of \textsc{Cckar} and \textsc{Greb1} without affecting other genes (Figure 3 and Figure 4C). Our list of genes may underrepresent estrous cycle-regulated transcripts since we prepared hypothalamic mRNA from several males or females. Regardless, sex hormones control dimorphic expression of most genes we have identified in adult males but not females.

Individual Genes Control Discrete Components of Male-Typical Behaviors

\textsc{Erz} is dimorphically expressed (Figure 4A, Figure S1, and Figure S2A) and controls dimorphic behaviors. We sought to determine whether other genes in our list also regulate such behaviors. Male and female mice null for \textsc{Brs3}, \textsc{Cckar}, \textsc{Irs4}, and \textsc{Syt4} are fertile but their behavior in standard tests of dimorphic displays is unknown (Ladenheim et al., 2008; Fantin et al., 2000; Gomi et al., 2005; Kopin et al., 1999). We first ascertained that sexual differentiation of brain regions in which these genes are dimorphically expressed is unaffected, at least as revealed by normal \textsc{Erz} expression in the mutant strains (Figure S6). We next examined these mutants for deficits in a range of sex-typical behaviors (Figure 5A).

Male mating is elicited with an estrus female, and it consists of chemoinvestigation (sniffing), ultrasonic vocalization (USV), mounting, and intromission (penetration), which can culminate in ejaculation. By contrast, an intruder male is sniffed and attacked by a resident male (Miczek et al., 2001). Male residents also mark territory with many urine spots (Desjardins et al., 1973). \textsc{Cckar} and \textsc{Irs4} mutant males were similar to WT siblings in these male-typical displays (Figures S4A–S4T), whereas \textsc{Brs3} (Table S2) and \textsc{Syt4} mutants exhibited behavioral deficits.

\textsc{Syt4}^-/- mice showed specific changes in some but not all mating parameters (Figures S5B–S5G). They sniffed females less but intromitted in more assays, differences that were also confirmed with additional statistical analyses (p < 0.01; data not shown). Although the females allowed intromission, males only ejaculated in a subset of assays as expected (Figures 5D and 5H). WT males who ejaculate show a reduced latency to intromit and intromit faster after the first sniff (Figures 5I and 5J). These differences are significant and an indicator of subsequent ejaculation. Although \textsc{Syt4}^-/^- WT males ejaculate equivalently, loss of \textsc{Syt4} function decorrelated mating pattern from ejaculation (Figures 5D, 5I, and 5J). \textsc{Syt4}^-/^- mice mated in a manner similar to ejaculatory WT males regardless of ejaculation.

\textsc{Syt4}^-/^- mutants do not have pervasive deficits. They attack males and mark urine like WT males (Figures S4U–S4Z). We also found no deficits in movement, general activity, and social interactions such as grooming (data not shown). \textsc{Syt4}^-/- mice emit USV to females but not males, indicating that they discriminate between the sexes (Figure 5C). Although \textsc{Syt4} regulates insulin release in vitro, \textsc{Syt4} mutants have normal insulin titer and a mild decrease in blood glucose (Wang et al., 1999; Gomi et al., 2005). There are also no overt changes in testosterone that could alter mating. The mean and the distribution of serum testosterone levels in male WT and \textsc{Syt4}^-/- mice were indistinguishable.
testosterone titer was similar between Sytl4 null and WT males (WT, 7.2 ± 2.7 nM; Null, 11.6 ± 4.9 nM; n ≥ 13; p > 0.9), with the titer always exceeding the receptor Kd. Testosterone levels were also similar between Sytl4 null and controls at a younger age (Table S3). Thus, Sytl4 controls specific components of male mating.

**Individual Genes Control Discrete Components of Female-Typical Behaviors**

We tested females null for Brs3, Cckar, Irs4, or Sytl4 for deficits in female-typical behaviors. Females reject male mating attempts except during a periovulatory period (estrus) when they are sexually receptive. Nursing females retrieve pups that wander from the nest and attack intruders in the cage (Gandelman, 1972). The mouse estrous cycle is ~5 days. Mutants and controls of each strain had 1 cycle within a 5 day period (n = 3–8 genotype/strain; p ≥ 0.5), indicating that these genes are not essential for estrous cyclicity. Brs3 or Sytl4 null females behaved similar to WT siblings (Figure S5). By contrast, Irs4 and Cckar mutants exhibited deficits in female-typical behaviors.

Irs4 mutants mated, delivered litters, and weaned them in a WT manner (Figure S5, Table S4). In tests of pup retrieval, control and mutant mothers retrieved pups and they did so with similar latencies (Figures 6A and 6B), but Irs4−/− mothers took longer to retrieve all pups (Figure 6C). Irs4−/− mothers were also impaired in maternal aggression such that fewer Irs4−/− mothers attacked intruders (Figure 6D, Movie S1, and Movie S2). However, when Irs4 mutants attacked, they did so in a WT fashion (Figures 6E–6I). Thus, the circuit for maternal aggression appears intact in Irs4−/− females, but it may be activated less frequently than in WT.

Irs4 mutants do not have systemic deficits. Irs4 is homologous to intracellular adaptor proteins essential for insulin receptor signaling (Burks and White, 2001). However, Irs4−/− females maintain normal weight and blood glucose and insulin titers (Fantin et al., 2000). The mutants also showed WT activity in social interaction and motor performance (Table S4). Thus, Irs4 is specifically required for maternal behaviors that may be essential for pup survival in nature because mouse pups are altricial and adults are infanticidal toward young of other mice.

Although Cckar, a cholecystokinin receptor, can control feeding and metabolism (Pirnik et al., 2010), Cckar null mice are normoglycemic and maintain normal body weight (Whited et al., 2006). Studies in rats have been inconclusive on the role

---

**Figure 5. Sytl4 Is Required for Patterning Male Sexual Behavior**

(A) Mice mutant for Brs3, Cckar, Irs4, or Sytl4 were tested for deficits in various sex-specific displays.

(B) Sytl4−/− residents (null) sniff WT female intruders less than Sytl4+/+ residents (control).

(C) USV Kinetics: Sytl4−/− residents (null) have more USV than Sytl4+/+ residents (control).

(D) Sexual behavior: Sytl4−/− residents (null) have a higher fraction of assays with mounting and intromission than Sytl4+/+ residents (control).

(E, F) Latency and number of events: Sytl4−/− residents (null) have a shorter latency and fewer events than Sytl4+/+ residents (control).

(G) Duration of events: Sytl4−/− residents (null) have a shorter duration of mounting and intromission than Sytl4+/+ residents (control).

(H) Successful mounts: Irs4−/− residents (null) have a higher proportion of successful mounts than Irs4+/+ residents (control).

(J) Sniff to intromission latency: Sytl4−/− residents (null) have a shorter latency than Sytl4+/+ residents (control).

Mean ± SEM; n ≥ 14 animals/genotype; *p < 0.04; **p < 0.01. See also Figure S4, Table S2, and Table S3.
of CCK and Cckar in the estrous cycle and female receptivity (Akesson et al., 1987; Hilke et al., 2007; Oro et al., 1988; Dornan et al., 1989; Babcock et al., 1988; Bloch et al., 1987; Holland et al., 1997; Mendelson and Gorzalka, 1984). In mice, we find a diminution in receptivity in Cckar\(^{-/-}\) females such that they do not readily permit intromission or ejaculation (Figure 7A). This deficit is not due to reduced interest by WT males who mounted all females equivalently (Figure 7A). Even in assays with intromission, Cckar mutants allowed fewer mounts to progress to intromission (Figure 7B, Movie S3, and Movie S4). Sexually experienced WT females permit more intromissions; although experienced Cckar\(^{-/-}\) females allowed more intromissions, they were always less receptive than controls (Figure 7C).

Cckar expression in the VMHvl requires ovarian hormones (Figure 3 and Figure 4C). Estrogen, which elicits receptivity via ER\(a\) in the VMHvl (Musatov et al., 2006), induces Cckar in the VMHvl (Figures 7H and 7I). By contrast, estrogen, which does not elicit receptivity in males, did not induce Cckar in the male VMHvl (Figures 7J and 7K). We do not yet know if Cckar expression in the VMHvl drives receptivity. Nevertheless, Cckar is induced in the female VMHvl by hormones that drive estrus and receptivity, and Cckar is essential for normal receptivity.

We next tested whether Cckar regulates receptivity in adults. We induced estrus in castrate WT females and injected devazepide or lorglumide, structurally distinct, specific, competitive Cckar antagonists (Berna et al., 2007). Strikingly, these antagonists reduced sexual receptivity (Figures 7E and 7G). In contrast to our findings with Cckar mutants, WT males intromitted and ejaculated normally with antagonist-treated females (Figures 7A, 7D, and 7F). This difference may reflect a developmental role of Cckar in the underlying circuit. The behavioral deficits observed in Cckar mutants or antagonist-treated females do not reflect sensorimotor obtundation because they displayed normal general mobility and social interactions (data not shown). Cckar mutant females also exhibited normal maternal behaviors (Figures S5P–S5U). Thus, our results show that Cckar functions in adult females to control sexual behavior.

### DISCUSSION

Our studies reveal a cellular and molecular representation of gender of inordinate complexity in the hypothalamus and amygdala. The genes we have identified provide an entry point for understanding the physiology of dimorphic neural circuits. Our findings also provide evidence for separable genetic programs that control particular components of sexually dimorphic behaviors.

### Sex Differences in Gene Expression in the Brain

In contrast to the rich array of dimorphisms in mammalian behaviors and neuroanatomy, few dimorphisms in gene expression have been identified in the brain, and the dimorphic function of most of these genes is unknown (De Vries, 1990; Simerly, 2002; McCarthy, 2008; Cahill, 2006; Gagnidze et al., 2010; Dewing et al., 2003). Since most neural functions are common to both sexes, molecular dimorphisms are likely embedded in shared neural circuits. This has made it difficult to prospectively identify dimorphisms in gene expression beyond genes such as those unique to the Y chromosome or genes involved in X-inactivation (Rinn and Snyder, 2005; Rinn et al., 2004). Many dimorphisms in gene expression have been observed in more homogenous tissues such as liver (van Nas et al., 2009; Clodfelter et al., 2006; Yang et al., 2006; Rinn et al., 2004). The Allen Institute of Brain Science (http://mousediversity.aleninstitute.org/) have examined expression of ~70 genes by ISH to reveal additional sexual dimorphisms in the adult mouse brain; this approach, while powerful, would require enormous resources if conducted with all genes. By contrast, we used microarrays to identify potential sex differences in gene expression followed by ISH validation; this approach has yielded a new set of sexually dimorphic genes. One drawback of our approach...
is the limited sensitivity of microarrays in detecting transcripts present at low abundance. Indeed, our list of dimorphic mRNAs does not include other dimorphically expressed genes such as the androgen receptor, aromatase, or ERβ (Shah et al., 2004; Wu et al., 2009; Roselli and Resko, 1987; Wolfe et al., 2005). Nevertheless, our results provide a general strategy to identify genes controlling dimorphic behaviors by first identifying genes expressed in a sexually dimorphic manner.

Many dimorphic genes we have identified harbor estrogen responsive elements (EREs; data not shown), and ERs may directly regulate transcription of such genes. Indeed, ERα is found on some of these EREs in breast cancer cells (Carroll et al., 2006). Sex chromosome linked genes can influence sexual differentiation of the brain independent of sex hormones (Arnold et al., 2003). All X-linked genes we have identified are regulated by sex hormones, suggesting that these represent a distinct set of X-linked genes. There are many imprinted genes in the mouse brain, with a subset being dimorphically imprinted (Gregg et al., 2010b, 2010a). One of these, Nnat, is expressed from the paternal allele (Kagitani et al., 1997; Gregg et al., 2010b). We show that Nnat is dimorphically upregulated in distinct hypothalamic areas in the two sexes, indicating significant complexity in the control of Nnat expression.

Adult castrates lose behaviors typical of their sex but do not behave like the opposite sex. Thus, a castrate male mouse does not attack males, but it does not display female-typical receptivity. We find that some, but not all, genes switch their sex-typical expression pattern following castration, thereby providing a molecular correlate in the brain of the intermediate behavioral state of castrates. These dimorphic genes may also control morphological plasticity controlled by adult sex hormones in regions such as the MeApd, VMHvl, and POA (Morris et al., 2008b; Cooke, 2006; Dugger et al., 2008, 2007; Balthazart et al., 2010; Konishi, 1989).

**Genetic Control of Sexually Dimorphic Behaviors**

Our findings, in conjunction with previous work (Wu and Shah, 2011), suggest a model in which sex hormones govern a sexually dimorphic behaviors, and this model can be further refined with additional genetic manipulations and behavioral tests.
dimorphic gene expression program such that individual genes regulate specific components of dimorphic behaviors. Such a model may be premature since we understand very little about how these genes influence behavior. For example, Sytl4 is upregulated in the male BNSTmpm and Sytl4 mutants, similar to male rats following surgical BNST lesions, mate aberrantly (Emery and Sachs, 1976). Nevertheless, Sytl4 expression in a small set of non-BNST neurons makes it difficult to conclude that Sytl4 functions in the BNST to control mating. In future studies, conditional genetic manipulations will permit a better understanding of gene function in discrete neuronal pools. The genes we have identified can also be used to engineer such conditional genetic manipulations. Thus, genes expressed in the VMHvl could potentially be used to identify neurons that control attacks in mice (Lin et al., 2011).

Individual genes can control complex behaviors as well as reflexive displays (Winslow et al., 1993; Brown et al., 1996; Scheller et al., 1983; Nishimori et al., 1996; Osborne et al., 1997; de Bono and Bargmann, 1998; Bendesky et al., 2011; Liu et al., 2011). We have identified many genes that control sex-specific behaviors. Mice mutant for these genes show deficits in specific behavioral parameters such that the sex-specific repertoire of behaviors is retained and other dimorphic interactions are unaffected. Our findings suggest that it may be possible to deconstruct all sex-specific displays into genetically separable behavioral components. Analogous genetic wiring of dimorphic behaviors may also operate in fruitflies and worms (Von Schilcher, 1976; Garcia et al., 2001), which employ nonhormonal mechanisms of sexual differentiation (Cline and Meyer, 1996; Manoli et al., 2006; Dickson, 2008). The notion that a behavior can be deconstructed into a suite of genetically encoded behavioral modules is similar to findings that complex neuropsychiatric conditions may also consist of discretely heritable traits (Kellenberger et al., 2009). Such studies have also revealed that different mutations in a gene can lead to distinct phenotypes (Zoghbi and Warren, 2010). It will be interesting to determine if mice mutant for the genes we have identified also exhibit other behavioral phenotypes.

Genetic wiring of components of dimorphic behaviors has intuitive appeal since these behaviors are subject to stringent selection. Such wiring allows evolutionary modulation of a reproductive behavior without disrupting it entirely. Social experience can modify sex-specific behaviors (Insel and Fernald, 2004), and it will be interesting to test if it alters sexually dimorphic expression of genes that control these behaviors. In summary, our findings suggest that sexually dimorphic displays may be a composite of behavioral routines that are genetically separable. It is possible that all innate social behaviors can be deconstructed similarly. In the case of sexually dimorphic displays, it will be important to identify all the underlying genes, and to understand how these genes act within neural circuits to influence social interactions.

EXPERIMENTAL PROCEDURES

Animals
Adult mice were used for all studies. Mice null for Brs3, Cckar, Irs4, and Sytl4 have been described previously (Kopin et al., 1999; Ladenheim et al., 2008; Fantin et al., 2000; Gomi et al., 2005). All studies were in accordance with IACUC protocols at UCSF.

Histology
We performed ISH on serial coronal sections spanning the rostrocaudal extent of the hypothalamus and MeA. To identify sex differences in gene expression, we performed ISH from male and female brains in parallel. This procedure precludes variability of signal:noise arising from lot-to-lot changes in reagents and permits direct sex comparison of expression.

Behavioral Assays
Testing was initiated, recorded, and analyzed as described previously (Junetti et al., 2010; Wu et al., 2009). We tested the role of Cckar in sexual receptivity once with sexually experienced females. Estrus was induced and, 20 min prior to being introduced to a sexually experienced WT male resident for 30 min, females were injected intraperitoneally with 50 μl of Devazepide (500 μg; Tocris) or Longlumide (500 μg; Sigma) resuspended in DMSO and saline, respectively. In preliminary studies we failed to observe any effect of Longlumide at a low dose of 50 μg/female. The antagonist doses we chose were based primarily on studies of gastric emptying and feeding with doses up to 250 μg/mouse. We increased this higher dose 2-fold to permit more antagonist to reach neurons and used 500 μg/adult mouse for further studies.

Statistical Analysis
Quantitation of data was performed blind to relevant variables, including sex, genotype, and drug treatment. Unless otherwise specified, we performed the following statistical tests. Categorical data was analyzed by a Fisher’s exact test. For other comparisons, we first tested the distribution of the data with Lilliefors’ goodness-of-fit normality test. Data not violating the test were analyzed with parametric tests (Student’s t test) and other data was analyzed with the nonparametric Wilcoxon rank sum test.

Microarray
MEEBO arrays (Verdugo and Medrano, 2006) were used for all hybridizations. Different normalization schemes for microarray analysis make distinct assumptions about the data. We performed various normalizations to generate a list-of-list of dimorphic genes that were robust to different normalizations and enriched in the hypothalamus compared to whole brain.

ACCESSION NUMBERS
The microarray data has been deposited in the NIH GEO database (accession #: GSE33307).

SUPPLEMENTAL INFORMATION
Supplemental Information includes Extended Experimental Procedures, four tables, four movies, and six figures and can be found with this article online at doi:10.1016/j.cell.2011.12.018.

ACKNOWLEDGMENTS
We thank R. Axel, H. Baier, H. Bourne, and S. Lomvardas for comments on the manuscript; D. Anderson, C. Bargmann, D. Julius, H. Zoghbi, and Shahlab members for discussions; and A. Barczak, P. Nittler, and E. Tumer for help with microarrays. This work was supported by NSF graduate fellowship (O.M.A.); Sandler postdoctoral fellowship (X.X.); Genentech graduate fellowship (C.F.Y. and J.K.C.); ARCS award (J.K.C.); Edward Mallinckrodt, Jr. Foundation, NARSAD, and NIH (N.M.S.; R01NS049488, DP1OD006425).

Received: June 4, 2011
Revised: September 22, 2011
Accepted: December 16, 2011
Published: February 2, 2012
REFERENCES


EXTENDED EXPERIMENTAL PROCEDURES

Animals
Mice were group-housed by sex at weaning and housed as described previously (Wu et al., 2009). Adult (9–24 weeks of age) mice were used for all studies. Null mutants for Cckar were obtained from matings between heterozygous parents. For X-linked genes (Brs3, Irs4, and Sytl4), null females and males were obtained from matings between heterozygous females and hemizygous males, or in the case of null males, from matings between heterozygous females and WT males. WT or heterozygous siblings generated from these crosses were used as control animals for all behavioral studies. Mice used to obtain tissue for mRNA, in situ hybridization, and behavior were on a C57Bl/6J background except WT intruder males for aggression (129/Sv), WT stud males for testing female sexual behavior (hybrid 129/Sv and C57Bl/6J), and mice bearing mutant alleles of Cckar (129/Sv) and Sytl4 (mixed 129/Sv and C57Bl/6J). Male and female mice were surgically castrated and supplemented with hormones as described previously (Wu et al., 2009). Briefly, the hormones were resuspended in sterile sesame oil (Sigma) and mice received the hormone or equivalent volume of vehicle subcutaneously. For testosterone supplementation, we dosed the males with 75 μg (50 μl oil) of testosterone propionate (Sigma) on alternate days. To induce receptivity in females, we injected 10 μg (100 μl oil) and 5 μg (50 μl oil) of 17β-estradiol benzoate (Sigma) 48 and 24 hr preceding the test, respectively; on the day of the test, we injected 50 μg of progesterone (Sigma; resuspended in 50 μl oil) 4–6 hr prior to the test.

Behavioral Assays
Animals were tested for behaviors only after they were 10 weeks of age and assays were initiated ≥ 1 hr after onset of the dark cycle. All experimental mice, males and females, were always exposed to intruders they had not encountered previously, and each assay was separated by ≥ 2 days. For male mice, singly housed residents were tested 3 times each for sexual behavior for 30 min with a WT estrus intruder and for aggression with a WT group-housed male intruder for 15 min. Performance in urine marking was tested once for 60 min in a fresh cage following social experience. A separate cohort of socially naive males was tested for ultrasonic vocalizations once for 3 min each to a WT male and female intruder introduced separately into the cage. Once behavioral testing commenced, it was completed within 3 weeks at which point the males were sacrificed and blood was collected to determine serum hormone levels as described previously (Wu et al., 2009). There can also be changes in testosterone titers across the day within a male. This is unlikely to impact our results since we did all testing within the same 2–3 hr window, and WT and mutant males were used randomly within this timeframe to remove bias resulting from the time of the test. Assays were performed after lights out since mice are nocturnal, and blood collection was done at a time at which testing would have commenced.

To test for sexual receptivity, females were castrated, and, subsequent to estrus induction with estrogen and progesterone, inserted singly into the home cage of a sexually experienced WT male for 30 min each in 3 assays. Pup retrieval and maternal aggression were tested in experimental females impregnated by a WT male and singly housed 3–5 days prior to parturition. At 2, 4, and 6 days after parturition, the dam was removed briefly from the cage and 3–4 pups were scattered across the cage floor away from the nest. The dam was returned to the cage and her pup retrieval ability was tested for 15 min. To test for maternal aggression, pups of postnatal age 8, 10, and 12 days were removed and a group-housed adult WT male intruder was inserted into the cage for 15 min. The pups were returned to the mother at the end of each assay.

For the rotarod test, we followed standard procedures described previously (Moldovan et al., 2011). In brief, the mice were acclimated once to an accelerated rotarod set-up (Ugo Basile) and then tested twice for 5 min each. We monitored the time each mouse could successfully remain on the rotarod, and its performance was assessed by the average of these two tests. For the cookie finding test, we followed a previously described protocol (Wysocki et al., 1982). Briefly, mice maintained on unrestricted food were starved for 18 hr and then placed into a fresh cage containing a cookie buried under the bedding. Their behavior was recorded for 3 min following which the assay was terminated. Each mouse was tested twice in this cookie finding assay and its performance was assessed by the average of these two tests.

Determination of Estrous Cyclicity
Daily vaginal smears were obtained from group-housed females, and the cytological characteristics of the smear were imaged using brightfield optics. An experimenter blind to the genotypes independently scored the stage of the estrous cycle.

Microarray Studies
MEEBO arrays, whose probe set provides ~84% gene coverage of the mouse genome, were printed at the UCSF Center for Advanced Technology. Sexually naive C57Bl/6J mice 10 weeks of age were singly housed for 7 days prior to tissue collection in order to reduce variability in gene expression resulting from social interactions. Freshly dissected brains were sectioned into 1 mm coronal slices using a tissue chopper (EMS). The hypothalamus, including the BNST, was identified by landmarks and coordinates from the mouse brain atlas (Paxinos and Franklin, 2003), dissected in ice-cold d-PBS (free of Ca²⁺ and Mg²⁺) with the aid of a dissecting stereomicroscope, and immediately frozen on dry ice. For each experiment, 4 males and 4 females were dissected to provide hypothalamic tissue that was pooled by sex; in addition, the whole brain (extending from the olfactory bulbs to the medulla oblongata) and pituitary were taken from 1 male and female each and pooled into a single “whole-brain reference” sample. mRNA was extracted in 3 steps: Trizol (Invitrogen) extracted RNA was column purified (Micro-to-Midi columns, Invitrogen), followed by mRNA purification...
(Fast Track, Invitrogen). The mRNA was incubated with DNase I (DNase I, Amplification Grade, Invitrogen) to remove residual genomic DNA and the DNase I was subsequently heat-inactivated. For each experiment, 0.5 μg of hypothalamic mRNA from each sex and 2 μg of whole-brain reference mRNA was reverse transcribed (StrataScript, Stratagene) with random nonamers and oligo dT and labeled with amino-allyl dUTP (Ambion). The hypothalamic samples were conjugated with Cy3 and the whole-brain reference sample was coupled to Cy5 using protocols supplied by the manufacturer (Amersham). Each hypothalamic sample from males and females was split and hybridized onto 2 arrays and the whole-brain reference sample was hybridized onto all 4 arrays. Thus, each experiment consisted of 2 technical replicates for both sexes, and we performed 3 such independent experiments (biological repeats). In an independent experiment, we used the T7 polymerase based amplification method (Amino allyl message AmpIl, Invitrogen) to amplify and label mRNA from male and female hypothalamic tissue as well as the whole-brain reference. We used 10 μg of the amplified Cy3-coupled hypothalamic sample of each sex and 20 μg of the Cy5-coupled whole-brain reference and performed 2 technical replicates as described above. All arrays were hybridized and washed using standard protocols. The arrays were subsequently dried and scanned with an Axon slide scanner 4000B in an ozone-reduced environment. Array images were analyzed with the GenePix 4.0 software to obtain hybridization intensity data for each printed spot on the array for both channels (Cy3 and Cy5).

Diverse computations with different assumptions about the distribution of the underlying data have been employed to interrogate microarray data (Smyth and Speed, 2003; Quackenbush, 2002; Holloway et al., 2002; Hoffmann et al., 2002; Ding and Wilkins, 2004). We reasoned that large sex differences in gene expression would withstand scrutiny by different mathematical approaches. Accordingly, we employed distinct schemes to compile a masterlist of genes whose expression revealed a consistent sexual dimorphism across several analyses. To obtain fold changes in gene expression, the male (m) versus female (f) expression analysis was performed either on the intensity (I) data for each sex (I_m/I_f) or on intensity data that were first compared to the whole-brain reference (wbr) intensity obtained within the same array [(I_m/wbr_m)/(I_f/wbr_f)]. I_wbr_m and I_wbr_f refer to the Cy5 intensity of the whole-brain reference sample for the array on which male and female Cy3-coupled cRNA samples were hybridized, respectively. This ratio of ratios can minimize variability from technical artifacts such as subtle differences in printing or hybridization. We performed normalization on these ratios (Global; Quantile) as well as on the intensity datasets (Quantile; Ubiquitin; Intensity Sum). The Matlab manorm function was used to execute Global normalization, which assumes that the majority of transcripts on an array are not different between the two samples and adjusts the calculated ratios accordingly. The Matlab quantilenorm function was used to perform Quantile normalization, which assumes that the same range of values exist among the samples and adjusts the range of raw intensities or ratios accordingly. For “Ubiquitin Normalization,” we assumed that the intensity of the hybridization for Ubiquitin C (Ubc), an ubiquitously expressed gene that is printed on 260 spots distributed across each array, would not be different between the samples being compared. Accordingly, we separately performed normalizations analogous to the Global and Quantile schemes described above. The “Intensity Sum Normalization” assumes that the overall intensity of the hybridization signal should be similar since equal amounts of labeled cRNA are loaded onto the reaction. Technical variability can result in different overall intensities, and the Intensity Sum Normalization introduces a correction so that the sum of all intensities on an array equals the average of the sum of all intensities of all arrays.

Lists of gene expression were generated with or without normalizing the fold changes obtained by ratiometric or direct intensity comparisons. The cutoff criteria for inclusion on the lists were: ≥ 1.2 fold sex difference in expression or ≥ 2 fold upregulated expression in the hypothalamus; absolute intensity ≥ 500; and, p ≤ 0.16. The p value was determined using the one sample t test with log transformed ratios. Subsequent to the generations of these lists, we generated masterlists of genes whose expression revealed up-regulation in either sex or the hypothalamus (compared to the whole-brain reference sample) such that the expression of any gene on the list was upregulated subsequent to ≥ 1 normalization scheme and ratiometric comparison (Table S1). We subsequently compiled a list-of-lists that included technical artifacts such as subtle differences in printing or hybridization. We performed normalization on the hypothalamus (Table S1). Our in situ hybridization analysis of these 70 genes identified 13 genes to be dimorphic at the cellular resolution afforded by this approach. We reasoned that genes expressed at low copy number or in small subsets of neurons within the hypothalamus would be missed with our approach. We therefore also screened our hypothalamic upregulated list for genes whose expression was not apparently sexually dimorphic from our microarray intensity data but that were expressed in small sets of neurons in the hypothalamus as determined by the data displayed on the Allen Institute for Brain Science (AIBS) website (http://www.brain-map.org/) (Table S1) (Ng et al., 2009). The studies by the AIBS have been largely restricted to the male brain, and we therefore screened this list of 14 additional genes by in situ hybridization on adult male and female C57Bl/6J mice as described in the methods. This screen identified 3 additional genes (Cckar, Dgkk, Gabrg1) to be expressed in a sexually dimorphic pattern.

RT-qPCR
We collected 200 μm thick coronal slices from acutely dissected 10–12 week old brains of C57Bl/6J mice using a vibrating microtome (Leica) into a dish containing ice-cold d-PBS (free of Ca2+ and Mg2+). The POA, BNST, VMH, PMV, and MeA were dissected from these slices using a dissecting stereomicroscope and immediately frozen on dry ice. Total RNA was extracted with Trizol, treated with DNase I as described above, and an equal quantity of RNA was subjected to first strand cDNA synthesis (SuperScript III) using random hexamers as well as oligo-dT primed reactions. qPCR was performed using the primers listed in Table S1 on an ABI 7900 machine and the 2XSYBR master mix (Fermentas). The sex difference in gene expression was calculated using the standard 2−ΔΔCt method and the 2XSYBR master mix (Fermentas). The sex difference in gene expression was calculated using the standard 2−ΔΔCt method.
method with Synapsin I as the reference gene. One sample t test was performed on log transformed ratios to determine significance. All primer pairs used for qPCR are listed in Table S1.

**Histology**

We analyzed gene expression by ISH on 100 μm thick serial coronal sections that spanned the entire rostrocaudal extent of the hypothalamus and MeA (1 mm rostral to bregma through 3.5 mm caudal to bregma) of age-matched socially naive mice. Sex differences in gene expression in forebrain areas not included in this series, such as the dimorphism reported in the nucleus accumbens for CART expression in rats (Fagergren and Hurd, 1999), were not evaluated in our studies. In situ hybridization was performed as described previously (Wu et al., 2009). Probes for in situ hybridization were generated from subcloned RT-PCR products obtained using primers listed in Table S1. Hybridization was visualized with the histochemical substrates BCIP and NBT subsequent to incubation with an alkaline phosphatase conjugated sheep anti-digoxigenin (Roche, 1:2,000) antibody. The mRNA labeling was imaged using bright-field optics and quantified using NIH ImageJ software. Fluorescent staining and imaging of immunolabeled sections (65 μm thick) has been described previously (Wu et al., 2009; Shah et al., 2004). The primary antisera used for fluorescent immunolabeling is rabbit anti-ERα (Millipore, 1:10,000). All brain regions were identified using standard landmarks defined by the mouse brain atlas (Paxinos and Franklin, 2003).

For ISH studies on castrated mice, adult males or females were surgically castrated and gene expression was compared with un-operated or sham-operated controls. These comparisons were performed 2–3 weeks following surgery, a time point by which sexually dimorphic behaviors are lost.

For comparison between the sexes or between castrate and control mice, ISH labeled sections were imaged with a 5× objective and brightfield optics. These color images were converted into grayscale (Adobe Photoshop), the region of interest (ROI) was outlined, and labeling intensity was quantitated (NIH ImageJ). We imaged and analyzed every histological section containing the ROI in this manner, including the POA, perIV, BNST, VMHvl, PMV, MeApd, and MeApv. In parallel, we also imaged and quantitated for each histological section the staining intensity in an adjacent region that contained no labeled cells, thereby providing us with an estimate of the background labeling (noise) for each probe. The pixel values representing noise were subtracted from the pixel values obtained from imaging the ROI, thereby yielding an estimate of the corrected signal for that region. The pixel values obtained by summing the corrected signal across a given brain region provides an estimate of the level of gene expression for that area. The fold-change in gene expression between different experimental conditions (male:female, male:castrate male, and female:castrate female) was log transformed and we performed the one sample t test to determine statistical significance.

**SUPPLEMENTAL REFERENCES**


Figure S1. Sexually Dimorphic Gene Expression in Specific Regions of the Hypothalamus, BNST, and MeA. Related to Figure 1

Representative coronal sections labeled for mRNA or protein expression are shown. (A and B) More Gabrg1 expression in the POA of the male than the female. (C–F) More Glr3a3 expression in the POA and BNSTmpm of the male than the female. (G–J) More Gpr165 expression in the POA and BNSTmpl of the male than the female. (K–R) More Gpib1 expression in the POA, BNSTmpm, MeApd, and VMHvl of the male than the female. (S–V) More Ca2t expression in the BNSTmpm and PMV of the male than the female. (W–B0) More Earl expression in the POA, BNSTmpm, and MeApd of the male than the female. (C0–F0) More Rps6ka6 in the BNSTmpm, MeApd, and MeApv (arrowheads) of the male than the female. (G0 and H0) More Chodl expression in the BNSTmpm of the male than the female. (I0 and J0) More Pak3 expression in the BNSTmpm of the male than the female. (K0–N0) More Nnat expression in the VMHvl of the female than the male. More Nnat expression in the PMV of the male than the female. (O0–R0) More Dgkk expression in the VMHvl of the female than the male. More Dgkk expression in the PMV of the male than the female. (S0–L0) More Earl a expression in the POA, BNSTmpm, MeApd, VMHvl, and PeriV of the female than the male.
n ≥ 3; scale bars represent 75 μm.
Figure S2. Summary of Sex Differences in Gene Expression, Related to Figure 4

(A) RT-qPCR quantitation of sex differences in gene expression normalized to the neuronal marker Synapsin I. The majority of sex differences we have identified represent sexual dimorphisms in mRNA expression per cell. 1, no sex difference in gene expression (dashed lines); ND, not determined. n = 3/sex; *p < 0.05, **p < 0.01, ***p < 0.001.

(B) Genes (individual rows) with sex differences in gene expression in adult brain regions are represented as orange cells. Also shown is the nondimorphic expression of genes within these regions (gray cells). Our screen selected for genes enriched in the hypothalamus compared to the rest of the brain and this appeared to be the case for all transcripts, except Pak3 which was expressed broadly in most brain regions, albeit at lower levels compared to its expression in the hypothalamus. Expression in other brain regions is not shown in this figure, but can be accessed from the AIBS website (http://www.brain-map.org/) (Ng et al., 2009). Note that many dimorphically transcribed genes were not expressed (white cells) in nuclei with overt dimorphism in the expression of other genes.
Figure S3. Control of Sexually Dimorphic Gene Expression by Testosterone in Males, Related to Figure 4
Adult castrate males were supplemented with vehicle or testosterone for two weeks. Their brains were micro-dissected to obtain tissue from the POA, BNST, VMHvl, PMV, and MeA, and processed for RT-qPCR for genes whose expression changed upon castration in males (Figure 4). Testosterone supplementation restores expression of the vast majority of genes to levels that resemble those seen in intact males. Fold change indicated by dashed lines represents no difference in gene expression between castrates treated with vehicle or testosterone. Cv, Castrate receiving vehicle (Circulating testosterone below level of detection); Ct, Castrate receiving testosterone (circulating testosterone = 14.7 ± 3.7 nM). n ≥ 3 for each cohort. *p < 0.04, **p < 0.01, ***p < 0.001.
Figure S4. Cckar, Irs4, and Sytl4 Are Not Required for Various Components of Male-Typical Behaviors, Related to Figure 5
(A–D and I–N) No difference between Null (Cckar\(--\)) and Control (Cckar\(+\+) or Cckar\(--\)) males in various parameters of mating (A–D) and territorial aggression and urine marking (I–N). n ≥ 8/cohort.
(E–H and O–T) No difference between Null (Irs4\(--\)) and Control (Irs4\(+\)) mice in various parameters of mating (E–H) and territorial aggression and urine marking (O–T). n ≥ 10/genotype.
(U–Z) No difference between Null (Sytl4\(--\)) and Control (Sytl4\(+\)) mice in territorial aggression and urine marking. n ≥ 10/genotype for aggression; n ≥ 7/genotype for urine marking.

Perimeter, perimeter of cage floor; center, rest of cage floor.
Figure S5. Brs3, Cckar, Irs4, and Sytl4 Are Not Required for Various Components of Female-Typical Behaviors, Related to Figure 6 and Figure 7

(A–G) No difference between Null (Brs3−/−) and Control (Brs3+/+) females in various parameters of pup retrieval, maternal aggression, and sexual receptivity. 
n ≥ 3/sex.

(H–N) No difference between Null (Sytl4−/−) and Control (Sytl4+/+) females in various parameters of pup retrieval, maternal aggression, and sexual receptivity. 
n ≥ 5/sex.

(O) No difference between Null (Irs4−/−) and Control (Irs4+/+) females in sexual receptivity. 
n ≥ 7.

(P–U) No difference between Null (Cckar−/−) and Control (Cckar+/+ or Cckar+/−) females in various parameters of pup retrieval and maternal aggression. 
n ≥ 5/cohort.
Figure S6. Sexual Differentiation of Brain Regions Expressing Brs3, Cckar, Irs4, or Sytl4 Appears Unaffected in Mice Mutant for These Genes, Related to Figure 5, Figure 6, and Figure 7

We assessed sexual differentiation of the POA, BNSTmpm, VMHvl, and MeApd with in situ hybridization for ERα, which is normally downregulated in males compared to females in these brain regions. We assessed sexual differentiation of the PMV with in situ hybridization for Dgkk, which is normally upregulated in males compared to females in this region. Representative coronal sections labeled for mRNA expression are shown.

(A and B) No difference between Sytl4 mutant and WT males in ERα expression in the BNSTmpm.

(C–F) No difference between Brs3 mutant and WT males in ERα expression in the BNSTmpm and MeApd.

(G–J) No difference between Irs4 mutant and control females in ERα and Dgkk expression in the VMHvl and PMV, respectively.

(K–R) No difference between Cckar mutant and WT females in ERα expression in the POA, BNSTmpm, MeApd, and VMHvl.

n ≥ 2/genotype. Scale bars represent 100 μm.