Published in final edited form as:
Cell. 2012 February 3; 148(3): 596–607. doi:10.1016/j.cell.2011.12.018.

Modular genetic control of sexually dimorphic behaviors

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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. 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

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directly regulate gene expression by binding DNA (Mangelsdorf et al., 1995), and they can initiate non-transcriptional 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 non-transcriptional 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 non-transcriptional hormone signaling. These 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).

We screened all 84 genes for sexually dimorphic expression by in situ hybridization (ISH) through the adult forebrain (Figure 1A–C“’). 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, Sytl4), a transcription factor (ERα), 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 ($p < 1\times10^{-4}$). 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 (Figures 2–4, 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 (Figures 2–4, 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 (preoptic area), VMHvl (ventrolateral area of the ventromedial nucleus), PMV (ventral premamillary nucleus), and periV (rostral periventricular region) in the hypothalamus (Figures 2–4, S1).

Of these 16 genes, 10 are male-upregulated, 2 are female-upregulated, and 4 exhibit a compound dimorphism such that each is upregulated in the female VMHvl and in ≥1 male brain region (Figures 2–4, S1). All 16 transcripts were also expressed in a non-dimorphic 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 (Figure 1B–C“’; 2A, B). 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; Shughrue et al., 1992; Koch and Ehret, 1989). Our results show unequivocal sex differences in ERα expression (Figures 4A, S1S’–L“, 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 (Figures 2, 4A, 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 (Figures 2, 3, 4B). Brs3 and ERα, which are normally female-upregulated, were upregulated in male castrates (Figures 2, 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 *Cckar* and *Greb1* without affecting other genes (Figures 3, 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

ERα is dimorphically expressed (Figures 4A, S1, 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 *Brs3, Cckar, Irs4,* and *Sytl4* 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 ERα 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). *Cckar* and *Irs4* mutant males were similar to WT siblings in these male-typical displays (Figure S4A–T), whereas *Brs3* (Table S2) and *Sytl4* mutants exhibited behavioral deficits.

Sytl4$^{-/Y}$ mice showed specific changes in some but not all mating parameters (Fig. 5B–G). 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 (Figure 5D, H). WT males who ejaculate show a reduced latency to intromit and intromit faster after the first sniff (Figure 5I, J). These differences are significant and an indicator of subsequent ejaculation. Although *Sytl4*$^{-/Y}$ and WT males ejaculated equivalently, loss of Sytl4 function decorrelated mating pattern from ejaculation (Figure 5D, I, J). *Sytl4*$^{-/Y}$ mice mated in a manner similar to ejaculatory WT males regardless of ejaculation.

Sytl4 mutants do not have pervasive deficits. They attack males and mark urine like WT males (Figure S4U–Z). We also found no deficits in movement, general activity, and social interactions such as grooming (data not shown). *Sytl4*$^{-/Y}$ mice emit USV to females but not males, indicating that they discriminate between the sexes (Figure 5C). Although Sytl4 regulates insulin release in vitro, Sytl4 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 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 peri-ovulatory 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 (Figure 6A, B), but $Irs4^{-/-}$ mothers took longer to retrieve all pups (Figure 6C). Irs4 mutants were also impaired in maternal aggression such that fewer $Irs4^{-/-}$ mothers attacked intruders (Figure 6D, Movies S1, S2). However, when Irs4 mutants attacked, they did so in a WT fashion (Figure 6E–I). 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 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). 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, Movies S3, 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 (Figures 3, 4C). Estrogen, which elicits receptivity via ERα in the VMHvl (Musatov et al., 2006), induces Cckar in the VMHvl (Figure 7H, I). By contrast, estrogen, which does not elicit receptivity in males, did not induce Cckar in the male VMHvl (Figure 7J, K). 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 (Figure 7E, G). In contrast to our findings with Cckar mutants, WT males intromitted and ejaculated normally with antagonist-treated females (Figure 7A, D, F). 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 (Figure S5P–U). 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.alleninstitute.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 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-typical repertoire of behaviors is retained and other dimorphic interactions are unaffected. Our findings suggest that it may be possible to deconstruct all sex-typical 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 non-hormonal 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 (Kellendonk 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 (Juntti 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 Lorglumide (500 µg; Sigma) resuspended in DMSO and saline, respectively. In preliminary studies we failed to observe any effect of Lorglumide 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 non-parametric Wilcoxon rank sum test.

Microarray

MEEBO arrays (Verdugo and Medrano, 2006) were used for all hybridizations and the data has been deposited in the NIH GEO database (accession #: GSE33307). 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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 (OMA); Sandler postdoctoral fellowship (XX); Genentech graduate fellowship (CFY, JKC); ARCS award (JKC); Edward Mallinckrodt, Jr. Foundation, NARSAD, and NIH (NMS; R01NS049488, DP1OD006425).

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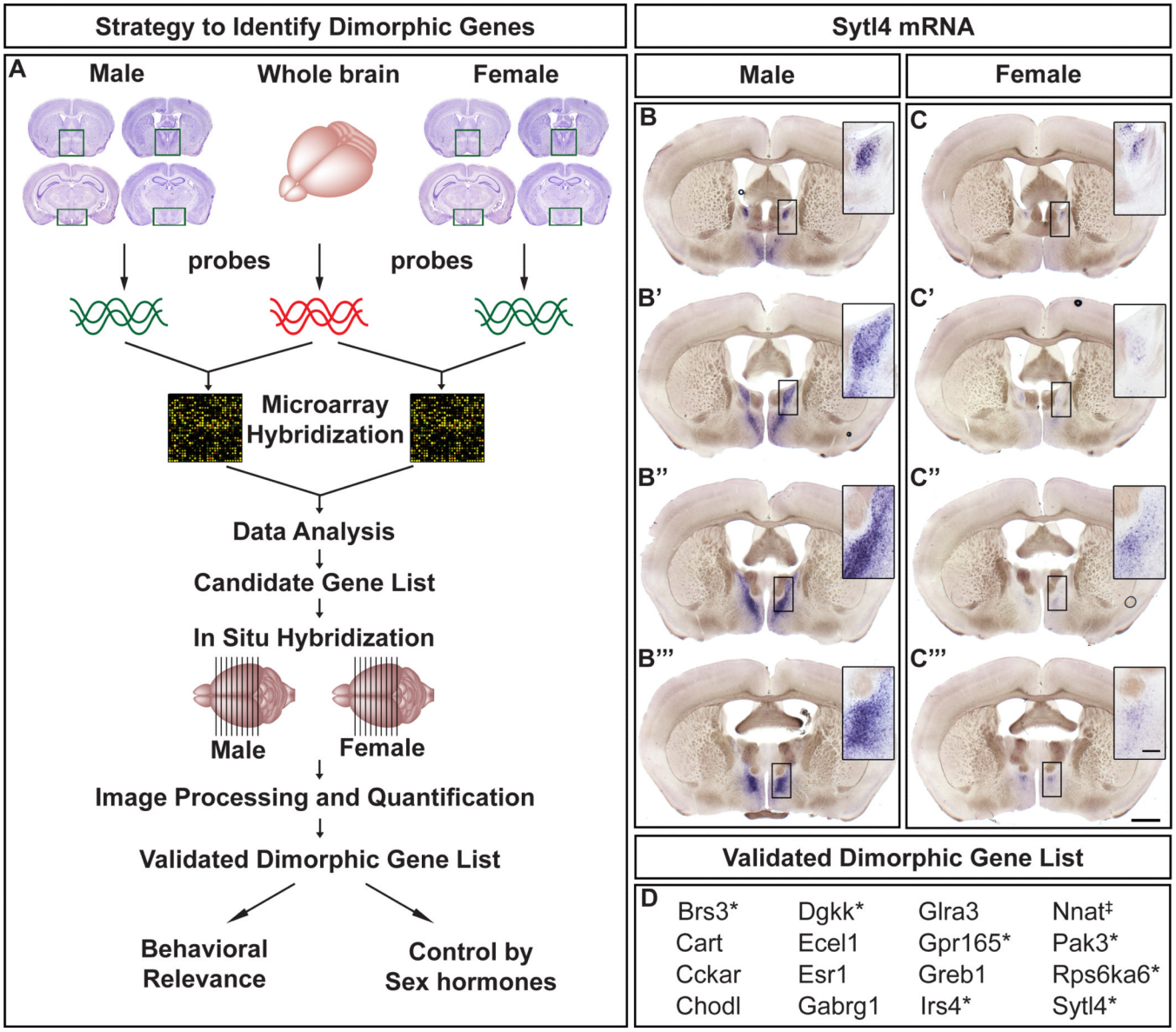

Figure 1. Identification of sexually dimorphic gene expression in the adult mouse brain

(A) Strategy to identify sex differences in gene expression. Boxed areas in Nissl stained coronal sections depict the dissected regions.

(B–C"') Expression of Sytl4 mRNA in serial coronal sections through the forebrain, with more rostral sections on top. Upregulated Sytl4 mRNA in the male BNST (insets).

(D) List of genes with sexually dimorphic expression.

*, X-linked gene; ‡, imprinted gene. Scale bar = 1 mm. Inset scale bar = 100 μm.

See also Figure S1 and Table S1.

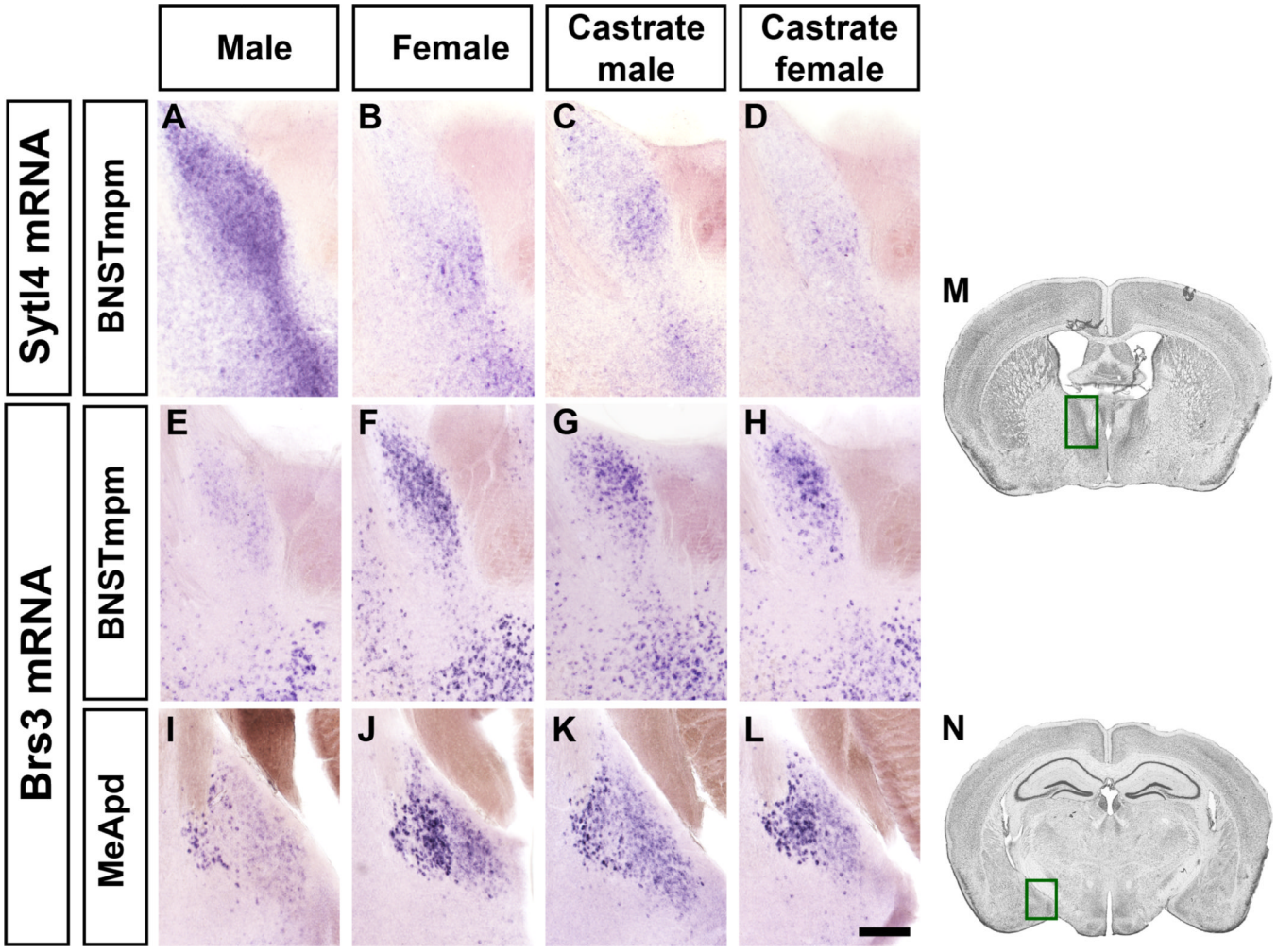

Figure 2. Sexually dimorphic expression of Sytl4 and Brs3

Sytl4 (A–D) and Brs3 (E–L) mRNA expression in coronal sections. Brains from male and female were processed in parallel whereas those from castrate male or female were processed in separate studies and are shown here (and Figure 3) for comparison purposes.

(A–D) More Sytl4 mRNA in the male BNSTmpm.

(E–L) Less Brs3 mRNA in the male BNSTmpm and MeApd.

(M, N) Boxed areas in Nissl stained sections outline the BNST (M) and MeA (N) regions shown in (A–H) and (I–L), respectively. Scale bar (A–L) = 100 μm.

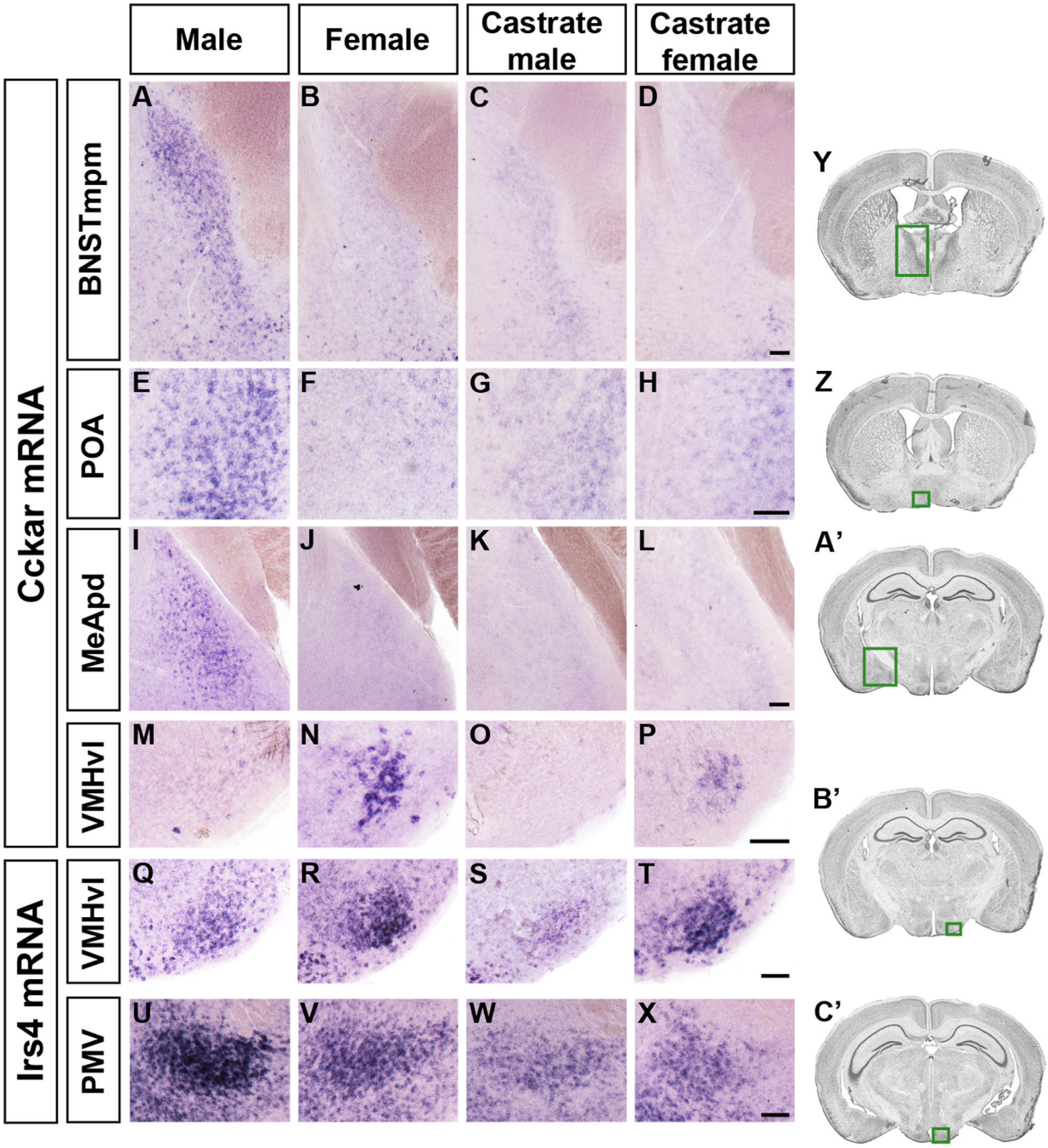

Figure 3. Sexually dimorphic expression of Cckar and Irs4

Cckar (A–P) and Irs4 (Q–X) mRNA expression in coronal sections.
(A–L) More Cckar mRNA in the male BNSTmpm, POA, and MeApd.
(M–P) More Cckar mRNA in the female VMHvl.
(Q–T) More Irs4 mRNA in the VMHvl of the female and castrate female.
(U–X) More Irs4 mRNA in the male PMV.
(Y, Z, A'–C') Boxed areas in Nissl stained sections outline the BNST (Y), POA (Z), MeA (A'), VMHvl (B'), and PMV (C'), respectively. Scale bars = 50 μm.

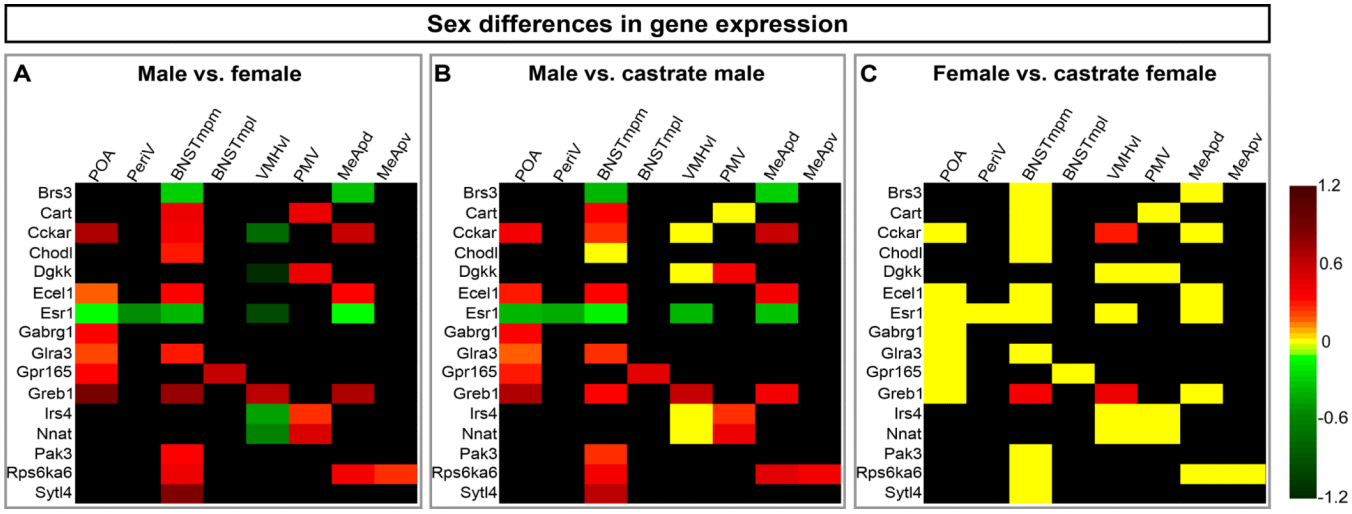

Figure 4. Sexual dimorphism in gene expression and its control by adult sex hormones

Heat map of $\log_{10}$-transformed fold differences in mRNA expression.

(A) Individual genes are upregulated in ≥1 brain region in one sex or in distinct regions in both sexes. A brain region can show upregulated expression of distinct genes in both sexes. Red = male-upregulated, green = female-upregulated.

(B) Most male-upregulated genes are downregulated after castration. Red = male-upregulated, yellow = no change, green = castrate male-upregulated.

(C) Most genes show similar expression in intact and castrate females. Red = female-upregulated, yellow = no change, green = castrate female-upregulated.

Heat map scale spans from red to green. Black = not sexually dimorphic or not expressed. $p < 0.05$ for all changes shown in green or red; $p > 0.05$ for yellow cells.

See also Figures S2, S3.

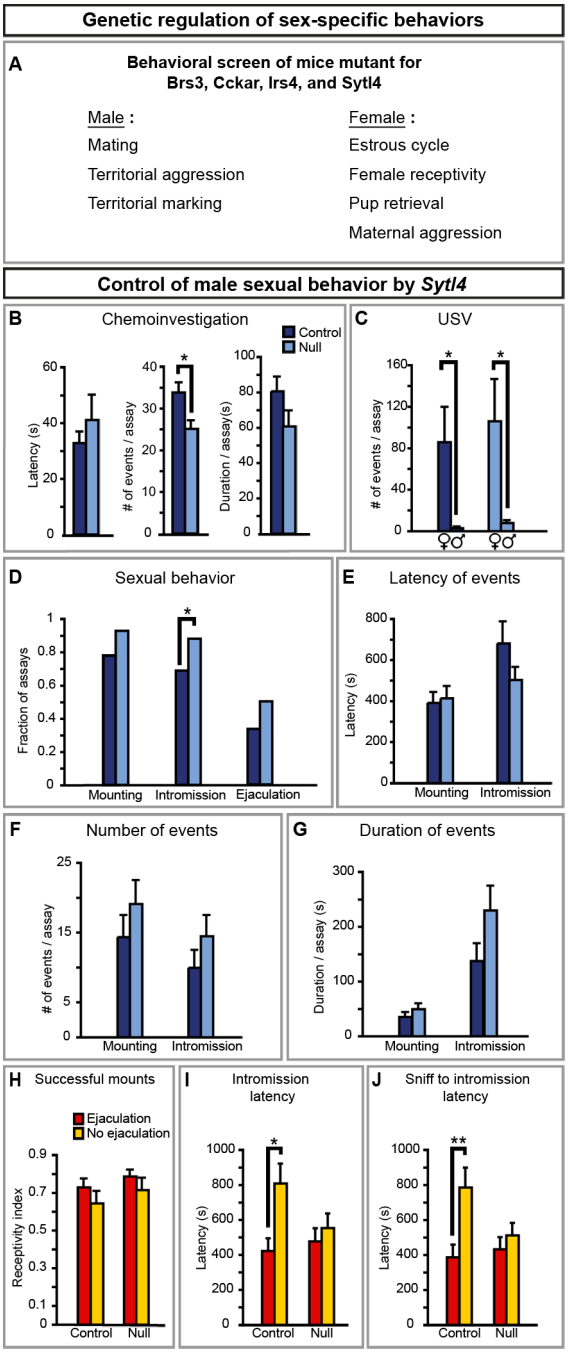

Figure 5. Sytl4 is required for patterning male sexual behavior

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

(B) *Sytl4*$^{-/Y}$ residents (Null) sniff WT female intruders less than *Sytl4*$^{+/Y}$ residents (Control).

(C) All residents vocalize more to WT female than to WT male intruders.

(D) *Sytl4*$^{-/Y}$ residents intromit WT female intruders in more tests.

(E–G) The latency, number, and duration of mounts and intromissions are unaffected in Sytl4 mutants.

(H) No difference in fraction of mounts that proceed to intromission between males that ejaculate and males that do not. Receptivity index = (# of mounts with intromission)/(# of all mounts).
(I, J) Control males that ejaculate show a shorter latency to intromit and proceed faster from the first sniff to the first intromission compared to controls who do not ejaculate. These behavioral parameters are decorrelated with ejaculation in null males.
Mean ± SEM; n ≥ 14 animals/genotype; * $p < 0.04$; ** $p < 0.01$.
See also Figure S4 and Tables S2, S3.

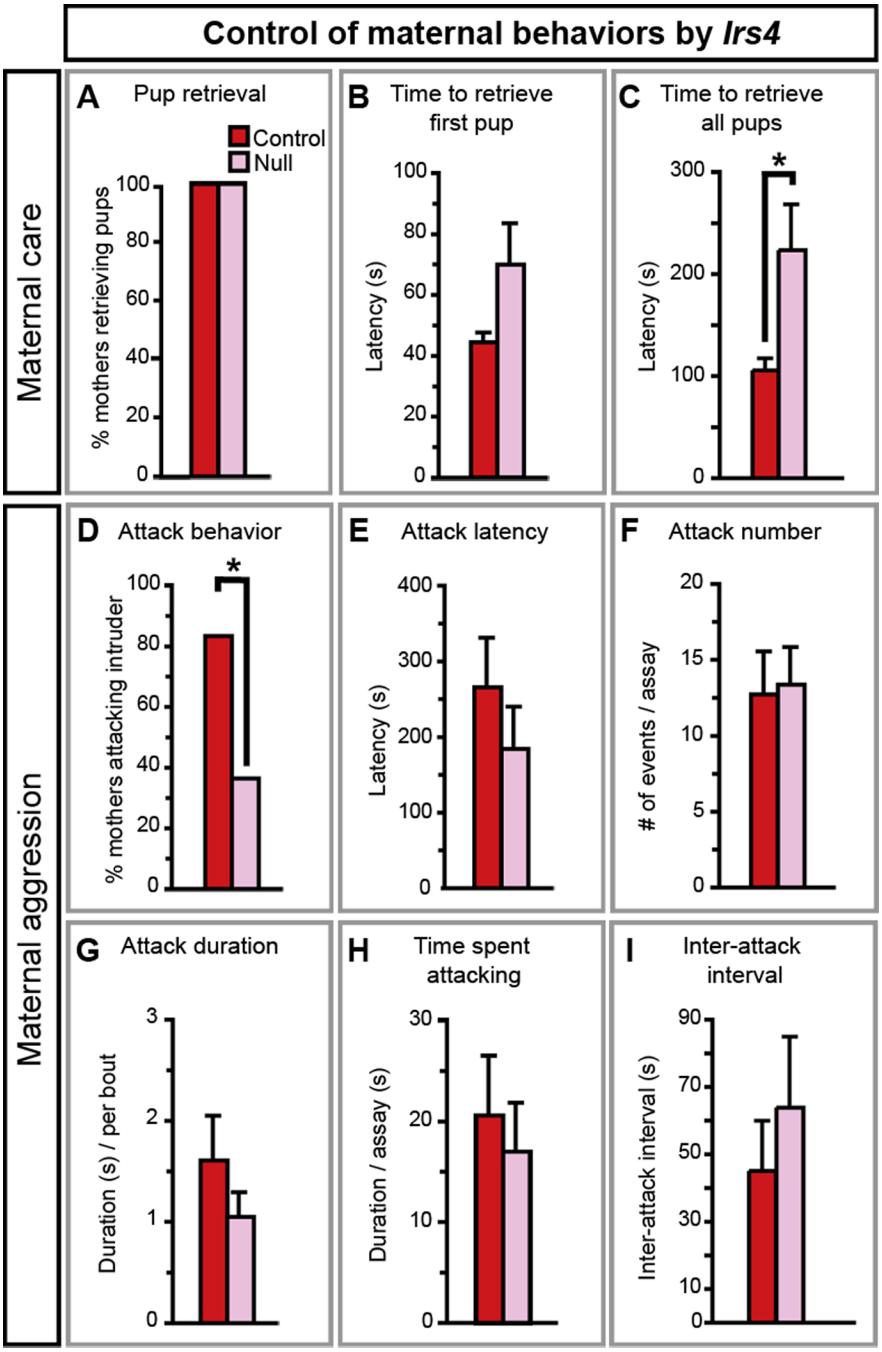

Figure 6. Irs4 is essential for maternal behaviors

(A, B) *Irs4*$^{-/-}$ (Null) and *Irs4*$^{-/+}$ (Control) mothers retrieve pups (A), and the latency to retrieve the first pup is similar between the two groups (B).

(C) Irs4 null mothers take longer to retrieve all pups.

(D) Fewer Irs4 null mothers attack intruder males.

(E–I) When Irs4 null mothers attack intruders they do so similar to control mothers.

Mean ± SEM; n ≥11/genotype; * $p < 0.04$.

See also Figure S5, Table S4, and Movies S1, S2.

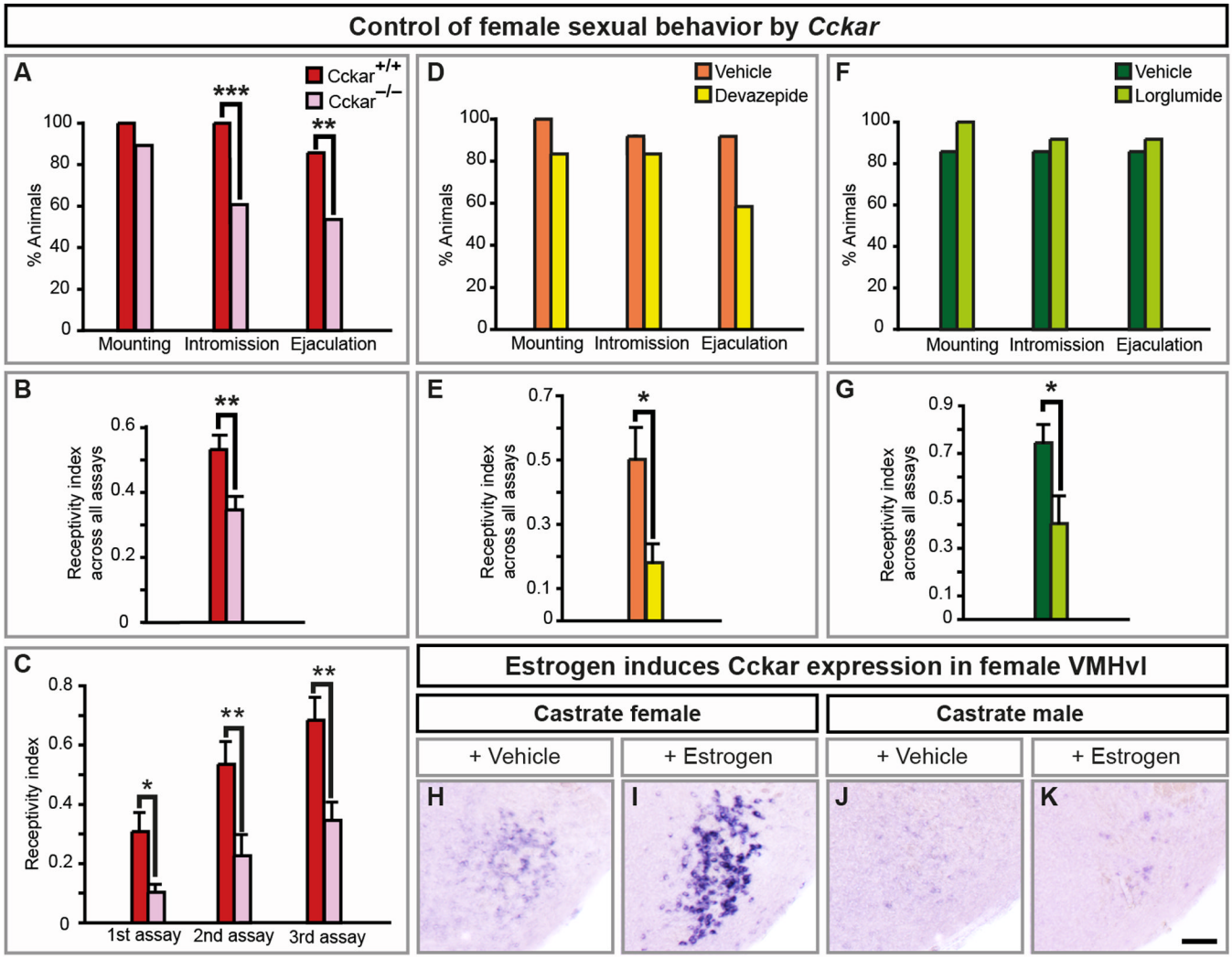

Figure 7. Cckar is essential for female sexual behavior

(A) WT males mount Cckar null or WT females equivalently, but fewer mutant females allow males to intromit or ejaculate.

(B) Lower receptivity index in *Cckar*$^{-/-}$ females.

(C) Lower receptivity index in sexually experienced *Cckar*$^{-/-}$ females.

(D, F) WT males mount, intromit or ejaculate equivalently with females treated with vehicle or Cckar antagonists.

(E, G) Both Cckar antagonists reduce sexual receptivity of females.

(H–K) Estrogen increases Cckar mRNA in the VMHvl of castrate females.

Mean ± SEM; n ≥ 21 (A–C); n ≥ 12/treatment (D–G); n ≥ 3/treatment (H–K); * $p < 0.05$, ** $p < 0.01$, *** $p \leq 0.001$. Scale bar = 100 μm.

See also Figures S5, S6, and Movies S3, S4.