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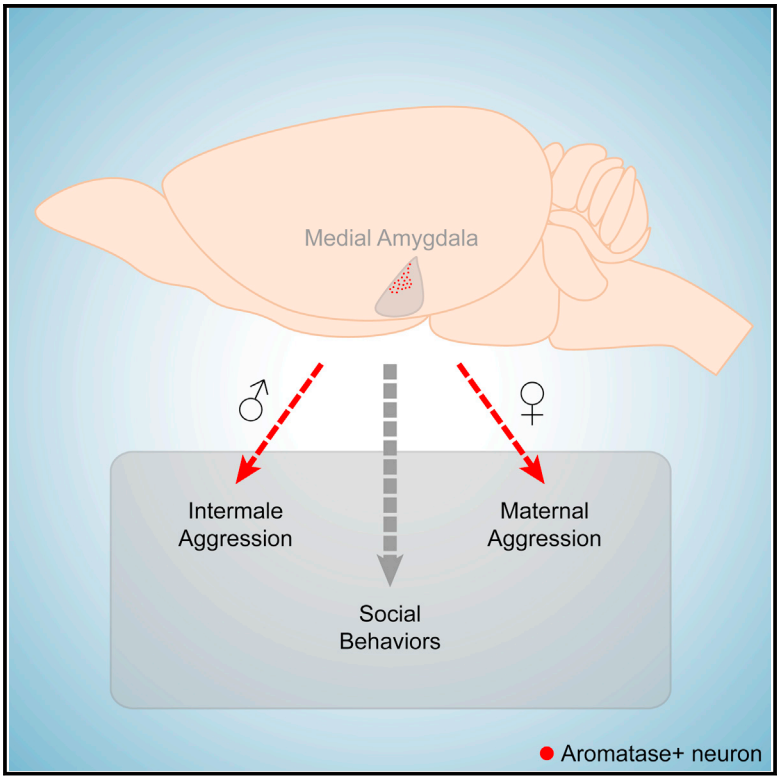
Medial Amygdalar Aromatase Neurons Regulate Aggression in Both Sexes

Graphical Abstract



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In Brief

Aromatase controls sexually dimorphic social behaviors in both sexes. Unger et al. show that the small population of aromatase-expressing neurons in the medial amygdala is required for intermale and maternal aggression, but not other sexually dimorphic behaviors. Thus, aromatase-expressing medial amygdalar neurons control complex social behaviors in a modular manner.

Highlights

- MeApd aromatase neurons regulate male aggression, but not marking, singing, or mating
- MeApd aromatase neurons regulate maternal aggression, but not female sexual behavior
- MeApd aromatase neurons regulate specific components of aggression in both sexes
- MeApd aromatase neurons control aggression in a modular manner

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Medial Amygdalar Aromatase Neurons Regulate Aggression in Both Sexes

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SUMMARY

Aromatase-expressing neuroendocrine neurons in the vertebrate male brain synthesize estradiol from circulating testosterone. This locally produced estradiol controls neural circuits underlying courtship vocalization, mating, aggression, and territory marking in male mice. How aromatase-expressing neuronal populations control these diverse estrogen-dependent male behaviors is poorly understood, and the function, if any, of aromatase-expressing neurons in females is unclear. Using targeted genetic approaches, we show that aromatase-expressing neurons within the male posterodorsal medial amygdala (MeApd) regulate components of aggression, but not other estrogen-dependent male-typical behaviors. Remarkably, aromatase-expressing MeApd neurons in females are specifically required for components of maternal aggression, which we show is distinct from intermale aggression in pattern and execution. Thus, aromatase-expressing MeApd neurons control distinct forms of aggression in the two sexes. Moreover, our findings indicate that complex social behaviors are separable in a modular manner at the level of genetically identified neuronal populations.

INTRODUCTION

Sexually reproducing species exhibit sex differences in social interactions such as courtship and aggression that are critical for reproductive success. Accordingly, such behaviors are developmentally programmed and can be elicited in naive animals without prior training. Within a species, each of these behaviors consists of many stereotyped components, thereby enabling sensitive detection of alterations in behavioral displays subsequent to functional manipulations. Although many brain regions are implicated in the control of sexually dimorphic behaviors in mammals, how these complex behaviors are encoded by such brain regions is poorly understood (reviewed in Yang and Shah, 2014).

Sex hormones essentially act as master regulators of the entire repertoire of sex-typical social interactions in most vertebrates (Arnold, 2009; Morris et al., 2004; Yang and Shah, 2014). Sex hormone signaling pathways therefore offer a functional entry point into neural circuits underlying these behaviors. As has been known for several decades (Ball, 1937), estrogen signaling controls sex-typical behaviors in both sexes in rodents and many other vertebrates (reviewed in McCarthy, 2008; Yang and Shah, 2014). In particular, it is critical for male-typical ultrasonic courtship vocalizations, sexual displays, aggression, and territory marking (Finney and Erpino, 1976; Kimura and Hagiwara, 1985; Matsumoto et al., 2003; Nunez et al., 1978; Ogawa et al., 2000; Wallis and Luttge, 1975; Wersinger et al., 1997). A role of estradiol in controlling male behaviors seems counterintuitive because it is essentially undetectable in the male mouse circulation. Estrogenic steroids in vivo are derived from testosterone or related androgens in a reaction catalyzed by aromatase, and aromatase+ cells in the male brain convert circulating androgens into estrogenic derivatives (Naftolin et al., 1971). It is this locally synthesized estradiol that is thought to control male-typical behaviors (Figure 1A) (MacLusky and Naftolin, 1981).

Aromatase+ cells represent <0.05% of neurons in the adult mouse brain, and they are sparsely distributed within a few brain regions thought to be important for sexually dimorphic behaviors (Wu et al., 2009). Given the role of estradiol in diverse male-typical behaviors, aromatase+ neuronal populations exert a profound effect on such behaviors. However, the behavioral function of individual aromatase+ neuronal pools is unclear. In one scenario, each aromatase+ population globally controls social interactions in males. Alternatively, individual aromatase+ populations regulate one or a subset of male-typical behaviors. Although there is little circulating testosterone in female mice and the female brain is exposed to circulating estradiol secreted by ovaries, there is aromatase expression in the female rodent brain, albeit at lower levels than in males (Roselli, 1991; Roselli et al., 1985; Wu et al.,

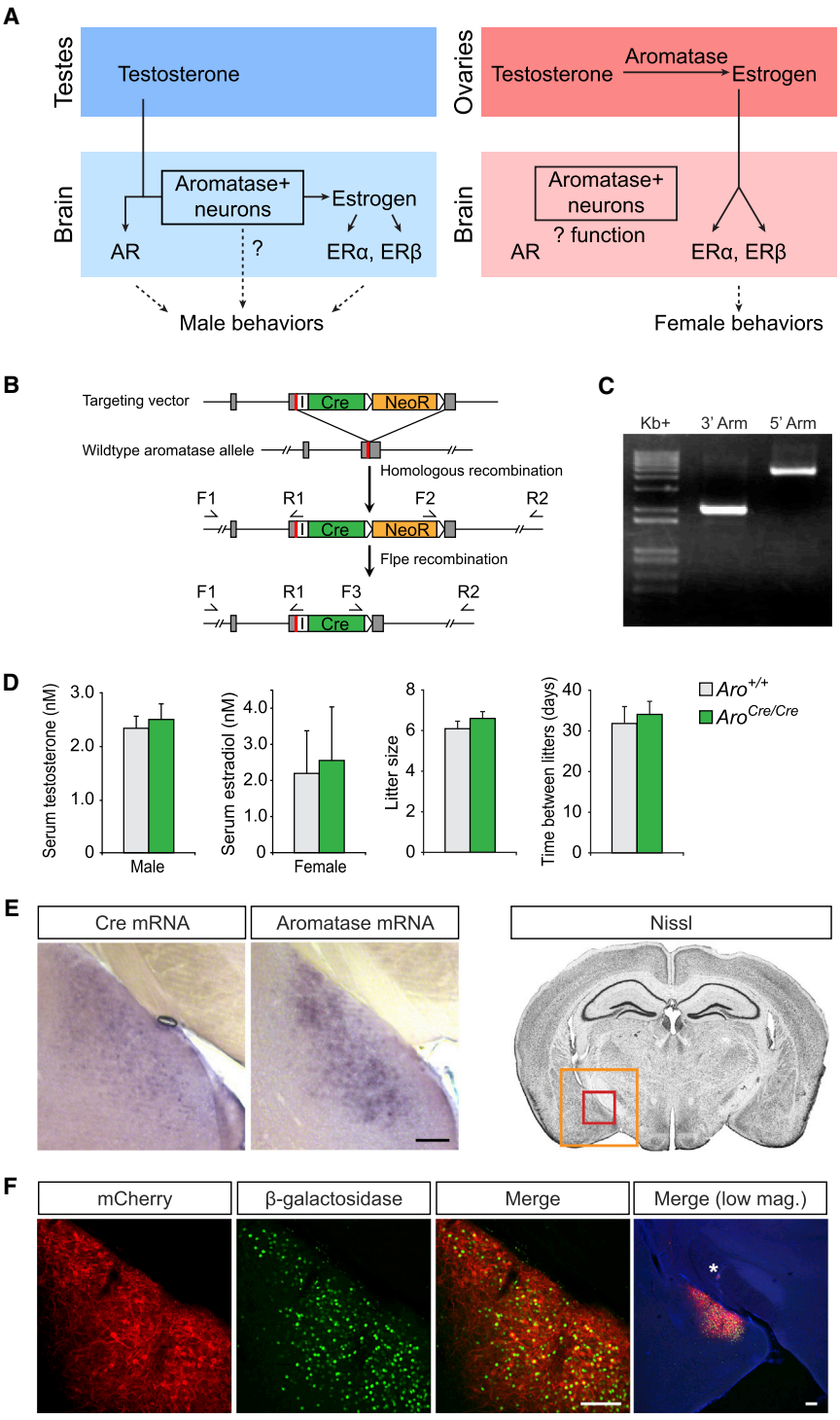

Figure 1. Generation and Characterization of Mice Expressing Cre Recombinase in Aromatase+ Cells

(A) Role of aromatase in sexually dimorphic behaviors. Aromatase+ neurons in the male brain produce estradiol that controls many male-typical behaviors, but the function of individual aromatase+ neuronal populations in these behaviors is unknown. Given that the female brain is exposed to circulating estradiol, the function, if any, of aromatase+ neurons is unclear in females.

(B) Generating the aro^{Cre} allele. Gray boxes represent last two exons of *aromatase*, and the red line in 3′ exon denotes stop codon. Primers (F1–F3 and R1 and R2) used for PCR-based genotyping are shown. Schematic not drawn to scale.

(C) PCR verification of homologous recombination at the *aromatase* locus from tail DNA of an $aro^{Cre/Cre}$ mouse.

(D) No difference in serum hormone titers, litter size, or time between litters between WT and $aro^{Cre/Cre}$ mice (mean ± SEM; n = 10 WT and 6 $aro^{Cre/Cre}$ of each sex).

(E) Cre mRNA expression mirrors that of aromatase mRNA in an adjacent section through the MeA. Boxed area in red in Nissl-stained coronal section highlights the MeApd (Paxinos and Franklin, 2003).

(F) Injection of AAV encoding a Cre-dependent mCherry into the MeA activates mCherry expression in aromatase+ neurons expressing β-galactosidase in $aro^{Cre/IPIN}$ mice. The low-magnification (mag.) merge panel is counterstained with DAPI (blue) to show that aromatase expression (and Cre-dependent reporter) is restricted to the MeApd and not to be found in neighboring regions; asterisk in the low-magnification panel denotes needle track. Boxed areas in red and orange in Nissl-stained coronal section (E) highlight the areas shown at high and low magnification, respectively.

Scale bars represent 200 μm.

2009). The function, if any, of aromatase+ neurons in female mice is unknown (Figure 1A).

We utilized genetic strategies to test the function of aromatase+ posterodorsal medial amygdala (MeApd) neurons in sexually dimorphic behaviors. Aromatase+ neurons comprise ~40% of neurons within the MeApd, a region important for reproductive behaviors and responsive to pheromone sources such as urine (Baum and Bakker, 2013; Bergan et al., 2014; Choi et al., 2005; DiBenedictis et al., 2012; Sokolowski and Corbin, 2012; Swanson, 2000; Wu et al., 2009). We find that aromatase+ MeApd neurons in males regulate the display of specific features of aggression, but not mating, courtship vocalization, or territory marking. In females, these neurons regulate specific features of maternal aggression, but not other aspects of maternal care or fertility and sexual behavior. Taken together, our findings reveal a role for aromatase+ MeApd neurons in aggression in both sexes, and furthermore, they demonstrate a surprising modularity in the neural control of sexually dimorphic behaviors.

RESULTS

A Genetic Strategy to Target Aromatase+ Neurons in Adult Mice

The medial amygdala (MeA) is a large structure that extends >1 mm rostrocaudally (Paxinos and Franklin, 2003) and influences diverse behaviors in rodents, including sexually dimorphic behaviors (Petrovich et al., 2001; Swanson, 2000; Swanson and Petrovich, 1998). In keeping with its size and functional diversity, the mouse MeA is molecularly heterogeneous (Carney et al., 2010; Choi et al., 2005; Keshavarzi et al., 2014; Xu et al., 2012; Yang et al., 2013). These considerations make it difficult to selectively target the small pool of aromatase+ MeApd neurons (Wu et al., 2009). In order to target these neurons, we knocked in an *IRES-Cre* transgene into the 3′ UTR of the *aromatase* locus (Figures 1B and 1C). As described before, this strategy does not disrupt function or expression of the modified locus (Yang et al., 2013), and it permits expression of Cre recombinase in aromatase+ cells. Indeed, in contrast to *aromatase*$^{-/-}$ mice (Matsumoto et al., 2003), mice bearing the *aromatase*^{Cre} (*aro*^{Cre}) allele are fertile and have wild-type (WT) levels of circulating sex hormones (Figure 1D). In situ hybridization for Cre shows this enzyme to be expressed in a pattern mirroring that of aromatase (Figure 1E). To validate functional Cre expression in aromatase+ MeApd neurons, we injected an adeno-associated virus (AAV) harboring a Cre-dependent reporter in mice doubly heterozygous for *aro*^{Cre} and the previously described knockin *aromatase*^{IPIN} (*aro*^{IPIN}) allele that drives nuclear β-galactosidase in aromatase+ cells (Wu et al., 2009). In these animals, we observed colocalization of β-galactosidase and mCherry, confirming functional expression of Cre in *aro*^{Cre} mice (Figure 1F and see below). In addition, these studies confirmed the highly restricted nature of aromatase expression within the MeA (Figure 1F) (Wu et al., 2009).

Aromatase+ MeApd Neurons Specifically Regulate Male Aggression

We tested the requirement of aromatase+ MeApd neurons in sexually dimorphic behaviors. We first ablated these neurons in adult mice via bilateral targeted delivery to the MeA of an AAV encoding a genetically modified caspase-3 whose activation requires Cre recombinase (Figure 2A) (Yang et al., 2013). Activation of this caspase-3 triggers apoptosis exclusively in Cre+ cells without bystander toxicity to neighboring cells not expressing Cre (Morgan et al., 2014; Nelson et al., 2014; Yang et al., 2013). Only those *aro*^{Cre} males with a substantive loss (>50% loss bilaterally) of aromatase+ MeApd neurons were included for analysis of behavioral performance (Figures 2B–2D and S1A–S1F). AAV-injected *aro*$^{Cre/IPIN}$ (experimental) and *aro*$^{+/IPIN}$ (control) males were allowed to recover for 4 weeks to ensure maximal cell loss, singly housed thereafter, and tested for behavioral performance (Figure 2A). Male mice exhibit a stereotyped routine of mating displays toward females, consisting of bouts of anogenital sniffing, mounting, and intromission (penetration) that can culminate in ejaculation (McGill, 1962). We observed no difference in these mating components between *aro*$^{Cre/IPIN}$ and control males when they were presented with a sexually receptive intruder female (Figure 2B). Male mice vocalize to females, and there was no difference between *aro*$^{Cre/IPIN}$ and control males in such courtship vocalization (Figure S1A). *Aro*$^{Cre/IPIN}$ males also directed their courtship vocalizations preferentially to females (Figure S1A), indicative of unaltered sex discrimination (Stowers et al., 2002). Taken together, our findings show that despite the importance of the MeApd in reproductive behavior (Baum and Bakker, 2013; Bergan et al., 2014; Choi et al., 2005; DiBenedictis et al., 2012; Sokolowski and Corbin, 2012; Swanson, 2000), most aromatase+ MeApd neurons are not essential for WT levels of male sexual behavior and courtship vocalizations in mice.

We next tested these resident males for aggression toward an unfamiliar adult WT intruder male. Both *aro*$^{Cre/IPIN}$ and *aro*$^{+/IPIN}$ residents chemoinvestigated, groomed, and attacked intruders, but *aro*$^{Cre/IPIN}$ males took significantly longer time to initiate aggression (Figures 2C and S1A). There was a corresponding decrease in the number of attacks directed toward the intruder by *aro*$^{Cre/IPIN}$ males (Figure 2C). The deficits in attacks correlated strongly with the extent of loss of aromatase+ MeApd neurons (Figure S1G). Resident males often tail rattle as a threat to intruder males, and we observed a significant increase in the latency to tail rattle in *aro*$^{Cre/IPIN}$ males (Figure 2C). In addition to attacking intruder males, male mice also mark their home territory with numerous urine spots. Despite the reduced aggression toward intruders, *aro*$^{Cre/IPIN}$ males marked their territory similar to controls (Figure S1C). We tested *aro*$^{Cre/IPIN}$ males to determine if they exhibited pervasive deficits that could contribute to the deficits in aggression. However, these males did not exhibit deficits in finding hidden food, anxiety-type behavior on an elevated plus maze, and locomotor activity, and they maintained body weight and circulating testosterone (Figures S1D–S1F). The anatomically restricted nature of aromatase expression (Wu et al., 2009) allows specific ablation of aromatase+ MeApd neurons (Figure 2D). It also ensures that other non-MeApd aromatase+ populations such as those in the bed nucleus of the stria terminalis (BNST) are distant from the injection site and are not infected by the stereotactically delivered virus. Indeed, the number of aromatase+ BNST neurons was unchanged (*aro*$^{Cre/IPIN}$, 660 ± 198; *aro*$^{+/IPIN}$, 565 ± 132; $n = 5$, $p > 0.1$) following viral delivery to the MeA. Ablation of progesterone receptor-expressing neurons in the ventromedial hypothalamus reduces all aspects of aggression, including the pattern of attacks as measured by inter-attack interval and mean duration of an attack bout (Yang et al., 2013). Although we cannot exclude a subtle role of aromatase+ MeApd neurons in all components of aggression, our findings reveal that ablation of a majority of these neurons reduces aggression without altering the pattern of attack (Figure S1B). It is possible that aromatase+ MeApd neurons relay pheromonal information relating to aggression rather than regulating different aspects of this behavior. If so, this would represent the identification of a class of molecularly specified MeA neurons that respond to pheromonal cues relating to aggression but not other social behaviors. Regardless of the underlying mechanism, our findings show that aromatase+ MeApd neurons are required for the display of WT levels of specific components of aggression.

Aromatase+ MeApd Neurons Acutely Regulate Male Aggression

We used a chemogenetic approach to acutely silence aromatase+ MeApd neurons (Sternson and Roth, 2014). Most

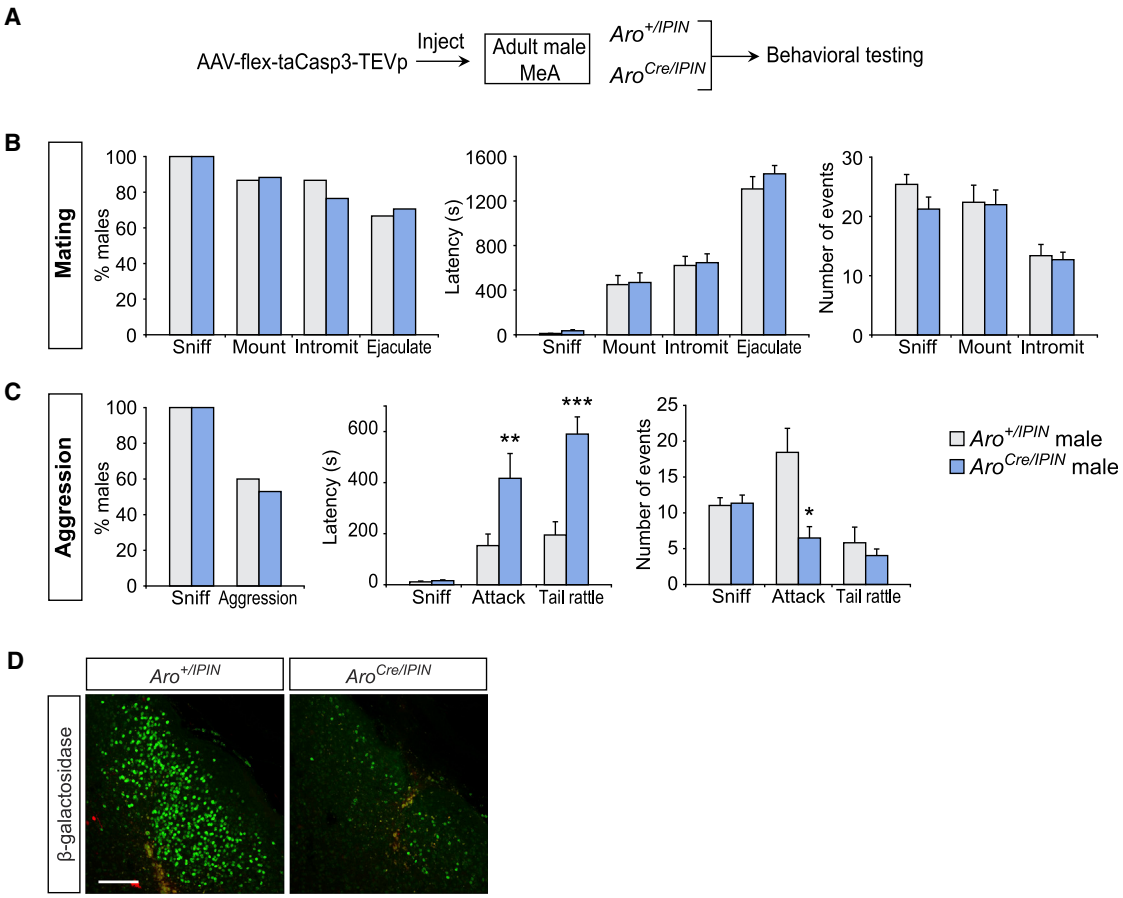

Figure 2. Ablation of Aromatase+ MeApd Neurons Reduces Specific Components of Male Aggression

(A) Experimental strategy to ablate Cre-expressing aromatase+ MeApd neurons and test for behavioral deficits. AAV encoding Cre-dependent caspase-3 was injected into the MeA of $aro^{Cre/IPIN}$ and $aro^{+/IPIN}$ males that were subsequently tested for mating and aggression.

(B) No difference between $aro^{Cre/IPIN}$ and control $aro^{+/IPIN}$ males in mating with a WT estrus female.

(C) Comparable percent of $aro^{Cre/IPIN}$ and $aro^{+/IPIN}$ males sniff and attack a WT intruder male. $Aro^{Cre/IPIN}$ males take significantly longer to attack and tail rattle, and they attack the intruder less.

(D) Histological verification of ablation of aromatase+ neurons in MeApd of $aro^{Cre/IPIN}$ males. Some autofluorescence is visible in the red channel.

Mean ± SEM; n = 14 $aro^{+/IPIN}$, n = 15 $aro^{Cre/IPIN}$; *p < 0.05, **p < 0.01, ***p < 0.005. Scale bar represents 200 μm. See also Figure S1.

aromatase+ neuroendocrine cells in the MeApd express glutamate decarboxylase 1 (GAD1), suggesting that they are GABAergic neurons (Figure S2A). Silencing these neurons allows us to test directly whether their neural activity is essential for social behaviors. Moreover, this approach permits testing whether the chronic loss of neurons following caspase-mediated ablation activates compensatory mechanisms that mask a role of these neurons in marking, mating, or courtship vocalization. We delivered AAVs encoding a Cre-dependent fusion protein consisting of a G_i-coupled DREADD (designer receptor exclusively activated by designer drug) and mCherry bilaterally to the MeA (Figure 3A). This DREADD-G_i is activated exclusively by the biologically inert small-molecule clozapine-N-oxide (CNO) such that CNO-bound DREADD-G_i hyperpolarizes neurons and silences them (Armbruster et al., 2007; Sternson and Roth, 2014). Only $aro^{Cre/IPIN}$ males where a majority of aromatase+ MeApd neurons coexpressed mCherry were included for behavioral analysis (Figures 3B–3D and S2B–S2G). We singly housed males 1 week after viral injection and began behavior testing 1 week later. They were tested in behavioral assays following CNO administration (Ray et al., 2011; Sasaki et al., 2011). There was no difference between $aro^{Cre/IPIN}$ males administered saline or CNO in assays of mating, courtship vocalization, urine marking, anxiety-type behavior, food finding, and locomotor activity (Figures 3B, S2B, S2D, and S2E); these males also maintained body weight and circulating testosterone (Figures S2F and S2G). Thus, control levels of neural activity of most aromatase+ MeApd neurons are not required for these aspects of male physiology or behaviors, including male sexual behaviors.

Comparable proportions of $aro^{Cre/IPIN}$ males administered saline or CNO sniffed and attacked a WT male intruder (Figure 3C). $Aro^{Cre/IPIN}$ males administered CNO had a significantly longer latency to initiate attacks and tail rattle toward intruder males (Figure 3C) without a change in the pattern of attack as measured by attack duration and interattack interval (Figure S2C). There was a strong correlation between the increased latency to attack in the

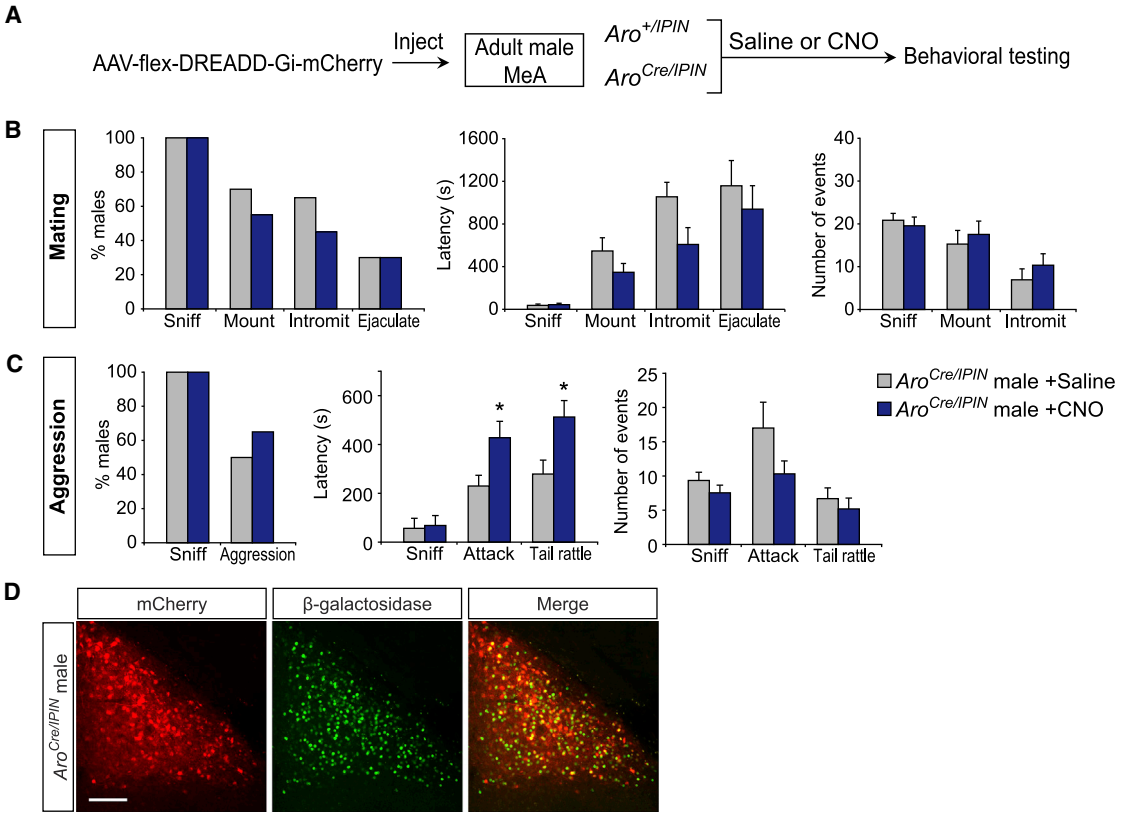

Figure 3. Inhibiting Aromatase+ MeApd Neurons with DREADD-G_i Reduces Specific Components of Male Aggression

(A) Experimental strategy to inhibit Cre-expressing aromatase+ MeApd neurons and test for behavioral deficits in males. AAV encoding Cre-dependent DREADD-G_i was injected into the MeA of $aro^{Cre/IPIN}$ males that were subsequently tested for behaviors following intraperitoneal CNO or saline administration.

(B) No difference between $aro^{Cre/IPIN}$ males administered saline or CNO in mating with a WT estrus female.

(C) No difference between percent $aro^{Cre/IPIN}$ males given saline and CNO that sniffed or attacked a WT intruder male. $Aro^{Cre/IPIN}$ males administered CNO took significantly longer to initiate attacks or tail rattle.

(D) Histological verification of DREADD-G_i (mCherry) expression in aromatase+ neurons in MeApd of $aro^{Cre/IPIN}$ males.

Mean ± SEM; n = 20 $aro^{Cre/IPIN}$; for parallel studies with $aro^{+/IPIN}$, please see Figure S2; *p < 0.05. Scale bars represent 200 μm. See also Figures S2 and S3.

presence of CNO and DREADD-G_i expression (Figure S2H). Importantly, $aro^{+/IPIN}$ control males injected with this AAV showed unaltered mating and aggressive behaviors even in the presence of CNO (Figures S2I and S2J), demonstrating that CNO itself does not modulate social behaviors. Our histological studies confirmed that DREADD-G_i expression was limited to the MeApd and did not spread to other aromatase-expressing locations such as the BNST (Figure S2K). Thus, acute silencing of aromatase+ MeApd neurons parallels the specific deficits in aggression observed with targeted ablation of these neurons. Importantly, activating these neurons with a G_q-coupled DREADD that increases neuronal activity (Sternson and Roth, 2014) did not alter sexual or aggressive displays at CNO doses previously shown by us and others to activate neurons and modulate behavior (Figure S3) (Anaclet et al., 2014; Sasaki et al., 2011). Indeed, CNO elicited a large increase in c-Fos+ neurons in the MeApd in vivo and depolarized and increased spike rate in aromatase+ MeApd neurons in acute brain slices (Figures S3A and S3B). The lack of behavioral modulation with activation of aromatase+ MeApd neurons may reflect that a larger subset of MeApd neurons needs to be activated to alter aggression. Alternatively, these neurons may relay chemosensory information to neurons that receive additional inputs required to drive mating or aggression. Indeed, fly sensory neurons that relay the presence of pheromones are required, but not sufficient, for WT levels of aggression (Wang et al., 2011). Regardless, our findings demonstrate that neural activity of a majority of aromatase+ MeApd neuroendocrine neurons is required for appropriate display of male aggression, but not other sexually dimorphic behaviors.

Aromatase+ MeApd Neurons Specifically Regulate Maternal Aggression

We tested the function of aromatase+ MeApd neurons in female-typical displays of receptivity and parental care by ablating them via targeted delivery of AAV encoding Cre-dependent caspase-3 (Figure 4A). As with males, we only analyzed females with a substantive loss (>50%) of aromatase+ MeApd neurons (Figures 4B–4D and S4A–S4F). To test for sexual behavior, female $aro^{Cre/IPIN}$ and $aro^{+/IPIN}$ mice were injected bilaterally into the

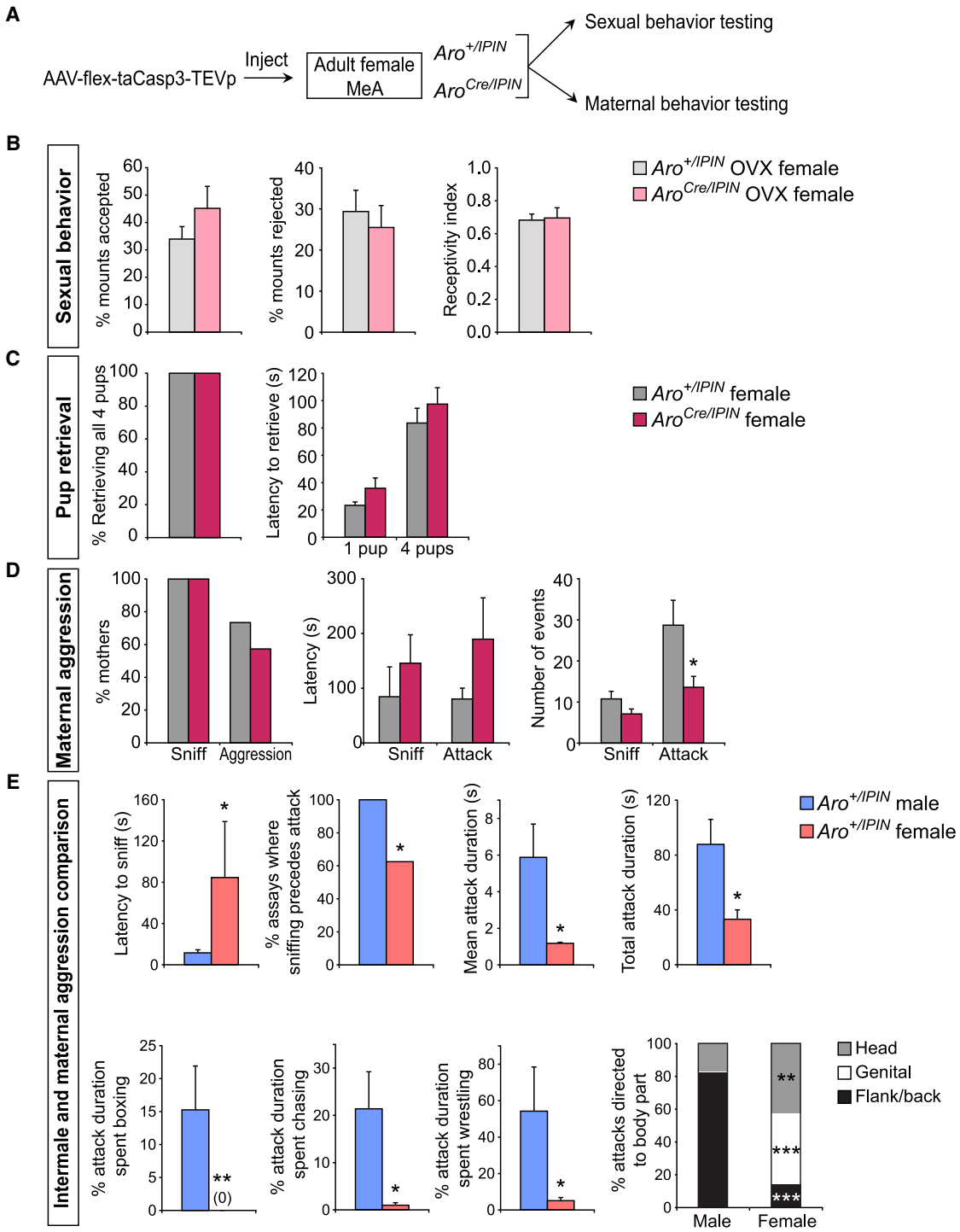

Figure 4. Ablation of Aromatase+ MeApd Neurons Reduces Specific Components of Maternal Aggression

(A) Experimental strategy to ablate Cre-expressing aromatase+ MeApd neurons and test for behavioral deficits. AAV encoding Cre-dependent caspase-3 was injected into the MeA of separate cohorts of $aro^{Cre/IPIN}$ and $aro^{+/IPIN}$ females for testing performance in mating and maternal behaviors.

(B) No difference in fraction of mounts that were accepted or rejected and no difference in receptivity index (# intromissions/# mounts) between $aro^{Cre/IPIN}$ and $aro^{+/IPIN}$ females.

(C) Vast majority of $aro^{Cre/IPIN}$ and $aro^{+/IPIN}$ females retrieved all pups to the nest, and they did so with similar latencies.

(D) Comparable percent of $aro^{Cre/IPIN}$ and $aro^{+/IPIN}$ females sniff and attack a WT intruder male. Significant decrease in number of attacks directed to intruder male by $aro^{Cre/IPIN}$ females.

(legend continued on next page)

MeA with virus, and their ovaries were removed to permit estrus induction on the day of testing. These mice were allowed to recover from surgery for 4 weeks, hormonally primed to be in estrus, and inserted into the home cage of sexually experienced WT males. We observed that sexual behavior of both groups of females was comparable, such that they stayed still rather than running away when the male approached and mounted them (Figure 4B). This receptive behavior permitted mounts to proceed to intromission (receptivity index) equivalently between these two sets of females (Figure 4B). WT males also appeared equally interested in $aro^{Cre/IPIN}$ and $aro^{+/IPIN}$ females as they investigated, mounted, and intromitted them with comparable latency and number (Figure S4A). These females did not exhibit deficits in finding food, anxiety-type behavior, and locomotor activity, and they maintained their body weight (Figures S4B and S4C). In summary, despite the importance of the MeA in female sexual behavior (DiBenedictis et al., 2012), a majority of aromatase+ MeApd neurons are not essential for this behavior.

To test whether aromatase+ MeApd neurons regulate maternal behaviors, we ablated them in a separate cohort of females that were subsequently mated with WT males to generate litters (Figure 4A). $Aro^{Cre/IPIN}$ and $aro^{+/IPIN}$ females were inseminated (as determined by the presence of a vaginal plug) and generated litters at equivalent rates (Figure S4D), indicating that ablation of aromatase+ MeApd neurons does not render females infertile. Upon parturition, $aro^{Cre/IPIN}$ as well as $aro^{+/IPIN}$ females ate their afterbirths, cleaned pups, and nursed them in their nest. Pups can crawl away from the nest, and dams efficiently retrieve them to the nest. Experimental removal of pups from their nest elicited pup retrieval with comparable efficiency between the two groups of females (Figure 4C). In addition, $aro^{Cre/IPIN}$ females did not exhibit deficits in finding hidden food, anxiety-type behavior on an elevated plus maze, and locomotor activity, and they maintained their body weight (Figures S4E and S4F). Thus, most aromatase+ MeApd neurons are not essential for these behaviors, including many components of maternal care.

Nursing mice attack unfamiliar intruder mice because such intruders can be infanticidal toward pups (Gandelman, 1972; Wu et al., 2014). We tested whether aromatase+ MeApd neurons were required for such maternal aggression by inserting a WT male into the cage of experimental and control nursing females. Both groups of females investigated and attacked intruders with equivalent probability (Figure 4D). There was a significant 2.4-fold decrease in the number of attacks by $aro^{Cre/IPIN}$ females that strongly correlated with the degree of cell loss within the MeApd (Figures 4D and S4G). Similar to males (Figure S1B), once aggression was initiated, there was no difference in the attack pattern between control and $aro^{Cre/IPIN}$ females (Figure S4D). Thus, ablation of aromatase+ MeApd neurons leads to a specific deficit in maternal aggression. We wished to test whether these neurons also acutely regulate maternal aggression by using DREADD-G_i mediated chemogenetic control of neural activity. However, we consistently observed only a few weakly expressing mCherry+ neurons within the MeApd of $aro^{Cre/IPIN}$ females (data not shown), presumably reflecting the lower levels of aromatase in the female brain. Given that caspase-3 is extremely cytotoxic (Morgan et al., 2014), it is not surprising that despite the lower efficiency of Cre recombination in females, we succeeded in ablating aromatase+ MeApd neurons. In any event, our findings demonstrate that aromatase+ MeApd neurons are required for WT levels of maternal aggression. Moreover, our studies reveal that aromatase+ neurons are, in fact, functional in females.

Different forms of fighting such as intermale and maternal aggression are thought to be controlled by different neural pathways (Moyer, 1968). Indeed, there are distinct sensory, hormonal, and molecular requirements for intermale and maternal aggression (Demas et al., 1999; Finney and Erpino, 1976; Gammie and Nelson, 1999; Gammie et al., 2000; McDermott and Gandelman, 1979; Nelson et al., 1995; Svare and Gandelman, 1975, 1976a, 1976b). Previous work suggested that these two forms of aggression may be executed differently (Gandelman, 1972; Scott, 1966). Given our finding that aromatase+ MeApd neurons regulate both intermale and maternal aggression, we sought to determine the nature of the differences in these two forms of fighting. We identified many quantitative differences between intermale and maternal aggression (Figure 4E). Moreover, there were striking qualitative differences between these two forms of aggression. Males typically attack the back and flank of the intruder, whereas dams attack the head and genital regions of the intruder and they rarely box, chase, or wrestle (Figure 4E; Movies S1 and S2). In summary, these findings show that aromatase+ MeApd neurons regulate distinct forms of aggression in the two sexes.

DISCUSSION

A Shared Neural Pathway for Distinct Forms of Aggression

It can be argued that intermale and maternal aggression are different forms of a common behavioral display, fighting. However, we observe dramatic differences between intermale and maternal aggression. Nevertheless, we find that aromatase+ MeApd neurons regulate both maternal and intermale aggression, demonstrating a hitherto unknown neural circuit link between different forms of aggression. More broadly, our findings suggest the possibility that a primordial neural pathway underlying aggression predates the divergent needs of the two sexes to

(E) A WT male intruder was inserted into the cage of a resident male or lactating female for 15 min. Mothers have a longer latency to sniff the intruder and, unlike resident males, can attack the intruder prior to chemoinvestigation. The duration of individual attacks as well as total duration of attacks initiated by mothers is significantly shorter. Mothers spend significantly less time chasing or wrestling with the intruder male. No boxing was observed in attacks initiated by mothers. Mothers bite the intruder significantly more on the head or anogenital region compared to resident males. Resident males bite the intruder significantly more on the back and flank compared to mothers. Data for these behavioral comparisons are taken from animals used to generate data for panels in Figures 2C, 4D, S1B, and S4D.

Mean ± SEM; n > 14 $aro^{+/IPIN}$ and n > 7 $aro^{Cre/IPIN}$ for tests of sexual and maternal behaviors each (A–D); n = 7 $aro^{+/IPIN}$ males and 6 $aro^{+/IPIN}$ mothers (E); *p < 0.05, **p < 0.01, ***p < 0.005; OVX, ovaries surgically removed. See also Figure S4.

fight in different contexts. Such a neural circuit could be modified during evolution and sexual selection such that it is activated by different stimuli and drives different motor programs in the two sexes.

Aromatase+ MeApd Neurons Subserve Distinct Behaviors in the Two Sexes

Most sexual dimorphisms in the vertebrate brain represent quantitative rather than qualitative differences in gene expression or neurocytological features. Indeed, there are significantly more aromatase+ MeApd neurons in males compared to females (Wu et al., 2009). It is possible that a sexually dimorphic neuronal pool is nonfunctional in one sex, drives distinct behaviors in both sexes, or functions to suppress a behavior of the opposite sex (De Vries and Boyle, 1998). We show here that aromatase+ MeApd neurons control distinct forms of aggression in males and females. The cell number difference in aromatase+ MeApd neurons could drive distinct behaviors (such as tail rattling or boxing) in the two sexes. The MeApd also expresses many other sex hormone-dependent genes in sex-specific patterns (Xu et al., 2012), and these may also permit aromatase+ MeApd neurons to regulate distinct behaviors in the two sexes. We have previously shown that sexually dimorphic progesterone receptor-expressing neurons in the ventromedial hypothalamus control distinct behaviors in males and females (Yang et al., 2013). Such functional bivalence may therefore be a general property of sexually dimorphic neuronal populations.

Global versus Modular Control of Complex Social Behaviors

Sex hormones exert global control over the display of sexually dimorphic social behaviors. For example, estrogen signaling in males governs vocalization, sexual displays, aggression, and territory marking. By contrast, we find that aromatase+ MeApd neurons are only required for WT levels of aggression. At least some components of the global control of vertebrate male behavior by estradiol likely result from paracrine signaling and masculinizing neural circuits during development (Balthazart and Ball, 1998; Forlano et al., 2006; McCarthy, 2008; Wu et al., 2009). In addition, specificity in behavioral control may be a general feature of molecularly defined neuronal populations such as those in the MeApd that are close to sensory input. In this case, aromatase+ neuronal populations more distant from sensory input, such as those in the hypothalamus, are predicted to control multiple features of different male-typical behaviors.

Our data show that it is possible to dissociate specific components of a complex social behavior such as aggression without altering other features of the behavior. In fact, the MeA itself is critical for aggressive as well as courtship displays in both sexes, whereas we find that the aromatase+ MeApd neurons regulate aggression, but not territory marking or sexual behaviors (Baum and Bakker, 2013; Bergan et al., 2014; Choi et al., 2005; DiBenedictis et al., 2012; Sokolowski and Corbin, 2012; Swanson, 2000; Wu et al., 2009). Aromatase+ MeApd neurons comprise ~40% of neurons within the MeApd and, we estimate, ~10%–20% of neurons within the MeA. Given the molecular heterogeneity within the MeA, we anticipate that other molecularly specified MeA neuronal subsets regulate other aspects of social interactions. Such exquisite modularity in behavioral control may be a general property of many similarly discrete neuronal populations in other brain regions. It will be interesting to understand how such neural modules underlying particular behavioral components are coordinated to generate apparently seamless behavioral displays. Presumably, neurons close to motor output pathways encode multiple features of a behavioral program. Indeed, neurons expressing progesterone receptor (and estrogen receptor α) in the ventromedial hypothalamus that are distant from sensory input are necessary and sufficient for multiple components of mating and aggression (Lee et al., 2014; Yang et al., 2013). Such modularity in the neuronal control of social behavior is likely to be a general mechanism whereby other complex behaviors such as feeding are regulated (Sternson, 2013). As discussed recently (Yang et al., 2013), such modular control of behavior resembles the modularity in signaling proteins and networks and may enable rapid evolution of behaviors.

Hong et al. (2014) recently published a study describing the contribution of MeA neurons to male mating, aggression, and grooming. Our study genetically identifies the aromatase+ subset of MeA neurons as specifically underlying male aggression and further shows that these neurons also regulate maternal aggression.

EXPERIMENTAL PROCEDURES

Generation of Mice Bearing the *aro^{Cre}* Allele

The *IRES-Cre* transgene was inserted into the 3′ UTR of the *aromatase* locus via homologous recombination as described previously (Wu et al., 2009; Yang et al., 2013). All experiments involving animals were performed in accordance with institutional animal care and use committee guidelines at University of California, San Francisco.

Viruses and Stereotaxic Surgery

The caspase-3 and DREADD-encoding cassettes and AAV viruses we used have been described previously (Alexander et al., 2009; Anaclet et al., 2014; Armbruster et al., 2007; Ray et al., 2011; Sasaki et al., 2011; Yang et al., 2013). We used AAV serotype 1 or 10 for delivering caspase-3 and AAV serotype 10 for all DREADD studies. Stereotaxic surgery was performed as described previously, and virus was delivered bilaterally at coordinates corresponding to the MeA (rostrocaudal, −1.6 mm; mediolateral, ±2.2 mm; depth of 5.15 mm) (Paxinos and Franklin, 2003).

Behavior

Testing for social behaviors was performed ≥1 hr after onset of the dark cycle and recorded and analyzed as described previously (Juntti et al., 2010; Wu et al., 2009; Xu et al., 2012; Yang et al., 2013). All tests were scored by an experimenter blind to the genotype and drug treatment of the mice, using a software package we developed in MATLAB (Wu et al., 2009).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.12.040.

AUTHOR CONTRIBUTIONS

E.K.U. and N.M.S. designed the experiments. E.K.U. and K.J. Burke performed the experiments. P.M.F. and C.F.Y. provided viral reagents. E.K.U., K.J. Burke, K.J. Bender, and N.M.S. analyzed the data. E.K.U. and N.M.S. wrote the paper.

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