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Social Control of Hypothalamus-Mediated Male Aggression

Highlights

- PR+ VMHvl neurons can drive aggression in singly housed, resident male mice
- This aggression can occur absent chemosensing, gonadal hormones, or opponents
- PR+ VMHvl neurons do not trigger aggression in socially housed intruder males
- Disabling chemosensing enables PR+ VMHvl-triggered aggression in these males

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In Brief

Yang et al. show that PR+ VMHvl neurons trigger aggression in solitary male mice independent of pheromone-sensing, gonadal hormones, opponents, or social context. By contrast, these neurons can trigger aggression in socially housed intruder males when their pheromone sensing is disabled.

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Social Control of Hypothalamus-Mediated Male Aggression

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SUMMARY

How environmental and physiological signals interact to influence neural circuits underlying developmentally programmed social interactions such as male territorial aggression is poorly understood. We have tested the influence of sensory cues, social context, and sex hormones on progesterone receptor (PR)-expressing neurons in the ventromedial hypothalamus (VMH) that are critical for male territorial aggression. We find that these neurons can drive aggressive displays in solitary males independent of pheromonal input, gonadal hormones, opponents, or social context. By contrast, these neurons cannot elicit aggression in socially housed males that intrude in another male's territory unless their pheromone-sensing is disabled. This modulation of aggression cannot be accounted for by linear integration of environmental and physiological signals. Together, our studies suggest that fundamentally non-linear computations enable social context to exert a dominant influence on developmentally hard-wired hypothalamus-mediated male territorial aggression.

INTRODUCTION

Social behaviors are essential for reproductive success and for success in other domains across diverse human societies. Many forms of social interactions are acquired traits that depend on learning. However, behaviors such as mating and aggression are primal, sexually dimorphic social interactions that are innate in the sense that they can be displayed without prior training, suggesting that the underlying neural circuits are developmentally programmed. Indeed, previous work shows that, in mice and many other vertebrates, sex hormones produced by the gonads during a critical developmental window control the organization of neural pathways underlying these behaviors in a sexually dimorphic manner (Arnold, 2009; Bronson and Desjardins, 1968; McCarthy, 2008; Peters et al., 1972; Phoenix et al., 1959; Wu et al., 2009; Yang and Shah, 2014). The extent to which social cues and internal signals modulate behavioral output elicited by such developmentally wired neural circuits in adult animals is poorly characterized (Fernald, 2015; Insel and Fernald, 2004; Wallen, 1996). In addition, how cues relating to social setting and prior experience are conveyed to neural circuits that control these innate behaviors is not well understood.

It can be difficult to tease apart the relative roles of social cues and internal physiological signals in shaping neural decisions underlying even innate behaviors. This is because salient sensory cues, ethological relevance of social setting, and molecularly defined neural pathways remain to be clearly determined for most behaviors, even in a laboratory setting. Hypothalamus-dependent male aggression in mice provides a suitable platform to study the relative contributions of environment and genetic hard-wiring. Wild male mice are naturally territorial across diverse settings; they attack other males even in the presence of abundant resources and without competition for mates (Berry and Bronson, 1992; Crowcroft, 1955, 1966; Crowcroft and Rowe, 1963; Quadagno, 1968). A successful territorial male will often drive other males into social cohabitation in small areas from which these males rarely attack the territorial male (Crowcroft, 1955, 1966; Crowcroft and Rowe, 1963). Male lab mice are also aggressive, and, as with wild mice, this aggression is purposive and flexible. It is purposive in the sense that a male

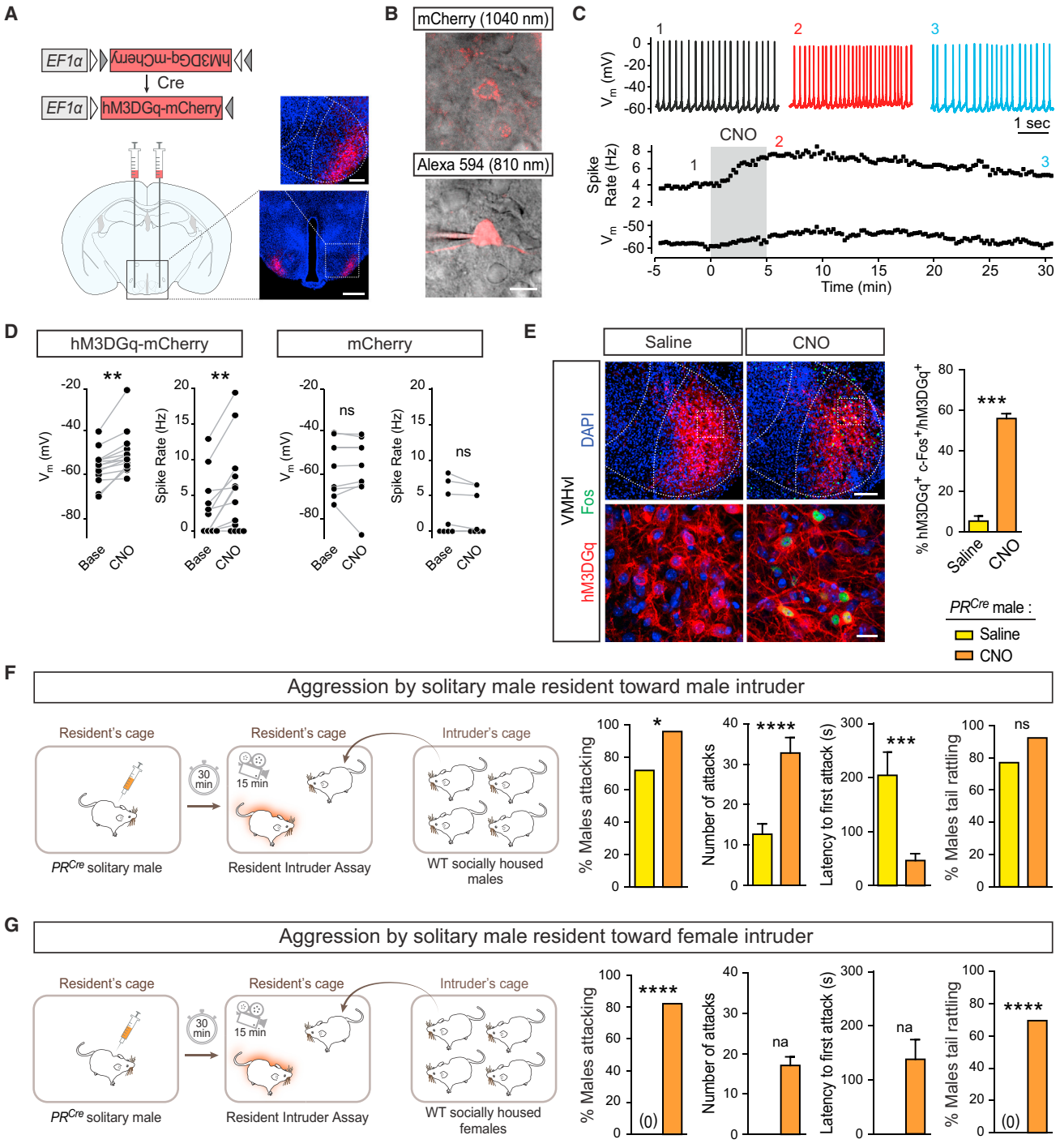


Figure 1. Chemogenetic Activation of PR+ VMHvl Neurons Triggers Aggression

(A–G) All mice were injected with an AAV encoding Cre-dependent hM3DGq-mCherry (A–G) or mCherry (D) into the VMHvl of adult PR^{Cre} males.

(A) Schematic showing bilateral stereotaxic delivery of the AAV to the VMHvl of PR^{Cre} mice. The inset shows mCherry+ cells in the VMHvl 10 days following injection.

(B) Top: mCherry+ (PR+, hM3DGq+) neuron in VMHvl targeted for patch-clamp recording using a 1,040-nm, two-photon excitation source. Bottom: a target neuron filled with Alexa 594 was visualized with 810-nm, two-photon excitation to confirm neuronal identity. Scanning differential interference contrast was visualized in both imaging configurations to guide recording.

(C) Example of such a patched mCherry+ neuron, where 5 min of CNO (1 μM, gray shading) depolarized the neuron and increased spiking activity. Numbers (1–3) highlight spiking during baseline, immediately after CNO, and 25 min following washout.

(legend continued on next page)

attacks a male but not a female. Male aggression is flexible in the sense that a resident male attacks a male intruding in his home-cage (Crowcroft, 1966; Miczek et al., 2001), whereas an intruder male who is cohoused with other males prior to being inserted into the resident's cage does not initiate attacks toward the resident male. Resident male aggression is triggered by intruder male pheromones such that residents disabled for pheromone sensing do not attack other males (Chamero et al., 2007; Leypold et al., 2002; Liberles, 2014; Mandiyan et al., 2005; Stowers et al., 2002; Yoon et al., 2005). However, the mechanisms that inhibit socially cohoused males from initiating attacks on residents are poorly understood. In addition to these external cues, resident male aggression in mice, as in many other vertebrates, also requires testosterone or its metabolites such that castration essentially abrogates fighting (Beeman, 1947). Finally, many studies have localized a hypothalamic center in or around the functionally heterogeneous ventromedial hypothalamus (VMH) of diverse vertebrates, including mice, that controls aggressive displays (Cheung et al., 2015; Lin et al., 2011; Silva et al., 2013; Yang and Shah, 2014). Indeed, optogenetic stimulation of the VMH elicits aggression by a resident male (Lin et al., 2011). We recently demonstrated that this center consists of a cluster of progesterone receptor (PR+) neurons in the ventrolateral subdivision of the mouse VMH (VMHvl) that is essential for resident male aggression (Yang et al., 2013). However, the functional relevance of PR+ VMHvl neurons in socially housed males that are not normally aggressive is unknown.

We set out to determine the relative influence of physiological signals and social context, defined here as the setting of the behavioral assay as well as prior experience, on PR+ VMHvl-modulated male aggression. We find that experimental activation of PR+ VMHvl neurons in solitary males elicits aggressive displays independent of social context, pheromone sensing, opponents, or circulating testosterone. By contrast, social housing with other males suppresses initiation of aggression by PR+ VMHvl neurons in intruder males. Genetic disabling of pheromone sensing in socially housed males enables PR+ VMHvl neurons to drive aggression in these males in this setting. Our functional network modeling studies show that, rather than linearly accumulating evidence from sensory modalities, physiological state, and social context, the neural circuit underlying aggression appears to utilize a fundamentally non-linear computation to generate behavioral output. In summary, we demonstrate that it is social setting and housing conditions rather than just the activation of a neural pathway necessary and sufficient for fighting that determine whether a male will initiate aggression.

RESULTS

PR+ VMHvl Neurons Acutely Regulate Male Aggression

We sought to define the role of PR+ VMHvl neurons in aggression by determining whether these neurons can drive this behavior independent of external sensory cues or internal physiological signals required for aggression in wild-type (WT) males. Accordingly, we sought to activate PR+ VMHvl neurons in an inducible manner. We had previously generated a PR-IRES-Cre (PR^{Cre}) knockin allele to drive the desired virally encoded transgenes in these neurons in a Cre-dependent manner (Yang et al., 2013). To activate PR+ VMHvl neurons, we expressed a Cre-dependent designer receptor exclusively activated by a designer drug (DREADD), which couples with the Gα protein Gq in these neurons, using an adeno-associated virus (AAV-flex-hM3DGq:mCherry) (Alexander et al., 2009; Ray et al., 2011; Sternson and Roth, 2014). Delivery of this virus into the VMH of PR^{Cre} mice results in reliable expression of the DREADD, as visualized by mCherry, which is fused to hM3DGq (Figures 1A and 1B). Importantly, there was no detectable expression of mCherry when the virus was injected in WT mice (Figure S1A). We sought to further define the specificity of DREADD expression in PR+ neurons. PR is expressed at very low levels in vivo, making it difficult to perform co-labeling studies with PR mRNA or protein. To visualize PR+ cells, we had previously generated a PR^{PL} knockin allele that drives expression of nuclear β-galactosidase (nβ−gal) in PR+ cells (Yang et al., 2013). Nuclear localization of β-gal enables detection of PR+ cells and co-expression studies. Upon injecting the virus into $PR^{Cre/PL}$ mice, we found that most mCherry+ cells were nβ−gal+ (91.7% ± 1.8; n = 331 mCherry+ neurons from 5 $PR^{Cre/PL}$ males); the ~8% mCherry+, nβ-gal− cells likely reflect the greater sensitivity in detection of functional Cre expression, which can recombine multiple copies of the virally encoded, flexed transgene, compared with single-copy expression of nβ-gal.

hM3DGq leads to membrane depolarization and increased spiking in neurons in the presence of its cognate ligand, CNO (clozapine N-oxide). We tested the functional expression of hM3DGq in the VMHvl of PR^{Cre} mice in acute brain slices through this region. We observed membrane depolarization as well as an increase in spike frequency in DREADD+ neurons exposed to

(D) Summary of electrophysiological studies on PR+ VMHvl neurons expressing hM3DGq-mCherry (left) or control mCherry (right). Each dot represents a single neuron, and lines connect baseline and CNO conditions within recordings. Data are from six hM3DGq-mCherry and four mCherry injected males.

(E) CNO injection increases Fos expression in PR+ VMHvl neurons expressing hM3DGq-mCherry. Top and bottom: histological images captured at 10× and 63× objectives, respectively. Quantification shows the percentage of hM3DGq-mCherry VMHvl neurons that express Fos following CNO or saline (control) injection. n = 3/condition.

(F) Resident solitary PR^{Cre} males attack WT socially housed intruder males with increased probability and intensity when injected with CNO. There is no difference in the percentage of resident males tail-rattling to an intruder when given CNO or saline. n = 25/condition.

(G) Resident solitary PR^{Cre} males tail-rattle to and attack WT socially housed intruder females primed to be in estrus only when given CNO. n = 28/condition.

na, not applicable. Only mice showing attack behavior were included in the analysis of non-categorical data, such as number of attacks and latency to first attack, in this and all other figures. If no or too few males given saline displayed attacks, it precluded statistical analysis of these attack parameters. See also Table S1 for this and all other figures where we have analyzed these non-categorical data following inclusion of all animals regardless of whether they displayed attacks. ns, not significant. Mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. Scale bars, 100 μm and 500 μm for the top and bottom insets, respectively (A), 10 μm (B), and 100 μm and 20 μm for the top and bottom insets, respectively (E).

CNO (Figures 1C and 1D; hM3DGq spike rate: 2.7 ± 1.1 Hz [baseline] and 5.5 ± 1.6 Hz [CNO], $p < 0.01$; mCherry spike rate: 2.7 ± 1.3 Hz [baseline] and 2.3 ± 1.1 Hz [CNO], $p = 0.1$; hM3DGq membrane potential [Vm]: −59 ± 1.8 mV [baseline] and −53 ± 2.3 mV [CNO], $p < 0.01$; mCherry Vm: −62 ± 2.7 mV [baseline] and −63 ± 3.8 mV [CNO], $p = 0.7$; hM3DGq, n = 14 cells from 6 males; mCherry, n = 8 cells from 4 males). Expression of DREADD did not alter the baseline properties of PR+ VMHvl cells because these were comparable in neurons expressing hM3DGq:mCherry or mCherry (spike rate, $p = 0.99$; Vm, $p = 0.35$). Consistent with excitation of PR+, hM3DGq+ VMHvl neurons by CNO in slices, we observed >10-fold induction of Fos, a surrogate of neural activity (Morgan and Curran, 1991), in these neurons when mice were given CNO (Figure 1E). Taken together with our findings that DREADD expression is Cre-dependent (Figure S1A) and that most mCherry+ cells are nβ-gal+ (a PR reporter in $PR^{Cre/PL}$ mice), these results indicate that CNO activates PR+, hM3DGq+ VMHvl neurons.

We next tested whether activating PR+, hM3DGq+ VMHvl neurons triggered aggression in PR^{Cre} males. Solitary (singly housed) male mice attack intruder males in their homecage without prior training; in these assays, the intruder male, who is from a group of socially housed males, does not initiate attacks (Miczek et al., 2001). CNO increased the probability of a solitary male initiating aggression toward a male intruder, and it also increased the intensity of the ensuing attacks (Figure 1F; Figure S1B). Male mice rattle their tail, presumably as a threatening display, in aggressive encounters (Scott, 1966). There is no 1:1 relation between individual episodes of tail rattling and physical attacks such as biting or wrestling, but most assays containing attacks also feature tail rattling (Figure 1F). Correspondingly, males given CNO also tail-rattled with a shorter latency (saline, 238.8 ± 51.4 s; CNO, 38.1 ± 22.1 s; n = 25 each; $p < 0.01$) and more frequently (saline, 6.5 ± 1.8; CNO, 20.9 ± 3.4; n = 25 each; $p < 0.01$). The majority (>92%) of PR+ VMHvl neurons express estrogen receptor alpha (ERα or Esr1) (Yang et al., 2013), and optogenetic activation of Esr1+ neurons also triggers attacks toward an intruder (Lee et al., 2014). Our findings extend this initial observation significantly because we assayed for aggression in a more naturalistic setting where resident solitary males were unencumbered and freely interacting with an intruder, thereby allowing us to rigorously assess the patterns of attack and effectively vary social housing conditions as described below.

This increase in aggression in solitary males complements our previous study showing that ablation of PR+ VMHvl neurons reduces fighting in male mice (Yang et al., 2013). We also confirmed behavioral deficits following chronic ablation of the neurons by acutely inhibiting their activity via bilateral delivery of AAV-flex-hM4DGi and administering CNO to a separate cohort of males. Binding of CNO to hM4DGi leads to membrane hyperpolarization and a reduction in neuronal activity (Sternson and Roth, 2014). Consistent with such inhibition of neuronal activity, intermale fighting was dramatically reduced in solitary males given CNO (Figure S1D). Ablation of PR+ VMHvl neurons also reduced male sexual behavior toward WT females, with a significant decrease in intromission (penetration) displays (Yang et al., 2013). We observed that males expressing hM4DGi and treated with CNO also reduced the number and duration of intromission events toward WT females (Figure S1F), demonstrating that these neurons acutely regulate both male mating and aggression. A previous study using optogenetic inhibition of Esr1+ VMHvl neurons did not observe deficits in male sexual behavior, and this could reflect ineffective silencing of these neurons (Lee et al., 2014). By contrast, our findings with chemogenetics here are in accord with those from chronic ablation of PR(Esr1)+ VMHvl neurons (Yang et al., 2013) and show that these neurons are required acutely as well as chronically for WT levels of male sexual behavior and aggression.

We next sought to test whether activating PR+ VMHvl neurons in males would alter sexual behavior. We found that males expressing hM3DGq in PR+ VMHvl neurons attacked females when given CNO, whereas they never did so when given saline (Figure 1G; Figure S1C). Increasing optogenetic stimulation of Esr1+ VMHvl neurons in male mice altered their behavior from mating to aggression (Lee et al., 2014). Given that PR+ VMHvl neurons also express Esr1, we wondered whether changing the dose of CNO would lead to a similar switch. Indeed, we observed that males administered lower doses of CNO mated with females or mated and attacked females, whereas they exclusively attacked females at higher doses (Figures S1E and S1G). Importantly, experimental triggering of aggression required expression of hM3DGq in PR+ VMHvl neurons because injection of AAV-flex-hM3DGq:mCherry into WT males did not lead to detectable mCherry expression or aggression (Figure S1A). Because we wished to study the influence of social context and internal physiological conditions on male aggression, we utilized CNO at a dose that reliably elicited this behavior (Figures 1F and 1G; Figure S1G). Indeed, we did not observe sexual behavior with this dose of CNO (0.3 mg/kg) in other behavioral assays unless otherwise mentioned. Moreover, this dose of CNO is within the range used in vivo to modulate neuronal activity in diverse brain regions (Ray et al., 2011; Sasaki et al., 2011; Unger et al., 2015).

Taken together with prior studies showing that PR+/Esr1+ VMHvl neurons, but not other neighboring neurons, are critical for triggering aggression in males (Lee et al., 2014; Yang et al., 2013), our results demonstrate that acute activation of PR+ VMHvl neurons is necessary and sufficient to elicit aggression from solitary males. We wondered whether this chemogenetically induced aggression resembles the aggression displayed by WT males or leads to novel patterns of fighting. We found that the patterns of CNO-elicited aggression were comparable with those shown by control males (Figure S1H). Consistent with this observation, CNO-elicited male aggression was also distinct from maternal aggression displayed by females defending their pups. These females do not tail-rattle, and they direct their bites largely to the neck and genital region (Unger et al., 2015); by contrast, males given CNO tail-rattled and largely directed bites to the flanks. Prior optogenetic studies employed arbitrary stimulation to elicit aggression during various phases of social interactions (Lee et al., 2014), thereby precluding analysis of patterns of aggression between freely interacting males. By contrast, our chemogenetic approach demonstrates that activation of PR+ VMHvl neurons in males elicits aggression that appears comparable with WT patterns of male-typical territorial aggression.

PR+ VMHvl Neurons Trigger Aggression in Singly Housed Males Independent of Pheromone Signaling

The observation that activation of PR+ VMHvl male neurons elicits aggression toward females suggests that such activation overrides the pheromonal input that would normally direct sexual displays toward females. If this is true, then it should be possible to drive aggression in solitary males genetically disabled for pheromone sensing. Alternatively, pheromonal input to other neuronal populations may be essential to drive aggression even when PR+ VMHvl neurons have been experimentally activated (Figure 2A). In the latter scenario, genetic disabling of pheromone sensing should abrogate aggressive displays. To distinguish between these possibilities, we activated PR+ VMHvl neurons in singly housed males genetically disabled for the major pheromone-sensing pathways, the vomeronasal organ (VNO) and the main olfactory epithelium (MOE). The cation channels Trpc2 and Cnga2 are essential for normal odor-evoked activity in VNO and MOE sensory neurons (Brunet et al., 1996; Leypold et al., 2002; Stowers et al., 2002), respectively, and for male aggression (Leypold et al., 2002; Mandiyan et al., 2005; Stowers et al., 2002). The deficit in male aggression in mice mutant for *Trpc2* or *Cnga2* likely reflects a requirement for Trpc2 or Cnga2 in VNO and MOE neurons rather than cells elsewhere because this phenotype is also observed in male mice mutant for other channels that are expressed in the VNO or MOE and essential for normal odor-evoked activity (Kim et al., 2012; Wang et al., 2006). As expected, few males disabled for pheromone sensing via the VNO ($PR^{Cre};Trpc2^{-/-}$) or MOE ($PR^{Cre};Cnga2^{-/Y}$) and expressing hM3DGq in the VMHvl were aggressive when given saline; the majority of these males, however, initiated aggression toward male or female intruders when given CNO (Figure 2B; Figures S2A and S2B). Moreover, we did not observe any sexual behavior by $PR^{Cre};Trpc2^{-/-}$ or $PR^{Cre};Cnga2^{-/Y}$ males given CNO, except that one male of each of these genotypes mated with a female intruder (compared to 7 of 12 $PR^{Cre};Trpc2^{-/-}$ males given saline, $p < 0.01$, and 1 of 8 $PR^{Cre};Cnga2^{-/Y}$ males given saline, $p = 1$, respectively). In summary, PR+ VMHvl neurons can elicit aggression in males genetically disabled for pheromone-sensing pathways that are otherwise essential for this behavior.

The foregoing results do not exclude the possibility that activation of PR+ VMHvl neurons elicits aggression as long as either the VNO or MOE is functional. *Cnga2* mutant pups have severe nursing defects, and adult mutants have difficulty mating and providing parental care (Brunet et al., 1996; Fraser and Shah, 2014; Mandiyan et al., 2005; Yoon et al., 2005), making it difficult to obtain males of the genotype $PR^{Cre};Trpc2^{-/-};Cnga2^{-/Y}$. However, the VNO and MOE are required non-redundantly for sex discrimination and sniffing (anogenital chemoinvestigation), which presumably enable access to aggression-eliciting pheromones in exocrine secretions, respectively (Chamero et al., 2007; Leypold et al., 2002; Mandiyan et al., 2005; Stowers et al., 2002; Yoon et al., 2005). Our findings show that PR+ VMHvl neurons can drive male aggression independent of these aspects of pheromone sensing.

Both the VNO and MOE sense conspecific pheromones and chemosensory cues emanating from other species, and recognition of conspecifics via either pathway may be sufficient for subsequent species-typical interactions such as aggression (Isogai et al., 2011; Kobayakawa et al., 2007; Li et al., 2012; Papes et al., 2010). Accordingly, we tested whether activation of PR+ VMHvl neurons in PR^{Cre} males WT for *Trpc2* and *Cnga2* can elicit aggression toward *Peromyscus*. *Peromyscus* is a rodent genus that last shared an ancestor with lab mice (*Mus*) ~25 million years ago (Ramsdell et al., 2008). Consistent with the notion that conspecific recognition is critical for meaningful social interactions (Fan et al., 2013), we observed that PR^{Cre} males rarely attacked *Peromyscus eremicus* in the presence of saline (Figure 2C; Figures S2C and S2D). By contrast, a majority of PR^{Cre} males attacked *P. eremicus* males and females when given CNO (Figure 2C; Figures S2C and S2D). Thus, activation of PR+ VMHvl neurons bypasses pheromone-mediated sex discrimination or conspecific recognition and elicits aggression.

PR+ VMHvl Neurons Can Elicit Limited Forms of Aggression-Related Responses

We next tested whether activating PR+ VMHvl neurons would provoke aggressive displays in the absence of a stimulus animal or animal-like object by inserting a mirror in the homecage. The use of a mirror was prompted by previous work showing that fish show aggressive displays to a mirror image (Desjardins and Fernald, 2010; Oliveira et al., 2016; Tinbergen, 1951). Strikingly, males given CNO did not physically attack the mirror, but they reliably tail-rattled to it (Figure 2D; Movies S1 and S2). Activation of PR+ VMHvl neurons also elicited tail rattling when males encountered the non-reflective surface of a mirror, although this was less effective than the reflective side (Figure 2D; Movies S3 and S4). CNO did not elicit tail rattling in the absence of a mirror, indicating that even this limited aggression-related display requires sensory input (Figure 2D). Males occasionally bit an inflated glove inserted into their homecage upon activation of PR+ VMHvl neurons (data not shown), but, as reported previously, this did not reach statistical significance (Lin et al., 2011). Nevertheless, a significant number of males tail-rattled to the glove when administered CNO (Figure 2D; Movies S5 and S6). These findings are in agreement with our observations that PR+ VMHvl neurons can elicit behavioral responses in a chemosensation-independent manner. Tail rattling (and, in the case of a glove, even biting) under these conditions upon stimulation of these neurons might result from a stress response. This is unlikely because optogenetic stimulation of VMHvl elicits time-locked biting of gloves, suggesting that this is not a result of elevated stress hormones (Lin et al., 2011); moreover, we have previously shown that ablation of PR+ VMHvl neurons does not affect anxiety-type behavioral responses (Yang et al., 2013). Consistent with this notion, chemogenetic stimulation of PR+ VMHvl neurons did not alter behavior in an elevated plus maze (Figure S2E). In summary, our findings demonstrate that mice can use visual cues to assess whether to initiate tail rattling, a display that usually accompanies aggressive behavior (Scott, 1966; Stowers et al., 2002). Moreover, our results demonstrate that activation of PR+ VMHvl neurons can elicit graded displays of aggression or related behaviors: none in the absence of a novel stimulus, some tail rattling to an object, increased tail rattling to a reflective surface, and tail rattling as well as physical attacks to intruders.

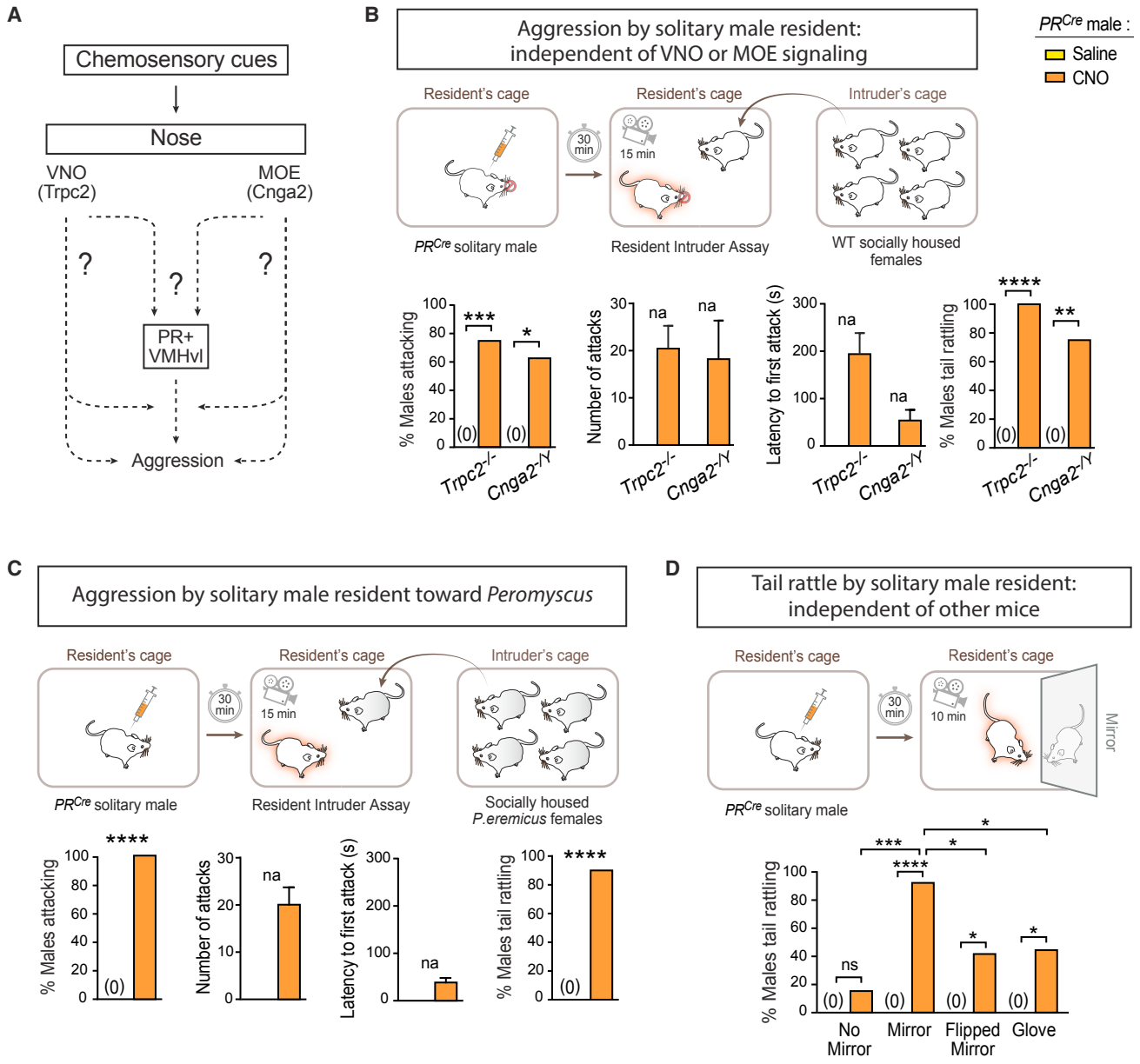


Figure 2. PR+ VMHvl Neurons Elicit Aggressive Displays Independent of Chemosensory Signaling

(A) Schematic showing that chemosensory cues detected by MOE or VNO neurons may elicit aggression by functioning in pathways upstream, downstream, or parallel to PR+ VMHvl neurons.

(B–D) An AAV encoding Cre-dependent hM3DGq-mCherry was delivered to VMHvl of PR^{Cre};$Trpc2^{-/-}$ (B), PR^{Cre};$Cnga2^{-/Y}$ (B), or PR^{Cre} (C and D) adult males.

(B) Solitary male residents null for *Trpc2* or *Cnga2* tail-rattled to and attacked socially housed female intruders only after being administered CNO. n = 12/condition ($Trpc2^{-/-}$) and 8/condition ($Cnga2^{-/Y}$).

(C) Solitary male residents tail-rattled to and attacked socially housed *P. eremicus* female intruders only after CNO injection. n = 10/condition.

(D) Solitary male residents tail-rattled more to inanimate objects after CNO administration. They also tail-rattled more to the reflective side of the mirror compared with its non-reflective side or to a partially inflated glove. n = 13/condition (no mirror, mirror), 12/condition (flipped mirror), and 13 (saline) and 9 (CNO) (glove). Mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Gonadal Hormones Are Not Required for PR+ VMHvl-Activated Male Aggression

In addition to chemosensory cues, testicular hormones are required for aggression in standard male lab mice (Figure 3A) such that solitary males castrated as adults do not attack other males (Beeman, 1947). This behavioral deficit can be corrected if the male is administered testosterone or its bioactive metabolites (Finney and Erpino, 1976; Yang and Shah, 2014). We tested whether chemogenetic stimulation of PR+ VMHvl neurons would bypass the need for testicular hormones in fighting. Accordingly,

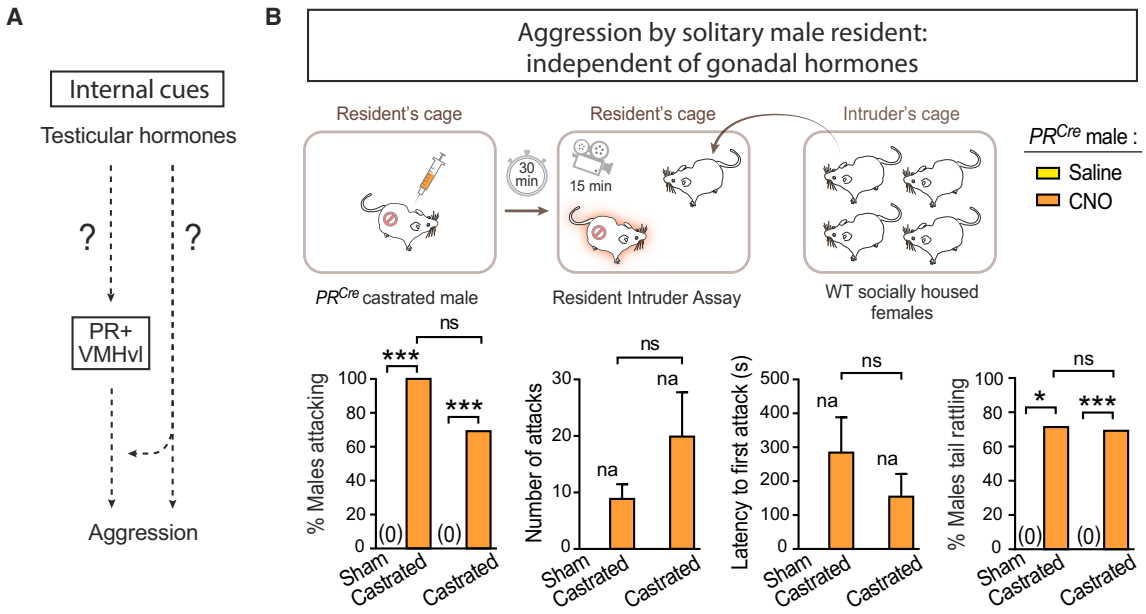


Figure 3. PR+ VMHvl Neurons Elicit Aggressive Displays Independent of Male Gonadal Hormones

(A) Schematic showing that testicular hormones may influence aggression by activating the physiological function of PR+ VMHvl neurons directly or that of the neural pathways upstream, downstream, or parallel to PR+ VMHvl neurons.

(B) An AAV encoding Cre-dependent hM3DGq-mCherry was delivered to VMHvl of PR^{Cre} adult males who were subsequently castrated or subjected to sham castration surgery. There were no differences between castrated and sham control males tail rattling to and attacking WT socially housed female intruders when injected with CNO.

Mean ± SEM, n = 7/condition (sham-castrated) and 13/condition (castrated). *p < 0.05, ***p < 0.001.

we infected PR+ VMHvl neurons in PR^{Cre} males with AAV-flex-hM3DGq as before, castrated them, and waited 3 weeks to allow the circulating testosterone to subside to undetectable levels. We then tested these males for aggression after administering saline or CNO. Castrated solitary males administered saline rarely showed aggression, consistent with undetectable levels of testosterone in their circulation (Figure 3B; Figures S3A–S3C; Movie S7). However, when given CNO, they attacked male or female intruders (Figure 3B; Figures S3A and S3B; Movie S8). The aggression elicited in the presence of CNO was comparable with that observed in sham-castrated control solitary males, demonstrating that PR+ VMHvl neuronal activation elicits male aggression in a gonadal hormone-independent manner.

These findings raise the possibility that stimulation of PR+ VMHvl neurons in females may also trigger aggression. Optogenetic stimulation of female Esr1+ VMHvl neurons does not elicit aggression, but it is unclear whether the females were singly housed in a manner similar to males (Lee et al., 2014). In acute brain slices through the female VMH, we found that bath application of CNO depolarized the membrane and increased spiking activity in PR+ VMHvl neurons (Figure S3D). We next delivered AAV-flex-hM3DGq:mCherry to the VMHvl of adult PR^{Cre} females, ovariectomized them, and housed them singly. None of these female residents attacked a male or female intruder in the presence of CNO (Figure S3E), indicating that stimulation of PR+ VMHvl neurons is not sufficient to trigger territorial aggression in females under these conditions. Our findings are in agreement with prior studies showing that the neural pathways underlying male territorial aggression are developmentally wired during a critical perinatal period (McCarthy, 2008; Wu et al., 2009; Yang and Shah, 2014). Such sexual differentiation could subsequently preclude the ability of PR+ VMHvl neurons to trigger territorial aggression in females. In contrast to these findings in females, our results show that stimulation of PR+ VMHvl neurons in male residents is sufficient to trigger aggression independent of circulating gonadal hormones. Testosterone-sensitive neurons are found all along the neuraxis, from the olfactory bulb to the spinal cord, including in the VMH (Shah et al., 2004; Simerly et al., 1990), and testosterone or its metabolites can elicit dramatic changes in neuronal morphology (Morris et al., 2004). Nevertheless, activation of PR+ VMHvl neurons bypasses these powerful effects of gonadal sex hormones and elicits aggression in castrate males.

Social Context and Housing Regulate the Ability of Males to Initiate Aggression

Given that stimulation of PR+ VMHvl neurons triggers aggression in solitary males under conditions that typically do not trigger fighting, we wondered whether such stimulation would enable socially housed males to initiate attacks. Socially housed males are, in fact, used as intruders into cages of singly housed males because they elicit but do not display aggression (Miczek et al., 2001). We injected socially housed PR^{Cre} males with AAV-flex-hM3DGq into the VMH and tested them as intruders. We found that few socially housed intruders tail-rattled or initiated attacks on the resident even when given CNO (Figures 4A; Figure S4A).

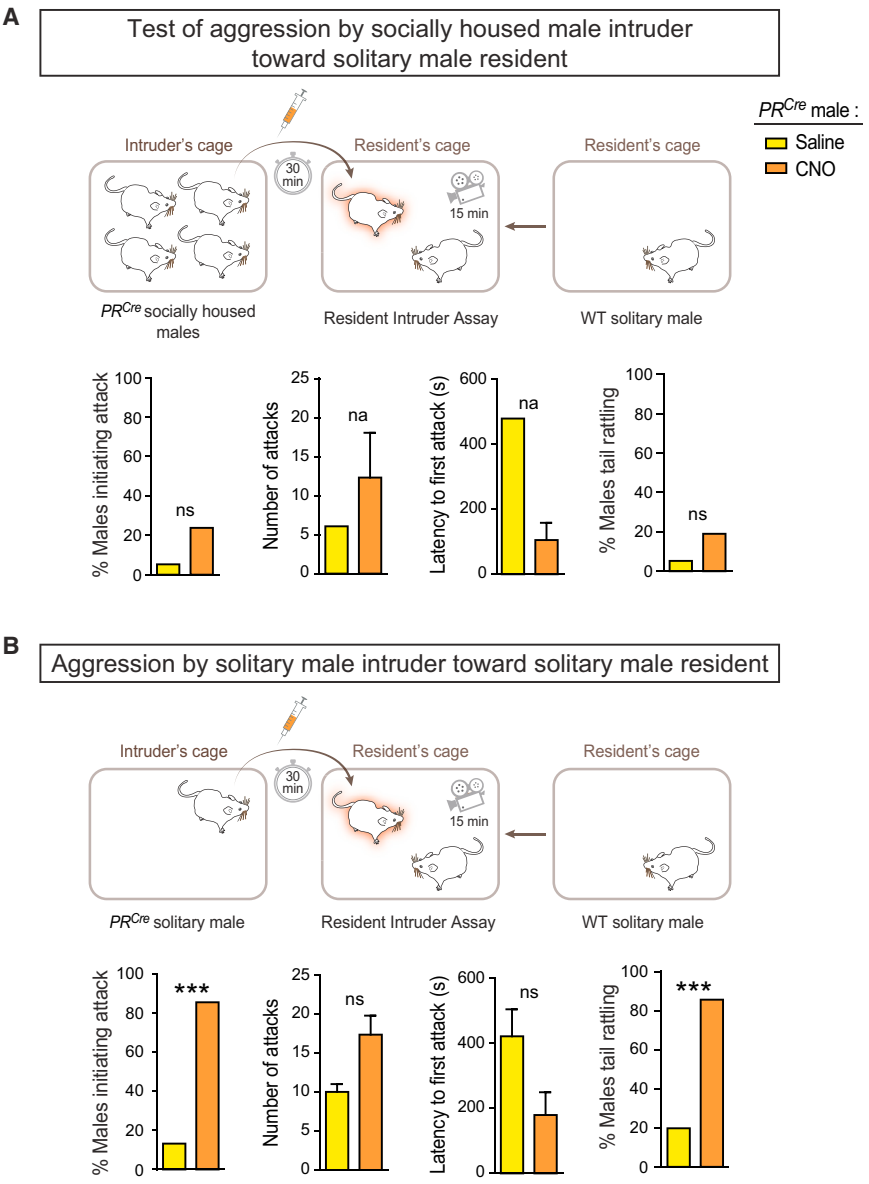


Figure 4. PR+ VMHvl Neuron-Elicited Aggression Is Dependent on Social Context

(A and B) An AAV encoding Cre-dependent hM3DGq-mCherry was delivered to VMHvl of PR^{Cre} adult males who were socially housed with other males (A) or housed singly (B) and used as intruders. Singly but not socially housed male intruders given CNO routinely tail-rattled to and initiated attacks toward a WT solitary male resident. Too few socially housed (n = 1) or solitary (n = 2) male intruders given saline attacked the resident, thereby precluding meaningful statistical analysis for non-categorical data.

Mean ± SEM; n = 19 (saline) and 21 (CNO) (A); n = 15 (saline) and 14 (CNO) (B). ***p < 0.001.

Thus, stimulation of PR+ VMHvl neurons is insufficient to initiate aggressive displays in most socially housed male intruders. However, when the WT solitary male resident started attacking, more socially housed male intruders given CNO defended themselves aggressively (saline, 5.3%; CNO, 61.9%; n = 19, 21, respectively; p < 0.001). We wondered whether the inability of socially housed male intruders to initiate aggression was dependent on social housing or being an intruder. Accordingly, we tested whether solitary males would initiate aggression in the context of being an intruder in a WT solitary resident male's cage. We injected PR^{Cre} males with AAV-flex-hM3DGq into the VMH, housed them singly, and then tested them as intruders. Few solitary male intruders given saline tail-rattled or attacked the resident male (Figure 4B; Figure S4B). By contrast, a majority of these intruders tail-rattled and initiated attacks when given CNO prior to being inserted into another resident male's cage (Figure 4B; Figure S4B). Importantly, although the latency to initiate attacks after CNO injection was comparable between socially housed and solitary male intruders (Figure 4; latency to initiate attack toward resident: socially housed intruder, 104.2 ± 52.8 s; solitary intruder, 179.7 ± 70.1 s; p = 0.36), significantly fewer socially housed intruders attacked the male resident (Figure 4; percentage of intruders that initiated attack toward resident: socially housed intruder, 23.8%; solitary male, 85.7%; p < 0.001). In summary, social housing suppresses initiation of male aggression otherwise triggered by PR+ VMHvl neurons.

The inability of socially housed intruders to initiate aggression contrasts with the performance of solitary intruders who attack and suggests the possibility that social housing abrogates initiation of aggression. We first tested whether CNO could activate PR+ VMHvl neurons in socially housed males, using Fos induction (Figure 1E) as a measure of neural activity. A similar percentage of hM3DGq/PR+ VMHvl neurons in PR^{Cre} males expressed Fos following CNO in solitary (43.6% ± 2.7%, n = 7) and social (44.0% ± 5.4%, n = 7; p = 0.94 for the comparison between these groups) housing conditions. These results do not exclude the possibility that CNO activates different subsets of PR+ VMHvl neurons in solitary and socially housed males, but they show that the inability of socially housed male intruders to initiate attacks is not due to a complete failure of CNO to activate these neurons. Consistent with the fact that CNO activates PR+ VMHvl neurons in socially housed males, we show below that CNO can elicit aggression in these males in specific social settings.

We tested whether socially housed males can initiate attacks in two paradigms in which solitary males initiate aggression without experimental activation of PR+ VMHvl neurons, as residents of their homecage (Figure 1F; Figure S1B) and as intruders in a clean cage (neutral arena) (Figure S5A). We tested separate

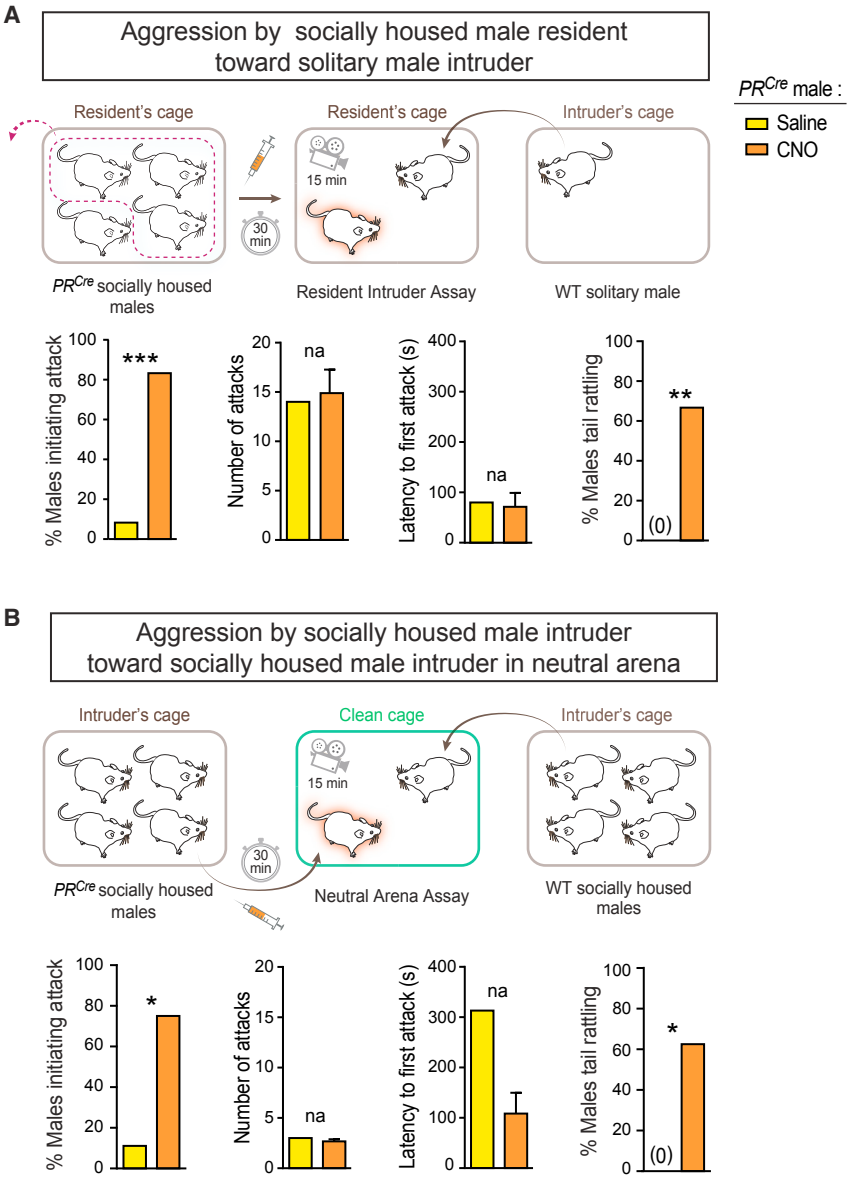


Figure 5. PR+ VMHvl Neurons Can Trigger Aggression in Socially Housed Males

(A and B) An AAV encoding Cre-dependent hM3DGq-mCherry was delivered to VMHvl of PR^{Cre} adult males who were socially housed with other males and used as residents (A) or intruders (B).

(A) Socially housed resident males were injected with CNO or saline, their cage mates were removed from the homecage, and a WT intruder male was inserted into the cage. Such socially housed residents tail-rattled to and attacked intruders significantly more with CNO compared with saline. n = 12/condition.

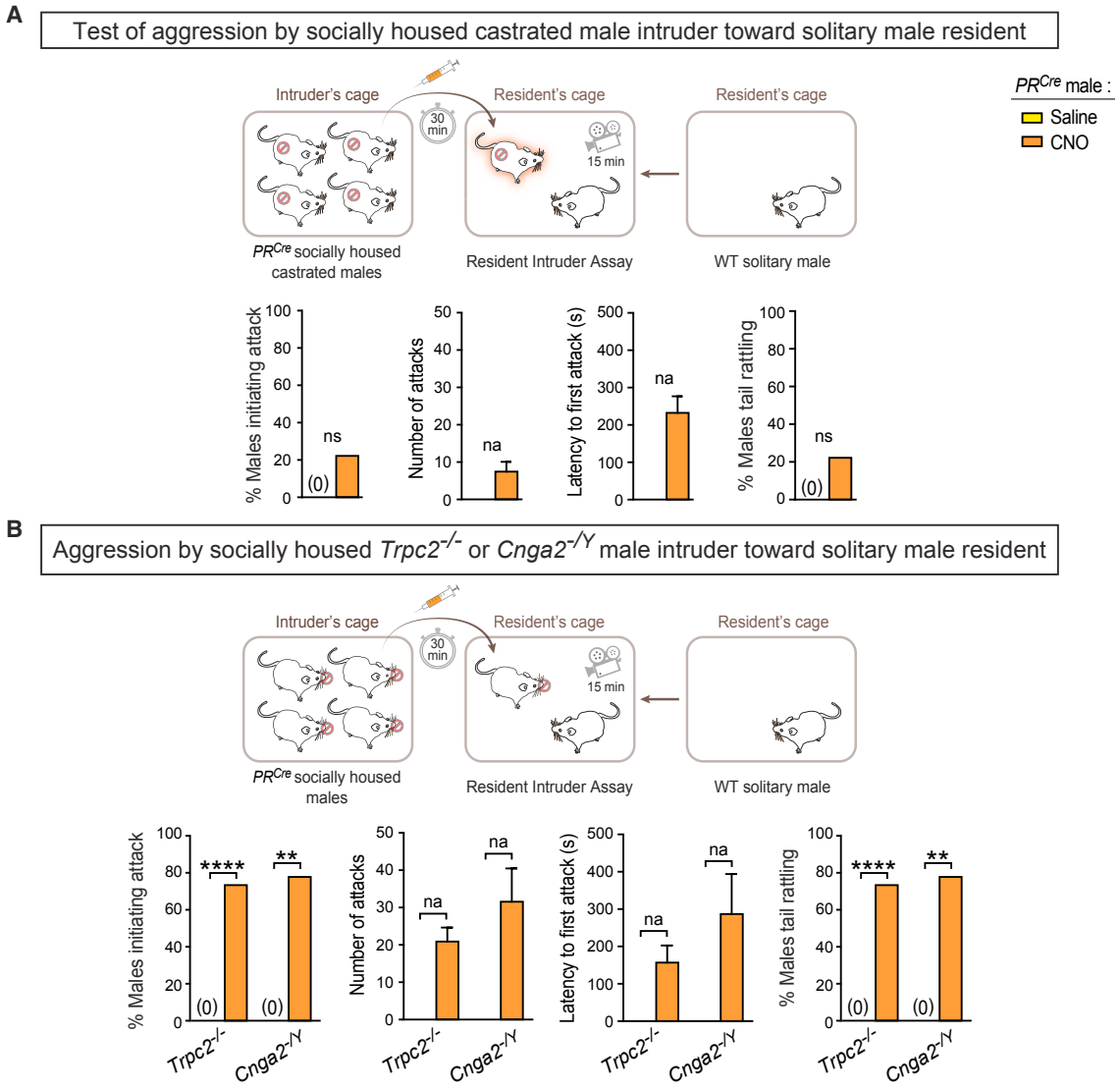
(B) Socially housed males injected with CNO or saline were inserted into a clean cage (neutral arena) along with a WT socially housed male. CNO-administered males tail-rattled to and attacked the WT male more than males injected with saline. n = 8/condition.

Too few socially housed males given saline initiated attacks in their homecage or a neutral arena (n = 1 each), thereby precluding meaningful statistical analysis for non-categorical data. Mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

cohorts of socially housed PR^{Cre} males in these two settings following injection of AAV-flex-hM3DGq into the VMH (Figure 5). For testing as a resident, a socially housed male was injected with saline or CNO and his cagemates were removed, and 30 min later, a WT solitary male intruder was inserted into the homecage. For testing in a neutral arena, a socially housed male was given saline or CNO and inserted 30 min later with an unfamiliar WT socially housed male into a clean cage with fresh bedding. In both settings, socially housed males given CNO initiated attacks and tail-rattled significantly more than males injected with saline, who displayed little aggression (Figure 5; Figures S5B and S5C). These findings show that there may be distinct modes of inhibiting aggression in socially housed males. In the absence of experimental activation of PR+ VMHvl neurons, these males do not initiate aggression in settings (as a resident or in a neutral arena) in which solitary males attack (Figure 5; Figures S5B and S5C). Such inhibition of aggression in socially housed males can be overcome by activating PR+ VMHvl neurons. By contrast, insertion as an intruder in a resident male's cage exerts a stronger inhibition on aggression. In this latter situation, most socially housed intruders do not initiate aggression even when PR+ VMHvl neurons have been stimulated (Figure 4A). Together, our findings show that suppression of attack in social housing is functionally upstream or downstream of PR+ VMHvl neurons in a social context-dependent manner.

Pheromone Sensing Inhibits Aggression by Socially Housed Males

We next sought to understand why socially housed male intruders did not initiate aggression. Social housing enables the emergence of an aggressive interaction-based hierarchy (Miczek et al., 2001), and we reasoned that a social hierarchy could suppress aggression in socially housed intruder males (Wang et al., 2011). In fact, the experimental solitary males we used had been housed socially prior to viral delivery of DREADDs and then housed singly after the injection for 10–12 days before being tested for aggression. In this situation, 10–12 days of solitary housing were clearly sufficient to erase any potential effects of a hierarchy within social housing. We wondered whether eliminating such aggression-based aspects of hierarchy in socially housed males would permit attacks when they were used as intruders. To eliminate aggressive interactions, we castrated all adult, co-housed males 1–2 days after injecting AAV-flex-hM3DGq into the VMH and then kept them socially housed for an additional 3 weeks to allow testosterone levels to subside. However, the majority



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of castrated, socially housed male intruders did not attack male residents even when given CNO (Figure 6A; Figures S6A and S6B). Thus, gonadal hormone-dependent aggressive interactions are not required to inhibit aggression in socially housed males. Castration also eliminates aggression-eliciting pheromones such as major urinary proteins (MUPs) (Chamero et al., 2007), and our findings therefore suggest that such pheromones are not required to suppress aggression in socially housed males.

We next tested whether pheromones secreted by residents inhibit aggression by socially housed intruders. We tested whether chemogenetic activation of PR+ VMHvl neurons in socially housed cohorts of PR^{Cre} males mutant for Cnga2 or Trpc2 would enable them to attack a male resident in his homecage. Indeed, we found that the majority of male intruders mutant for Cnga2 or Trpc2 tail-rattled and initiated attacks against resident males when given CNO (Figure 6B; Figure S6C). These findings support our hypothesis that pheromones secreted by resident mice inhibit aggression by intruder males; moreover, both the VNO and MOE are non-redundantly required to detect such inhibitory pheromones. We wondered whether pheromones secreted by either male or female residents can inhibit aggression by socially housed male intruders. We therefore tested whether activating PR+ VMHvl neurons in socially housed PR^{Cre} male intruders WT for Cnga2 and Trpc2 would enable aggression toward a female resident. We found that, upon administration of CNO, socially housed male intruders both tail-rattled and initiated attacks on female residents (Figure 6C; Figure S6D). This is in contrast to the lack of aggression toward male residents (Figure 4A) and in agreement with the notion that intruder males are inhibited from initiating attacks by pheromones produced by the resident male.

Modeling Male Aggression with Logic Circuits and Neural Networks

Our findings demonstrate that many features of the external world and internal state modulate male territorial aggression. We wished to capture these variables succinctly within a logic circuit model that represented various paths leading to aggression toward conspecifics. We therefore first collected these features and their influence on aggression in a truth table (Table 1) and then set about building a model that incorporated studies involving *Mus* opponents. This resulted in a binary output variable (attack) for all remaining experiments and allowed us to assemble a logic circuit that includes every binary input variable (social experience, social setting, opponent, pheromone sensing, circulating sex hormones, and VMHvl stimulation) we tested (Figure 7A). This schematic utilizes basic logic gates (and, or, not) to represent our findings in a concise manner. Starting from the output variable, our Boolean model shows two paths to aggression so that if either pathway is active, then the male will attack. In the absence of experimental stimulation of VMHvl activity (bottom pathway, Figure 7A), all other input variables need to be active or switched on; that is, the male needs to be solitary, in his homecage or neutral arena, have circulating testosterone, sense pheromones, and face a male opponent. By contrast, when the VMHvl is stimulated (top pathway, Figure 7A), any one of four conditions is needed to make the male fight; that is, the male needs to be solitary, or in his homecage or neutral arena, or have pheromone sensing disabled, or face a female opponent. This logic circuit highlights our finding that VMHvl neurons are not command neurons and that stimulation-elicited aggression is independent of circulating testosterone.

We next wondered how our Boolean logic circuit could be recapitulated within the modeling framework of a neuron-like network. Such modeling should potentially provide insights into the underlying mechanisms at the algorithmic (Marr, 1982) but not necessarily the physical implementation level of the neural circuit underlying aggression. Moreover, neural networks can provide a biologically plausible implementation of logic circuits as well as insights into the complexity of the underlying Boolean function. We first tested whether a single-layer network could model the developmentally hard-wired nature of male territorial aggression, as schematized in the logic circuit (Figure 7A). Strikingly, we find that there is no such single-layer network (Figure 7B). This finding rules out a mechanism whereby a single integrator network generates aggression by linearly combining evidence from neuronal populations representing the different input variables shown in the logic circuit (Figure 7A). Although not all Boolean functions are realizable by single-layer networks (Minsky and Papert, 1969), adding additional layers or non-linear features to a single-layer network can fit any such function (Bishop, 1995; Hornik, 1991), given enough neurons or features. Indeed, we find that adding only two units in an additional (hidden) layer is sufficient to train the network to capture all responses (Figure 7B). Our neural network modeling shows that our experimental findings are not well modeled by a linear classifier. Rather, a cascaded or nonlinear classifier has to be invoked to model the modulation of male aggression by features of the external world and internal state. This cascading or nonlinear classifier is required to model aggression because of our finding that male pheromones inhibit aggression in socially housed intruder males (Figures 6B, 6C, and 7B; Table 1). The antagonistic functions of pheromones in promoting and inhibiting aggression could be implemented within the VMHvl itself or elsewhere within the neural circuit underlying aggression.

Figure 6. Male Pheromones Inhibit Aggression Elicited by PR+ VMHvl Neurons

An AAV encoding Cre-dependent hM3DGq-mCherry was delivered to VMHvl of PR^{Cre} adult males who were socially housed with other males and used as intruders.

(A) Castrated male intruders injected with CNO did not tail-rattle to or initiate attacks toward WT solitary resident males significantly more than when they were injected with saline. n = 15/condition.

(B) Male intruders genetically disabled for chemosensory signaling via the VNO or MOE (PR^{Cre}; $Trpc2^{-/-}$ or PR^{Cre};$Cnga2^{-/Y}$), injected with CNO but not saline, tail-rattled to and attacked WT solitary resident males. n = 15/condition ($Trpc2^{-/-}$) and 8/condition ($Cnga2^{-/Y}$).

(C) Male intruders injected with CNO but not saline tail-rattled to and attacked WT solitary resident females. n = 13/condition.

Mean ± SEM. **p < 0.01, ****p < 0.0001.

Table 1. Summary of Findings Relating to Male Aggression toward *Mus* Opponents

	Experience	Environment	VMH PR+ Neural Activity	Pheromone Sensing via the VNO or MOE	*Mus* Opponent	Gonads	Response	Experiments
1	solitary	resident	stimulated	intact	male	intact	attack	Figure 1F; Figure S1A
2	solitary	resident	saline (control)	intact	male	intact	attack	Figure 1F; Figure S1A
3	solitary	resident	inhibited	intact	male	intact	nothing	Figure S1C
4	solitary	resident	stimulated	intact	female	intact	attack	Figure 1G;Figures S1B and S1F
5	solitary	resident	saline	intact	female	intact	nothing	Figure 1G; Figures S1B and S1F
6	solitary	resident	stimulated	disabled	male	intact	attack	Figure S2B
7	solitary	resident	saline	disabled	male	intact	nothing	Figure S2B
8	solitary	resident	stimulated	disabled	female	intact	attack	Figure 2B; Figure S2A
9	solitary	resident	saline	disabled	female	intact	nothing	Figure 2B; Figure S2A
10	solitary	resident	stimulated	intact	male	castrated	attack	Figure S3B
11	solitary	resident	saline	intact	male	castrated	nothing	Figure S3B
12	solitary	resident	stimulated	intact	female	castrated	attack	Figure 3B; Figure S3A
13	solitary	resident	saline	intact	female	castrated	nothing	Figure 3B; Figure S3A
14	social	intruder	stimulated	intact	male	intact	nothing	Figure 4A; Figure S4A
15	social	intruder	saline	intact	male	intact	nothing	Figure 4A; Figure S4A
16	solitary	intruder	stimulated	intact	male	intact	attack	Figure 4B; Figure S4B
17	solitary	intruder	saline	intact	male	intact	nothing	Figure 4B; Figure S4B
18	social	resident	stimulated	intact	male	intact	attack	Figure 5; Figures S5B and S5C
19	social	resident	saline	intact	male	intact	nothing	Figure 5; Figures S5B and S5C
20	social	intruder	stimulated	intact	male	castrated	nothing	Figure 6A; Figure S6A
21	social	intruder	saline	intact	male	castrated	nothing	Figure 6A; Figure S6A
22	social	intruder	stimulated	disabled	male	intact	attack	Figure 6B; Figure S6C
23	social	intruder	saline	disabled	male	intact	nothing	Figure 6B; Figure S6C
24	social	intruder	stimulated	intact	female	intact	attack	Figure 6C; Figure S6D
25	social	intruder	saline	intact	female	intact	nothing	Figure 6C; Figure S6D

Note that the experiments summarized in rows 18 and 19 describe socially housed males that were either residents in their homecage or encountered a male in a neutral arena.

DISCUSSION

We have varied external sensory and internal physiological signals to delineate their influence on the role of PR+ VMHvl neurons in intermale aggression. Given that we have identified a genetically defined neural center that is necessary and sufficient for aggression in solitary male residents, one might expect that these neurons can elicit unbridled aggression in all other contexts. In contrast to this expectation, our studies show that social context can override a developmentally hard-wired neural pathway that is necessary and sufficient for aggression in solitary males. Moreover, our studies have uncovered inordinate richness in the rules of social engagement between male mice, rules that inhibit aggressive displays in most social contexts in WT males and that are different for solitary and socially housed males.

Function of PR+ VMHvl Neurons in Male Aggression

We previously showed that genetically targeted ablation of PR+ VMHvl neurons reduces male aggression (Yang et al., 2013). Our current findings reveal that activation of PR+ VMHvl neurons is acutely necessary and sufficient for this behavior and, moreover, that these neurons can trigger the full complement of male-typical aggression. VMHvl PR+ neurons co-express Esr1, and these Esr1+ cells are also necessary and sufficient for male aggression (Lee et al., 2014; Yang et al., 2013). Moreover, knockdown of Esr1 in the VMH reduces male aggression (Sano et al., 2013), indicative of a signaling role of this receptor in this behavior. In addition, PR (Esr1)+ neurons constitute the functionally relevant population for male aggression in this region, and VMHvl neurons are active during fighting (Falkner et al., 2014; Lee et al., 2014; Lin et al., 2011; Yang et al., 2013). Together, these findings from complementary approaches demonstrate a physiological role for PR (Esr1)+ VMHvl neurons in male territorial fighting. Chemogenetic (our study) or optogenetic stimulation that reliably triggers male aggression induces Fos in more VMHvl neurons than observed in unmanipulated males that attack (Lee et al., 2014). However, unmanipulated males do not always show aggression (see Figure 1F for example). We speculate that experimental activation of more PR/Esr1+ VMHvl neurons reliably triggers male aggression because it may elicit network dynamics that ensure aggression or stimulate a subset of PR/Esr1+ VMHvl neurons whose activation always drives aggression. Regardless of these considerations, these observations regarding male aggression were made in singly housed resident males. As we show here, activation of these neurons is not

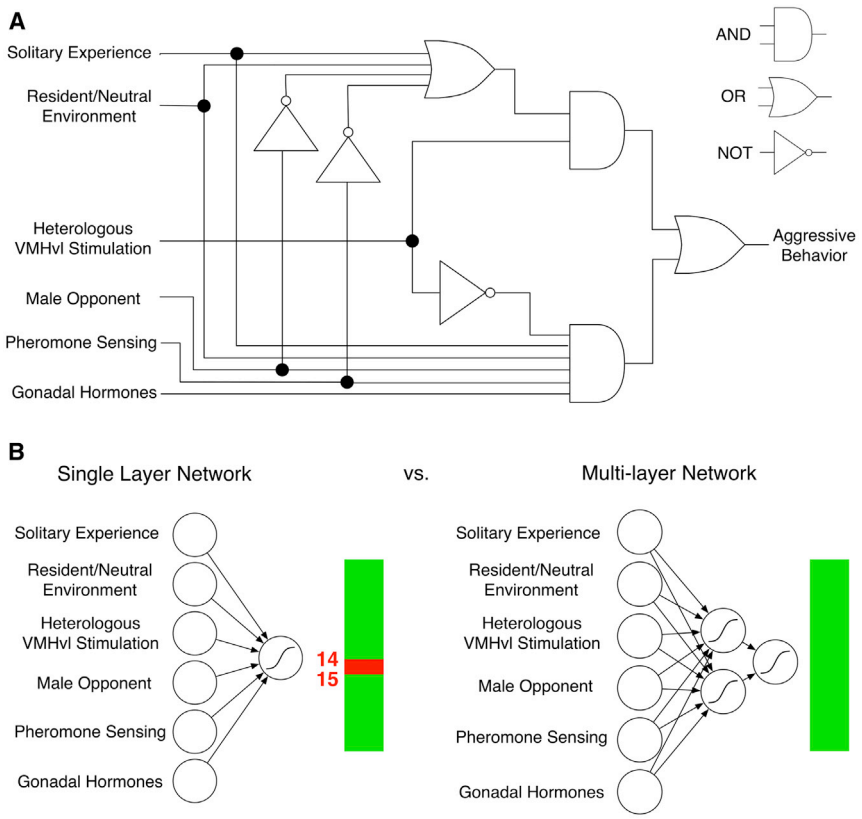

Figure 7. Modeling Male Aggression with Logic Circuits and Neural Networks

(A) Schematic of a logic circuit that summarizes results in which the output variable is attack and the opponent is a lab mouse.

(B) A two-layer but not a single-layer network adequately captures the findings summarized in the logic circuit outlined in (A) and Table 1. We determined the fewest experiments that need to be excluded for the logic circuit to be implementable by a single-layer network. This search showed that removal of only one experiment, outlined in rows 14 and 15 (Table 1), is sufficient for the logic circuit to be realized by a linear classifier. The experiment outlined in these two rows demonstrates that socially housed intruder males do not initiate attacks toward resident males even upon stimulation of PR+ VMHvl neurons. The green bars indicate findings from rows in Table 1 that can be implemented by the neural network, whereas findings from numbered rows (red) cannot be implemented by the network.

sufficient to elicit aggression in socially housed intruders, providing a fundamentally new perspective into their function in ethologically relevant settings.

What might be the function of PR+ VMHvl neurons in male territorial aggression? These cells are not command neurons (Kupfermann and Weiss, 1978) for aggression because their ability to trigger behavior is contingent on sensory input or social context. These neurons also do not encode or relay a complete motor plan for aggression because their activation elicits tail rattling but not attacks to a mirror. It is possible that PR+ VMHvl neurons are part of a decision network (Gold and Shadlen, 2007; Newsome, 1997) that accumulates evidence in a non-linear manner from various sensory modalities, physiological state, and social experience prior to relaying signals to downstream centers about whether the animal should fight. In this scenario, our results are in contrast to the classic "diffusion to bound" model in which decision dynamics are well approximated by a circuit that linearly integrates evidence from a sensory modality (Gold and Shadlen, 2002). Our findings also do not exclude the possibility that PR+ VMHvl neurons constitute one of several more or less independent neural pathways that feed on to a downstream command-type neural center whose activation state is obligatorily linked to aggression. In this instance, non-linear integration of particular input variables could occur within the VMHvl or other pathways, including a command-type center, that modulate aggression. Our modeling efforts in this study make simplifying assumptions in that we excluded findings from encounters with gloves, mirrors, and *Peromyscus* and focused on interactions with conspecifics. Such simplifying assumptions in other models (e.g., Hopfield, 1982) have nevertheless yielded important insights in the past and, in our case, reveal a non-linearity in the network that modulates innate male territorial aggression. In future studies, it will be interesting to incorporate other features into the model that enable the animal to respond to inanimate objects or other species.

Social Context Controls Territorial Aggression

In keeping with the notion that male aggression is developmentally hard-wired, many genetic loci controlling male aggression have been identified (Miczek et al., 2001; Nelson and Trainor, 2007). This hard-wiring of territorial aggression is also evident from studies showing that, barring particular strains, wild or lab female mice typically do not display such aggression, including upon stimulation of VMHvl neurons (Figure S3E; Crowcroft, 1966; Ferrari et al., 1996; Lee et al., 2014; Miczek et al., 2001; Scott, 1966). Nevertheless, male territorial aggression is sensitive to social cues and experience so that males intruding in another male's territory do not initiate fights. Despite decades of studies in mice, the mechanisms underlying these dichotomous male territorial behaviors have remained poorly understood.

Solitary males are aggressive in their homecage or a neutral arena but not when inserted in another male's cage. These observations suggest that pheromones from a male's cage inhibit attacks by an intruding solitary male. A test of this hypothesis will require molecular identification of such pheromones and their cognate receptors because disabling all pheromone sensing by the MOE or VNO also abrogates male aggression (Leypold et al., 2002; Mandiyan et al., 2005; Stowers et al., 2002; Yoon et al., 2005). We find that activating PR+ VMHvl neurons enables solitary males to attack even as intruders, thereby placing these neurons functionally downstream of potential

pheromones that inhibit their aggression. Activation of PR+ VMHvl neurons also enables solitary males to show aggressive displays in every situation we have tested. Under these conditions, therefore, activation of PR+ VMHvl neurons in solitary males appears to correspond with a decision to fight.

The control of aggression in socially housed males is more complex. Absent activation of PR+ VMHvl neurons, these males do not attack. Chemogenetic activation of these neurons elicits aggression in the homecage, in a neutral arena, and to female residents. This inhibition of aggression by social housing that can be chemogenetically bypassed may be controlled by pheromones or other sensory cues present in social housing. In flies, exposure to pheromones in social housing over the course of a few days inhibits aggression elicited by presence of a desirable resource, such as food, in a neutral chamber (Liu et al., 2011). Whether such pheromonal inhibition regulates aggression in flies in other ethologically relevant settings is unknown. More importantly, it is unclear whether manipulating the activity of central neural pathways underlying aggression in flies can override the suppression of aggression mediated by pheromones in social housing. Regardless, our findings place PR+ VMHvl neurons functionally downstream of any aggression-suppressing cues or social interactions in social housing when the male is tested as a resident or in a neutral arena or encounters a female resident. By contrast, activation of these neurons in socially housed intruders does not trigger attacks to male residents unless pheromone sensing is disabled. Thus, PR+ VMHvl neurons are effectively upstream of pheromone sensing in this particular social context. It will be interesting to determine the mechanisms whereby MOE and VNO pathways inhibit aggression in socially housed male intruders.

Nature and Nurture in the Display of Male Aggression

How nature and nurture—in the form of genetic underpinnings and social context—influence neural pathways underlying the instinctual display of male territorial aggression is an important question (Stowers and Liberles, 2016). We find that social context can effectively veto a neural pathway whose activation is otherwise necessary and sufficient for male territorial aggression. Social housing reduces aggression in many animals, indicating that it may be a general strategy to enable otherwise aggressive animals to cohabit. Distinct sensory modalities may underlie social housing-mediated changes in aggression in different species, and this reduced aggression can also be accompanied by changes in gene expression (Korzan et al., 2014; Maruska and Fernald, 2011; Maruska et al., 2013; Wang et al., 2008). In the naturally territorial male mouse, we have identified a neural locus in PR+ VMHvl neurons whose ability to drive aggression is dictated by the social setting of the encounter and prior experience. Intriguingly, the logic circuit summarizing our data shows that an elevated level of activity in PR+ VMHvl neurons is sufficient to unmask aggression when even one of the several variables normally restricting aggression is modified. It is possible that heightened activity in these neural pathways contributes to violent outbursts seen in animals with rage syndrome or humans with intermittent explosive disorder in situations that do not typically provoke aggression (Dodman et al., 1992; Rosell and Siever, 2015; Scott et al., 2016).

More generally, our study shows the importance of exploring ethologically meaningful social settings and prior experience of test animals to understand the behavioral relevance of a population of neurons. Previous work on VMH neurons had deemed them necessary and sufficient for aggression without exploring their role in different social contexts. Our study shows that, in fact, the social context in which aggression is assayed can override the sufficiency of these neurons in triggering aggression, thereby forcing a re-evaluation of their role in male aggression. We intuit that territorial aggression in animals of other species, inhabiting distinct ecological niches, is responsive to social setting and prior experience in a species-specific manner. It will be interesting to understand how sensitivity to social context is genetically selected for during evolution.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - CNO
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  - Hormone Assays
  - Building Logic Circuits and Neural Network Models for Male Aggression
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, one table, and eight movies and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2017.06.046.

AUTHOR CONTRIBUTIONS

T.Y., C.F.Y., M.D.C., and N.M.S. designed the experiments. T.Y., C.F.Y., M.D.C., M.B., S.I., and M.C.C. conducted all experiments except the electrophysiological experiments, which were conducted by K.J.B., Jr. and K.J.B. T.Y., C.F.Y., K.J.B., Jr., K.J.B., and N.M.S. analyzed the findings. T.Y., N.M., S.G., and N.M.S. designed and discussed modeling strategies in light of the findings, and N.M. implemented the subsequent modeling studies. T.Y. and N.M.S. wrote the manuscript.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rat anti-RFP	Chromotek	Cat # 5f8-100; RRID: AB_2336064
Rabbit anti-Fos	EMD Millipore	Cat # PC38; RRID: AB_2106755
Chicken anti-β-galactosidase	Abcam	Cat # 9361; RRID: AB_307210
Donkey anti-rat IgG secondary antibody, Cy3 conjugate	Jackson ImmunoResearch	Cat # 712-165-150; RRID: AB_2340666
Donkey anti-rabbit IgG secondary antibody, Alexa 488 conjugate	Invitrogen	Cat # R37118; RRID: AB_2556546
Donkey anti-chicken, Alexa 488 conjugate	Jackson ImmunoResearch	Cat # 703-545-155; RRID: AB_2340375
Bacterial and Virus Strains		
Recombinant adeno-associated virus: AAV2-EF1α-flex-hM3DGq-mCherry	UNC viral vector core	Addgene number: 50460
Recombinant adeno-associated virus: AAV2-EF1α-flex-hM4DGi-mCherry	UNC viral vector core	Addgene number: 50461
Recombinant adeno-associated virus: AAV2-EF1α-flex-mCherry	UNC viral vector core	Addgene number: 50477
Chemicals, Peptides, and Recombinant Proteins		
DAPI	Sigma-Aldrich	Cat # D9542; CAS 28718-90-3
Clozapine N-oxide (CNO)	Enzo Life Sciences	Cat # BML-NS105-0005
Estradiol	Sigma-Aldrich	Cat # E8875;CAS 50-28-2
Progesterone	Sigma-Aldrich	Cat # P0130; CAS 57-83-0
Critical Commercial Assays		
Testosterone ELISA kit	DRG	Cat # EIA-1559
Experimental Models: Organisms/Strains		
Mouse: C57BL/6J	The Jackson Laboratory	Cat # 000664; RRID: IMSR_JAX:000664
Mouse: 129/SvEvTac	Taconic Biosciences	Cat # 129SVE-M
Mouse: PR^{Cre}	Yang et al., 2013	Jax Stock 017915; RRID: IMSR_JAX:017915
Mouse: PR^{PL}	Yang et al., 2013	Jax Stock 022517; RRID: IMSR_JAX:022517
Mouse: *Trpc2*$^{-/+}$	Leypold et al., 2002	N/A
Mouse: *Cnga2*$^{-/+}$	Brunet et al., 1996	Jax Stock 002905; RRID: IMSR_JAX:002905
Peromyscus eremicus	University of South Carolina Peromyscus Stock Center	Cat # EP
Recombinant DNA		
AAV2-EF1α-flex-hM3DGq:mCherry	Gift from Bryan Roth	Addgene number: 50460
AAV2-EF1α-flex-hM4DGi:mCherry	Gift from Bryan Roth	Addgene number: 50461
AAV2-EF1α-flex-mCherry	Gift from Bryan Roth	Addgene number: 50477
Software and Algorithms		
ImageJ	NIH	https://imagej.nih.gov/ij/index.html; RRID: SCR_003070
MATLAB	MathWorks	https://www.mathworks.com/products.html; RRID: SCR_001622
GraphPad Prism 6	GraphPad Software	https://www.graphpad.com/scientificsoftware/prism/; RRID: SCR_002798
Python with the scikit-learn library	Pedregosa et al., 2011	http://scikit-learn.org/

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Nirao Shah (nirao@stanford.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

Mice were bred in a barrier facility at UCSF and Stanford University (PR^{Cre}, PR^{PL}, $Trpc2^{-/+}$, $Cnga2^{-/+}$, *P. eremicus*) or purchased from Jackson (C57BL/6J for WT female stimulus animals) and Taconic (129/SvEvTac for WT socially housed male stimulus animals). All animals were housed under a reversed 12:12 hr light:dark cycle and controlled temperature and humidity, and water and food were available ad libitum. All animal studies were performed following Institutional Animal Care and Use Committee guidelines and protocols at UCSF and Stanford.

METHOD DETAILS

Viruses

AAV2/1-EF1α-flex-hM3DGq:mCherry, AAV2/1-EF1α-flex-hM4DGi:mCherry, and AAV2/1-EF1α-flex-mCherry were purchased from the UNC Vector Core or custom packaged by the UNC Vector Core with maxi-prepped plasmid DNA that was originally purchased from Addgene. Virus titers were 1.5-2.5 × 10^{12} genomic copies/mL.

Stereotaxic Surgery

Viruses were stereotaxically delivered to the brain of male or female mice at 2-6 months of age exactly as described previously (Yang et al., 2013). Mice were allowed to recover in a clean cage on a heated pad, closely monitored, and returned to their home cage when ambulatory. Animals were allowed at least 10 days of recovery following surgery prior to being tested in behavioral assays.

Histology

We validated viral delivery by confirming that virally delivered DREADDs (mCherry+) were expressed within the VMHvl in all experimental animals using standard histological procedures (Yang et al., 2013). We had previously established the stereotaxic coordinates and viral serotype (AAV2/1) for reliable infection of PR+ VMHvl neurons such that we observed very little infection of other PR+ neurons elsewhere in the hypothalamus (Yang et al., 2013). Using these stereotaxic coordinates and the identical serotype for our DREADD-encoding AAVs, we once again reliably achieved bilateral infection of VMHvl neurons in our PR^{Cre} animals. The specificity of this infection was demonstrated by lack of DREADD (mCherry) expression in WT animals following injection of these viruses (Figure S1A). In rare cases (< 5%), some animals were found dead in their cage and we could not histologically verify DREADD expression in the VMHvl; in these instances, behavioral data from these animals was not included for further analysis. For histological analysis following termination of behavioral studies, animals were perfused with 4% paraformaldehyde, and the brains were dissected and post-fixed for at least 2 hr in 4% paraformaldehyde. Brains were sectioned at 65 μm thickness using a vibrating microtome (Leica) and immunostaining was performed as described previously (Yang et al., 2013). The primary antisera used are rat anti-RFP (Chromotek; 1:2,000), rabbit anti-Fos (Calbiochem; 1:2,000), and chicken anti-β-galactosidase (Abcam; 1:3,000). The secondary antisera used are: Cy3 donkey anti-rat (Jackson ImmunoResearch; 1:800), Alexa Fluor 488 donkey anti-rabbit (Invitrogen; 1:300), and Alexa Fluor 488 donkey anti-chicken (Jackson ImmunoResearch; 1:300). The sections were also counterstained with DAPI (0.2 μg/mL). To examine Fos induction by hM3DGq, a subset of animals was perfused 1 hr after intraperitoneal (IP) administration of saline or 0.3 mg/kg of CNO. This Fos induction paradigm closely mirrors the procedure we followed for behavioral studies in which we tested the behavior of animals 30 min after CNO injection; these behavioral studies were conducted for 15 min, and unless otherwise mentioned, we also used 0.3 mg/kg of CNO. Sections were imaged via confocal microscopy (LSM780 or LSM880, Zeiss). Neurons expressing hM3DGq, Fos, or nβgal were imaged using confocal microscopy and quantified using NIH ImageJ software as described previously (Unger et al., 2015; Yang et al., 2013).

Electrophysiology

Electrophysiological response of PRCre neurons to CNO was determined as described previously (Unger et al., 2015). Briefly, AAV2-EF1α-flex-hM3DGq:mCherry or AAV2-EF1α-flex-mCherry was injected bilaterally into the VMH of PR^{Cre} males or females 8 weeks of age. The mice were allowed to recover > 10 days from surgery and then sacrificed for brain slice preparation. 225 μm-thick coronal slices were cut in ice-cold HEPES buffer solution, incubated for 15 min at 33°C in NMDG recovery solution, and maintained for 1-5 hr at room temperature in HEPES buffer solution. HEPES solution composition (in mM): 92 NaCl, 2.5 KCl, 1.2 NaH2PO4, 30 NaHCO3, 20 HEPES, 25 Glucose, 5 Na^+ Ascorbate, 2 Thiourea, 3 Na^+ Pyruvate, 10 MgSO4, 0.5 CaCl2, 305 mOsm, 7.3-7.4 pH. NMDG solution was identical to HEPES, with an equimolar replacement of NaCl for NMDG. Neurons were then selected for recording based on mCherry expression visualized with 2-photon or conventional fluorescent microscopy. Whole cell current-clamp recordings were made using the following recording solutions (in mM): *Internal*: 9 HEPES, 113 K-Gluconate, 4.5 MgCl2, 0.1 EGTA, 14 Tris-phosphocreatine, 4 Na2ATP,

0.3 Tris-GTP, 10 Sucrose, 290 mOsm, 7.2-7.25 pH ; *External*: 125 NaCl, 2.5 KCl, 1 MgCl2, 1.25 NaH2PO4, 25 NaHCO3, 25 Glucose, 2 CaCl2, 305 mOsm, 7.25-7.30 pH. Reported Vm was not corrected for junction potential (12 mV). Cells with an unstable baseline membrane potential, defined as a significant ($p < 0.05$) linear regression in Vm > ± 0.5 mV/min, were excluded from analyses.

CNO

CNO stock solution was prepared by dissolving CNO (Enzo) in sterile saline at 5 mg/mL, kept as frozen aliquots, and freshly diluted with sterile saline prior to intraperitoneal (IP) administration. The final dose of CNO for hM3DGq and hM4DGi studies was 0.3 mg/kg and 3 mg/kg, respectively, unless mentioned otherwise. Saline (vehicle) or CNO were administered in a randomized manner to each animal during successive trials. Assays were initiated 30 min after IP injection of CNO or saline.

Behavioral Assays

Behavioral assays were performed in the dark cycle (≥1 hr after lights out), recorded using camcorders (Sony) under infrared illumination, and analyzed as previously described (Yang et al., 2013). All experimental males were group housed by sex after weaning until they were to be injected with virus. Thus, solitary males were singly housed for > 10 days prior to behavioral testing. These males had been socially housed prior to stereotaxic viral injections and were subsequently singly housed. They were tested for mating for 15 min with a WT socially housed stimulus female that was hormonally primed to be in estrus as described previously (Wu et al., 2009). Solitary males were tested for aggression with WT socially housed stimulus males for 15 min as described previously (Yang et al., 2013). Solitary males to be tested as intruders in another solitary male's homecage were injected with saline or CNO and, 30 min later, were inserted into the resident's homecage for 15 min. Tests of aggression toward inanimate objects lasted 10 min with the object in the homecage; a custom sized mirror that fit against the short wall of the cage or partially inflated glove was inserted into the cage of a solitary male that had been administered saline or CNO. Solitary mice were tested once each with saline and CNO in a randomized manner, with the assays separated by 2-3 days. CNO or saline was administered to the solitary male in his homecage 30 min prior to the beginning of the assay.

Socially housed males had never been housed singly and, after recovery from surgery, continued to cohabit in same sex groups of 4-5 mice/cage. Prior to testing these males in behavioral assays, they were injected with saline or CNO, immediately moved to a clean cage for 30 min to preclude any social interactions with cagemates, and then inserted into a solitary male's cage (as an intruder in resident-intruder test) or clean cage (neutral arena) or returned to their homecage after removing the other cagemates (as a resident in resident-intruder test). Solitary males who received an experimental intruder male (PR^{Cre}) in their homecage were experienced fighters. Tests of aggression lasted 15 min. Socially housed males were tested once each with saline or CNO in a randomized manner.

Females used as stimulus animals in mating assays were ovariectomized as described previously (Wu et al., 2009) and estrus was induced via subcutaneous administration of 10 μg of 17 β-estradiol benzoate (Sigma) in 100 μL of sesame oil on day −2, 5 μg of 17 β-estradiol benzoate in 50 μL of sesame oil on day −1, and 50 μg of progesterone (Sigma) in 50 μL of sesame oil on day 0, the day of behavioral testing.

In some cases, PR^{Cre} males were surgically castrated and allowed to recover for ≥ 3 weeks to eliminate gonadal hormones from the circulation. These males had been previously injected with virus into the VMH. Some males were singly housed following castration whereas others continued to be socially housed; these males were used as solitary residents or socially housed intruders as described in the main text.

In experiments where we used PR^{Cre} females as residents, these females had been socially housed with other females post-weaning. During stereotaxic delivery of the virus into the VMH, these females were ovariectomized and allowed to recover in social housing for 2 weeks. They were subsequently singly housed for 10-12 days and then tested for behavioral responses for 15 min to male or female intruders 30 min after intraperitoneal injection of saline or CNO.

Behavior in elevated-plus maze was tested as described previously (Unger et al., 2015). In brief, 30 min following saline or CNO administration, a solitary male was placed in the common center area of the elevated plus maze. His behavior was recorded for 5 min and analyzed subsequently as described before (Unger et al., 2015).

Hormone Assays

Serum testosterone level was measured using an ELISA kit (EIA1559, DRG International) following the manufacture's instruction. Cardiac blood from castrated and control males were collected at the time of perfusion and serum was obtained as described previously (Wu et al., 2009).

Building Logic Circuits and Neural Network Models for Male Aggression

To model the experimental results, each feature (experience, environment, VMHvl stimulation, opponent, pheromone sensing, and circulation of hormones) and the response (attack behavior) was treated as a binary variable ('0' or '1'). The Boolean circuit was designed by manual inspection of the truth table, ensuring that it satisfied each of the rows (experiments). Other Boolean circuit configurations could presumably also account for our findings.

The single layer network was trained as a logistic regression model, where the model prediction consisted of a weighted sum of the binary features, passed through a sigmoidal threshold. The multi-layer network consisted of two cascaded layers of the single layer architecture. To train each model, we minimized the model prediction error plus a small penalty to constrain the weight scale (all

results did not depend on the choice of this penalty). This minimization was done via batch gradient descent using the scikit-learn software package. To identify the experiment(s) that precludes linear classification from explaining the data, we iteratively removed each experiment from the table and re-trained the single layer model to determine if removing a particular example would allow the rest to be linearly classified. For neural network modeling, we treated each input variable (such as pheromone sensing or opponent) as a single categorical entity. However, we note that even splitting up pheromone sensing into two, one inhibiting aggression and the other promoting it, during modeling did not yield a single layer network capable of capturing all the findings in the truth table.

QUANTIFICATION AND STATISTICAL ANALYSIS

Recorded videos and histological samples were analyzed blind to relevant variables, including administrated solution, genotype, sex, prior housing condition, surgical procedure, and virus injected. Videos were played at 30 frames per second and manually annotated using custom software as previously described (Wu et al., 2009). In particular, anogenital investigation (sniff), mounting, repeated pelvic thrust (intromission), and ejaculation were scored for male sexual behaviors. Aggression was scored when a physical attack (episodes of biting, wrestling, or chasing) was observed, and various parameters of attack (latency, number, inter-attack interval, and duration) were subsequently calculated. Importantly, tail rattling was always scored as a distinct event such that a tail rattle was not considered as an episode of physical attack. Similarly, we only included physical attacks for analysis in our modeling studies. Distinct events during physical attacks such as biting the neck, biting the flank, chasing, or wrestling were also annotated separately for a subset of animals to analyze specific patterns of attack for Figure S1H.

Statistical analysis was performed using MATLAB or GraphPad PRISM (GraphPad Software). To compare categorical data, including percentage of males initiating attack and percentage of males tail rattling, Fisher's exact test was performed from a 2x2 contingency table. For non-categorical data, behavioral parameters such as number of attack, latency to first attack, mean duration of attack, total duration of attack, and inter-attack interval were analyzed only from animals who displayed behaviors. We first determined if the data values came from a normal distribution with D'Agostino-Pearson omnibus normality test. In experiments with paired samples, we used a paired t test and Wilcoxon matched-pairs signed rank test for parametric and non-parametric data, respectively. In all other experiments, we used a t test, t test with Welch's correction for unequal standard deviation, or ANOVA for parametric data and a Mann-Whitney or Kruskal-Wallis test for non-parametric data.