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Sexually dimorphic neurons in the ventromedial hypothalamus govern mating in both sexes and aggression in males

Cindy F. Yang[1,2], **Michael Chiang**[2], **Daniel C. Gray**[3], **Mahalakshmi Prabhakaran**[2], **Maricruz Alvarado**[2], **Scott A. Juntti**[1], **Elizabeth K. Unger**[4], **James A. Wells**[3,5], and **Nirao M. Shah**[1,2,4]

[1]Program in Neuroscience, University of California, San Francisco, San Francisco, CA 94158

[2]Dept. of Anatomy, University of California, San Francisco, San Francisco, CA 94158

[3]Program in Chemistry and Chemical Biology, University of California, San Francisco San Francisco, CA 94158

[4]Program in Biomedical Sciences, University of California, San Francisco San Francisco, CA 94158

[5]Depts. of Pharmaceutical Chemistry and Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco, CA 94158

SUMMARY

Sexual dimorphisms in the brain underlie behavioral sex differences, but the function of individual sexually dimorphic neuronal populations is poorly understood. Neuronal sexual dimorphisms typically represent quantitative differences in cell number, gene expression, or other features, and it is unknown if these dimorphisms control sex-typical behavior in one sex exclusively or in both sexes. The progesterone receptor (PR) controls female sexual behavior, and we find many sex differences in number, distribution, or projections of PR-expressing neurons in the adult mouse brain. We have ablated one such PR-expressing neuronal population located in the ventromedial hypothalamus (VMH) using a novel genetic strategy. Ablation of these neurons in females greatly diminishes sexual receptivity. Strikingly, the corresponding ablation in males reduces mating and aggression. Our findings reveal the functions of a molecularly-defined, sexually dimorphic neuronal population in the brain. Moreover we show that sexually dimorphic neurons can control distinct sex-typical behaviors in both sexes.

INTRODUCTION

Males and females show sex differences in many behaviors, including mating and aggression, that result from sexually dimorphic development or activation of the underlying neural circuits. Gonadal sex hormones exert a profound influence on vertebrate sex-typical

*Correspondence: nms@ucsf.edu.

behaviors by controlling sex differences in the brain (Cooke et al., 1998; Dewing et al., 2003; Gagnidze et al., 2010; Jazin and Cahill, 2010; McCarthy and Arnold, 2011; Morris et al., 2004; Simerly, 2002; De Vries, 1990; Xu et al., 2012; Yang et al., 2006). Most behaviors and neural circuits are shared between the sexes such that sexually dimorphic neuronal clusters represent a small fraction of the neurons within larger brain regions. It has therefore been difficult to discern which dimorphic, hormone-responsive neurons in the brain control each of the various sex differences in physiology and behavior. In addition, neuronal sex differences usually represent quantitative rather than allor-nothing dimorphisms in gene expression or cytological features. It is presently unclear whether such groups of dimorphic neurons regulate gender-typical behaviors in one or both sexes.

Progesterone controls female reproduction, including sexual receptivity, by signaling via its cognate receptor, PR (Levine et al., 2001; Mani et al., 1997). PR is widely distributed in the brain, and the PR+ neurons that regulate sexual receptivity remain to be identified unambiguously (Blaustein and Feder, 1979; Olster and Blaustein, 1990; Quadros et al., 2008). The VMH, which contains a small pool of PR+ neurons in its ventrolateral division (VMHvl), is well characterized for its relevance to female mating in mammals (Blaustein, 2008; Cohen and Pfaff, 1992; Flanagan-Cato, 2011; Rubin and Barfield, 1983). Studies with c-Fos suggest that many VMHvl neurons, including a subset of PR+ neurons, are activated following female mating (Flanagan-Cato et al., 2006). However, lesions or manipulations of neuronal activity of the VMH can lead to no change, a decrease, or an increase in female sexual behavior (Goy and Phoenix, 1963; Kow et al., 1985; Leedy and Hart, 1985; Mathews and Edwards, 1977a, 1977b; Musatov et al., 2006; Pfaff and Sakuma, 1979a, 1979b; Robarts and Baum, 2007; La Vaque and Rodgers, 1975). Some studies also report a concurrent increase in body weight, suggesting a complex role of this region in feeding and mating (King, 2006; Musatov et al., 2007). This phenotypic diversity is likely due to manipulations that variably affect the heterogeneous neuronal subsets within the VMH (Kurrasch et al., 2007), adjacent brain regions, and fibers of passage. Given these challenges, the identity and function of VMHvl neurons that specifically influence female mating remain unclear.

In accord with the notion that the VMHvl influences female sexual behavior, the VMHvl exhibits quantitative cell and molecular sex differences (Dugger et al., 2007; Grgurevic et al., 2012; Matsumoto and Arai, 1983, 1986; Patisaul et al., 2008; Wu et al., 2009; Xu et al., 2012). Intriguingly, lesions or manipulations of neural activity of the VMH or the surrounding neurons have long suggested an important role of this region in controlling aggression (Hess and Akert, 1955; Kruk et al., 1979; Reeves and Plum, 1969; Wheatley, 1944). In fact, this region is activated during male aggression, and correspondingly, electrical activation or inhibition elicits or inhibits fighting, respectively (Kollack-Walker and Newman, 1995; Lin et al., 2011; Veening et al., 2005). However, as with VMH neurons that regulate female receptivity, the identity of VMH neurons that influence aggression is unknown. In principle, these behaviors may be regulated by a single set or by non-overlapping sets of neurons.

We utilized genetic strategies in mice to visualize PR+ neurons and to assess their contributions to mating and aggression. We find many sex differences in PR+ neurons in the adult brain, including in the VMHvl. We have developed a Cre-loxP strategy to ablate any

molecularly defined neuronal population via targeted viral delivery of a genetically engineered caspase. Using this approach, we have ablated PR+ VMHvl neurons in adult females and observe a dramatic reduction in sexual receptivity. The corresponding ablation in males reduces mating and territorial aggression. Thus our results define a role of PR+ VMHvl neurons in sex-typical behaviors. Moreover, we establish that a discrete, sexually dimorphic neuronal population influences sexually dimorphic behaviors in both sexes.

RESULTS

Visualizing PR expression in the mouse brain

We wished to identify PR+ neurons at high cellular resolution. We inserted an *IRES-PLAP-IRES-nuclear LacZ* (*PL*) reporter into the 3' UTR of *PR* using gene targeting (Figures 1A, B). As described previously (Shah et al., 2004), this cassette permits expression of placental alkaline phosphatase (PLAP), which labels neuronal processes, and nuclear targeted β-galactosidase (β-gal) in PR+ cells. This strategy maintains the expression and function of PR and permits examination of PR+ neurons in otherwise wildtype (WT) mice. Accordingly, and in contrast to $PR^{-/-}$ mice (Chappell et al., 1997; Lydon et al., 1995), $PR^{PL/PL}$ females were similar to WT females in fecundity and also maintained normal sex hormone titers (Figure 1C–G).

In the forebrain, we observed β-gal activity in pools of neurons in specific hypothalamic nuclei, posterodorsal medial amygdala (MeApd), medial division of the posteromedial bed nucleus of the stria terminalis (BNSTmpm), various cortical areas, basal ganglia, and dentate gyrus (Figures 1H–M, 2, S1). This distribution of cells mirrors the expression pattern of PR mRNA in adjacent sections (Figure 1H–M). In regions such as the basal ganglia with low level PR expression that precludes visualization by in situ hybridization, we can detect PR message by RT-qPCR (Figure S1A). The distribution of β-gal+ cells is in accord with histological and pharmacological studies (Becker, 1999; Blaustein and Feder, 1979; Olster and Blaustein, 1990; Quadros et al., 2008). In the case of the basal ganglia, our studies localize PR expression to sparsely distributed neurons across the rostrocaudal axis (Figure S1B–D). In addition, we find unreported PR+ neuronal pools scattered within the basal forebrain (Figure 2), an observation confirmed by RT-qPCR from this region (Figure S1A). The ~1 week $t_{1/2}$ of β-gal in neurons precludes detection of PR mRNA changes across the 4–6 day estrous cycle (Allen, 1922; Smith et al., 1995). However, the long $t_{1/2}$ and superb signal:noise of β-gal labeling allows sensitive detection of PR expression. Together, the PR^{PL} reporter mouse confirms and extends previous reports of PR expression in the mouse brain.

Widespread sex differences in the distribution and cell number of PR+ neurons

We observed previously unreported as well as known sex differences in PR+ cells in the adult PR^{PL} brain (Figures 2, S2A, and Table S1). We found more PR+ cells in the female preoptic area (POA), the adjacent anteroventral periventricular hypothalamic nucleus (AVPV), arcuate nucleus, and VMHvl (Figure 2A–L). The VMHvl contains cells expressing the estrogen receptor alpha (ERα or Esr1) (Xu et al., 2012), and we find that >92% PR+ neurons co-label for ERα in both sexes (Figure S2B). We asked whether PR+ VMHvl

neurons expressed Cckar, a GPCR required for sexual receptivity and expressed in the female but essentially absent in the male VMHvl (Xu et al., 2012). We observed that 67% ± 3 (Mean ± SEM) of PR+ VMHvl cells co-labeled with Cckar whereas 96% ± 0.2 of Cckar+ VMHvl cells were PR+ (n = 3 $PR^{PL/PL}$ females, ≥500 cells analyzed/brain) (Figure S2C–E). Thus PR+ neurons represent the vast majority of VMHvl neurons that express *Cckar*, a gene required for female mating.

We observed many clusters of PR+ cells (~15–40 cells/cluster) in the male but not female basal forebrain (Figure 2M–P). Together with a sex difference in androgen receptor expression in this region (Shah et al., 2004), our findings suggest an unappreciated role of the basal forebrain in responding to sex hormones. We also found more PR+ cells in the male BNSTmpm and MeApd (Figure 2Q–X). This increased PR expression is surprising because there is little circulating progesterone in males; our findings are nevertheless consistent with studies indicating a role of PR in male behaviors (Phelps et al., 1998; Schneider et al., 2005, 2009; Witt et al., 1995). As suggested previously (Mani et al., 1994a; Power et al., 1991; Tsutsui, 2012), PR may function in a progesterone- independent manner or locally synthesized progesterone may activate PR in males. Consistent with these sex differences in PR expression, the POA, BNSTmpm, MeApd, arcuate nucleus, and VMHvl have been implicated in sex differences in behavior or physiology (Cooke et al., 1998; Morris et al., 2004; Simerly, 2002), and PR+ neurons in these regions could contribute to such sexually dimorphic output.

We find that the dimorphic PR+ cells co-label with pan-neuronal markers (Figure S2F). However, within any given brain region expressing PR dimorphically, only a subset of neurons is PR+. Even within the VMHvl, only 49% ± 4 of NeuN+ cells co-label with PR (n = 3 brains, ≥10^3 NeuN+ cells analyzed for PR/brain). There is a sex difference in the soma size of thionin-labeled neurons within the rat VMHvl (Dugger et al., 2007). However, there was no such sex difference in PR+ VMHvl neurons (Figure S2G), suggesting a species difference or that other VMHvl neurons account for this dimorphism. The sex differences in PR expression cannot result solely from sex differences in neuronal numbers. Indeed, no sex difference in neuronal number has been reported in the basal forebrain or VMHvl, and in the POA and arcuate nucleus, which contain more neurons in males (Gorski et al., 1980; Leal et al., 1998), we find more PR+ neurons in females. Finally, the 3–4 fold more PR+ neurons in the male BNSTmpm and MeApd exceeds the <2-fold more neurons in these regions in males (Morris et al., 2008; Shah et al., 2004; Wu et al., 2009). Thus, our studies confirm known sex differences (POA, VMHvl, arcuate nucleus, MeApd) (Blaustein et al., 1980; Brown et al., 1996; Grgurevic et al., 2012; Kudwa et al., 2009; Quadros et al., 2002) and reveal new sexual dimorphisms in PR expression (basal forebrain, BNSTmpm) in the mammalian brain.

Visualizing sex differences in projections of PR+ neurons

We determined whether sexually dimorphic PR+ neurons projected to distinct locations in the two sexes. Consistent with PR expression in interconnected regions such as the POA, BNST, MeA, and VMHvl, we observed a rich distribution of PLAP+ fibers in the $PR^{PL/PL}$ forebrain (data not shown) that precluded identification of dimorphic projection patterns.

We devised a genetic strategy to visualize the projections of any subset of PR+ neurons. We first targeted an *IRES-Cre recombinase* cassette to the 3' UTR of *PR* (Figures 3A, S3A, B). As expected, these *PR-IRES-Cre* (PR^{Cre}) mice, like PR^{PL} mice, are viable and fertile, and Cre expression mirrors that of PR in the brain (Figure S3C–F). We also designed a lentiviral vector that expresses PLAP in a Cre-dependent manner (Lenti-lxlplap, Figures 3A, S3G). This lentivirus is replication-incompetent and integrates into the host genome, properties that restrict PLAP expression to Cre+ cells for the life of the cells. This virus infects cells in both WT and PR^{Cre} mice, but we only observe PLAP expression in PR^{Cre} mice (Figure 3B–E).

The VMH has been implicated in sex-specific behaviors, and we therefore traced the projections of PR+ VMHvl neurons in adults. We initially determined that we could visualize maximal expression of PLAP 7–8 days following delivery of Lenti-lxlplap into the VMH (CFY, unpublished observations). Such injections revealed the soma and local arbors of PR+ VMHvl neurons (Figure 3F–I). In contrast to the wide-ranging projections of the entire VMH (Saper et al., 1976; Krieger et al., 1979), we observed PLAP+ projections of PR + VMHvl neurons in the AVPV and adjacent periventricular area, POA, and periaqueductal gray (PAG) (Figure 3J–U). Unlike PR+ VMHvl projections in the guinea pig (Ricciardi and Blaustein, 1994), mouse PR+ VMHvl neurons did not appear to project appreciably to the BNST or MeA, suggesting subtle species differences in these cells. Although we observed similar localization of PLAP+ projections of PR+ VMHvl neurons in both sexes (Figures 3J–W, S3H, and Table S2), there was a striking, previously unreported 7-fold increase in PLAP+ fibers in the female AVPV (Figure 3J–M). This sex difference cannot solely result from the dimorphism (~30%) in PR+ VMHvl cell number. In fact, we even observed the dimorphic AVPV projection in PR^{Cre} females in whom a few PR+ VMHvl neurons had been infected. Thus, more PR+ female VMHvl neurons project to the AVPV or their axonal termini arborize more extensively. The AVPV is thought to control ovulation, and the PAG can regulate sexual receptivity in females (Sakuma and Pfaff, 1979; Simerly, 2002). In summary, PR+ VMHvl neurons project to a subset of VMH targets, their efferents are sexually dimorphic, and each of their targets can influence sexually dimorphic behaviors or physiology.

A novel genetic approach to ablate adult neurons in vivo

We determined the requirement of PR+ VMHvl neurons in sex-typical behaviors by targeting Cre-dependent, virally encoded toxins to the VMHvl of PR^{Cre} mice. Initial studies suggested that virally encoded diphtheria toxin A or tBid (Jiang and Wang, 2004; Maxwell et al., 1986) were partially effective in ablating PR+ neurons in vivo even though they were effective in tissue culture cells (CFY, unpublished observations). We therefore employed a genetically engineered caspase-3, a caspase whose activation commits a cell to apoptosis, to kill adult neurons in vivo (Figure 4A) (Gray et al., 2010). Endogenous caspase-3 normally exists as pro-caspase-3, and apoptotic signals activate upstream caspases that cleave pro-caspase-3 into its active form (Figure 4A). Our designer pro-caspase-3, pro-taCasp3, lacks the cleavage site for upstream caspases and encodes a cleavage site for the heterologous enzyme Tobacco Etch Virus protease (TEVp). Provision of TEVp activates pro-taCasp3 into the apoptosis-inducing taCasp3. We generated an adeno-associated virus (AAV) to drive

expression of pro-taCasp3 and TEVp in a Cre-dependent manner (Figures 4B, S4A) (Atasoy et al., 2008). This virus (AAV-flex-taCasp3-TEVp) utilizes the T2A peptide encoding sequence to ensure bi-cistronic expression of pro-taCasp3 and TEVp. Importantly, taCasp3 triggers cell-autonomous apoptosis, thereby minimizing toxicity to adjacent non-Cre+ cells (Gray et al., 2010).

Infection of HEK293T cells with this virus leads to rapid Cre-dependent cell death (Figure 4C, D). We next tested whether this virus could ablate adult PR+ neurons by stereotaxically targeting it to the VMHvl of adult $PR^{+/PL}$ or $PR^{Cre/PL}$ mice. PR+ VMHvl neurons appeared unaffected in controls but were essentially completely lost in $PR^{Cre/PL}$ mice 2–4 weeks following viral delivery (Figures 4E, F, S4B). We tested whether the taCasp3-encoding AAV targeted to the VMHvl diffused to and ablated PR+ cells in distant hypothalamic regions. We therefore enumerated PR+ cells along the rostrocaudal extent of the hypothalamus in a cohort of virally injected control and PR^{Cre} mice. This analysis revealed no difference in PR+ cell counts between PR^{Cre} and control females (number of PR+ cells: Control, 619 ± 60 and PR^{Cre}, 679 ± 150; n = 5/cohort, p = 0.7). Thus taCasp3-mediated ablation appears restricted to the vicinity of the injection site. We observed local spread of the virus to the arcuate and present these findings below. In separate experiments, we found that stereotaxic delivery of the taCasp3-encoding virus ablated Cre+ neurons in different brain regions (CFY, EKU, and MC, unpublished observations), indicating that we have devised a general strategy for targeted ablation of Cre+ cells.

The dimorphic PR+ VMHvl cluster of neurons regulates female sexual behavior

We tested the role of PR+ VMHvl neurons in female mating. We targeted AAV-flex-taCasp3-TEVp bilaterally to the VMHvl of adult PR^{Cre} and control females (Figure 5A). To assure optimal sexual receptivity, females were ovariectomized at the time of viral injection and, following recovery, hormonally primed to be in estrus when tested with WT males.

We observed a marked diminution of female sexual behavior in such PR^{Cre} females (Figure 5B–G, Movies S1 and S2). As in many vertebrates, female mating in mice is stereotyped and includes permitting the male to approach and mount and dorsiflexing the neck and back (lordosis) upon sensory stimulation to the dorsum (Harvey, 1651; McGill, 1962). This allows the males to intromit (penetrate, as determined by his thrust pattern) and attempt ejaculation. PR^{Cre} females rejected mount attempts by kicking or running away (Figure 5B), thereby reducing the fraction of mounts that progressed to intromission (receptivity index, Figure 5C). In sharp contrast to controls, PR^{Cre} females walked around during intromission, lordosed rarely, and with a >20-fold reduction in lordosis duration (Figures 5D–F). This reduced sexual behavior of PR^{Cre} females affected the WT male partner's performance (Figure 5H–J). Males were interested in both PR^{Cre} and control females, initiating anogenital sniffing, mounting, and intromission equivalently, but were less successful in ejaculating with the former (Figures 5H, S5A, S5B). Accordingly, males intromitted only briefly with PR^{Cre} females even though they mounted the females more and for longer duration (Figure 5I, J). Correspondingly the total duration of intromission per assay was also reduced (Control, 279 s ± 41 and PR^{Cre}, 121 s ± 19; n ≥ 10, $p = 3\times10^{-3}$). In summary,

targeted ablation of adult PR+ VMHvl neurons leads to a significant diminution in female mating.

We next assessed the ablation of PR+ VMHvl cells in these PR^{Cre} females. We observed that most (97% ± 1; n = 10 control and 16 PR^{Cre} females) PR+ VMHvl neurons were ablated upon injection of the taCasp3-encoding AAV into PR^{Cre} females (Figure 5G). Co-injection of this AAV and a constitutively expressed EGFP-encoding AAV revealed spread to the adjacent arcuate nucleus, which contains PR+ neurons (Figure 2I–L) and controls feeding and the estrous cycle (Atasoy et al., 2012; Simerly, 2002). Consistent with the lack of estrous cycle or body weight phenotypes in PR^{Cre} mice (see below and Figure S5), our injections spared most PR+ arcuate neurons in PR^{Cre} females (74% ± 12 of controls). There was no correlation in the extent of loss of PR+ arcuate neurons and reduced sexual receptivity ($R^2 = 5\times10^{-3}$, p = 0.8). Moreover, we found that PR^{Cre} females (n = 7) in whom the number of PR+ arcuate neurons was indistinguishable from controls also rejected males and displayed reduced sexual receptivity (Rejections/assay: Controls, 1 ± 1 and PR^{Cre} females, 35 ± 7; $p \leq 6 \times 10^{-5}$, n ≥ 7; Receptivity Index: Controls, 0.5 and PR^{Cre} females 0.2 ± 0.1, $p \leq 3 \times 10^{-3}$, n ≥ 7). Thus PR+ VMHvl neurons are required for normal female sexual behavior.

We tested the specificity of the behavioral deficit in PR^{Cre} females following ablation of PR + VMHvl neurons. Despite their reduced sexual receptivity, these mice sniffed and groomed males normally (Figure S5C, D) (Groom duration: Control, 2 s ± 1 and PR^{Cre}, 5 s ±1, n ≥ 10, p ≥ 0.3). There were no overt deficits in tests of anxiety, motivated behavior, motor coordination, and locomotor activity (Figure S5E–H). In contrast to the weight gain subsequent to a VMH lesion (Dhillon et al., 2006; Hetherington and Ranson, 1940; King, 2006; Majdic et al., 2002), PR^{Cre} females maintained body weight similar to controls upon ablation of PR+ VMHvl neurons (Figure S5I). Thus, we have partitioned the VMHvl to reveal that PR+ VMHvl neurons are required for normal levels of female sexual receptivity but not for all social or other behaviors and physiology.

In separate studies we ablated PR+ VMHvl neurons but left the ovaries intact to examine whether other female-typical behaviors are regulated by these neurons. This ablation did not disrupt the estrous cycle as assayed by vaginal cytology (Figure S5J). To test for maternal behaviors, we obtained litters from PR^{Cre} and control females by co-housing them with WT males. Similar to control females, PR^{Cre} females displayed various elements of maternal care toward their litters, including pup retrieval and aggression toward unfamiliar intruders in their cage (Figure S5K–O). Our results therefore show that ablation of PR+ VMHvl neurons reduced female sexual displays without overt disruption of other female-typical behaviors and physiology.

PR regulates female mating (Lydon et al., 1995), and our findings suggest that it functions in the VMHvl to do so, consistent with prior work (Mani et al., 1994a, 1994b; Ogawa et al., 1994; Pollio et al., 1993). Cckar is also required for female mating (Xu et al., 2012). Most Cckar+ VMHvl neurons are PR+ (Figure S2C–E), resulting in a near-complete loss of these cells upon ablation of PR+ VMHvl neurons (Figure 5K, L). It is possible that PR or Cckar act elsewhere to control female mating, and these genes only mark a pool of VMHvl

neurons that controls this behavior. We favor a more parsimonious model in which PR and Cckar function in the VMHvl to regulate female mating. In any event, our findings show that PR+, Cckar+ VMHvl neurons are essential for high, WT levels of female sexual behavior.

The dimorphic PR+ VMHvl cluster of neurons regulates mating and aggression in males

The VMHvl has been implicated in regulating female mating and male fighting. PR+ neurons represent ~50% of VMHvl neurons and these regulate female mating (Figure 5), but fighting could be controlled by PR+ or PR- VMHvl cells. We tested whether PR+ VMHvl neurons regulate male behaviors by ablating them with the taCasp3-encoding AAV (Figure 6A). PR^{Cre} and control males were allowed to recover for 4 weeks following viral delivery, singly housed, and tested for mating and fighting.

PR^{Cre} and control males initiated mounting intruder females equivalently, but PR^{Cre} males were less likely to intromit (Figures 6B and S6A). The reduced intromissions likely resulted from the fewer mounts exhibited by PR^{Cre} males (Figure 6C). Even when these males intromitted, there was a decrease in the number and duration of intromissions (Figures 6C, D, S6B). The decreased intromission count was significant ($n \geq 16$/cohort; $p = 5\times10^{-3}$) even when normalized to the fewer mounts. Thus, ablation of male PR+ VMHvl neurons leads to specific deficits in consummatory elements of mating. This phenotype is not accompanied by deficits in presumptively appetitive behaviors such as sniffing (Figure 6B, S6C–E), sex discrimination, or territory marking. There was no difference between PR^{Cre} and control males in sex discrimination as shown by predominantly female-directed ultrasonic vocalization (Figure 6E) (Nyby et al., 1977). Both PR^{Cre} and control males also marked their territory equivalently (Figure 6F, G) (Desjardins et al., 1973; Kimura and Hagiwara, 1985). Together, PR+ VMHvl neurons are essential for the normal display of male sexual behavior.

We tested whether ablation of PR+ VMHvl neurons disrupted aggression toward a WT male intruder. PR^{Cre} males exhibited a >2-fold reduction in the probability of initiating aggression compared to controls (Figure 7A). Even when PR^{Cre} males fought, they attacked less, for a shorter duration, and with a longer inter-attack interval (Figure 7B–D). Male fighting includes tail rattles and overt attacks such as biting. Control and PR^{Cre} residents rattled their tails equivalently, but PR^{Cre} males bit the intruders over 3-fold less (Figure 7E). Thus ablation of PR+ VMHvl neurons significantly reduces male aggression.

We assessed the ablation of PR+ VMHvl neurons in males tested behaviorally. Most of these neurons (95% ± 1; n = 14 control and 35 PR^{Cre} males) were ablated in PR^{Cre} males (Figure 7F–H) whereas PR+ arcuate neurons were largely spared (92% ± 12 of controls). There was no correlation in the extent of loss of PR+ neurons in the arcuate and the reduced mating or fighting (mating, $R^2 = 4\times10^{-4}$, $p = 0.9$; fighting, $R^2 = 2\times10^{-2}$, $p = 0.7$). PR^{Cre} males (n = 15) in whom the number of PR+ arcuate neurons was indistinguishable from controls also exhibited deficits in mating and fighting (Percent males intromitting: Controls, 67% and PR^{Cre} males, 27%, $n \geq 15$, $p = 0.02$; Percent males attacking: Controls, 75% and PR^{Cre} males, 20%, $n \geq 15$, $p = 1\times10^{-3}$). Taken together, our findings demonstrate that PR+ VMHvl neurons control the normal display of male mating and fighting.

We tested the specificity of the deficits in PR^{Cre} males following ablation of PR+ VMHvl neurons. Despite deficits in mating and fighting, these males sniffed and groomed intruders in a WT manner (Figures 6B, 7A, S6C–E, S7A–C). PR^{Cre} males performed at WT levels in assays of anxiety, motivated behavior, motor coordination, and locomotor activity (Figure S7D–G). These males maintained normal body weight, and there was no change in the weight of gonads, seminal vesicles, and serum testosterone titers (Figure S7H–J). Thus, PR+ VMHvl neurons are specifically required in males for the high, WT levels of mating and aggression.

DISCUSSION

We have identified a small, sexually dimorphic cluster of ~2,000 PR+ hypothalamic neurons that is essential for the normal display of sexual receptivity in females and sexual and aggressive behaviors in males. Our findings directly demonstrate that sexually dimorphic neurons in the brain influence dimorphic behaviors. Moreover these PR+ neurons are functionally bivalent in that they regulate distinct dimorphic behaviors in the two sexes.

Control of social behaviors by the VMH

Experimental studies and clinical observations have suggested that the VMH or adjacent hypothalamic regions regulate aggression and female mating (Bard, 1928; Blaustein, 2008; Clemente and Chase, 1973; Colpaert and Wiepkema, 1976; Grossman, 1972; Hess and Akert, 1955; Kow et al., 1985; Kruk et al., 1979; Lin et al., 2011; Olivier and Wiepkema, 1974; Pfaff and Sakuma, 1979a, 1979b; Reeves and Plum, 1969; Swaab, 2003; La Vaque and Rodgers, 1975; Wheatley, 1944). Despite intense scrutiny, the neurons that control these behaviors remained unidentified. In fact, whether separate or overlapping neuronal groups control these innate behaviors was also unknown. Our studies reveal the molecular identity of the long sought-after neurons in or around the VMH that influence male fighting and female mating. While other neighboring neurons may also influence these behaviors, we show that PR+ VMHvl neurons are required for the normal display of mating in females and fighting in males. These PR+ neurons also regulate male mating. Non-targeted inhibition of neurons in this region disrupts male fighting but not mating (Lin et al., 2011), suggesting partial inactivation or incomplete targeting of the neurons that regulate male mating. By contrast, our ablation of the PR+ VMHvl population revealed a role for these cells in male mating. Generalized arousal systems may feed into the VMH to enhance social interactions (Schober et al., 2011). We do not observe altered locomotor activity, sensorimotor coordination, or general social interactions in mice lacking PR+ VMHvl neurons, suggesting that these neurons are unlikely to exert a major influence on neural pathways that increase such arousal. In summary, we show that PR+ VMHvl neurons are required for the normal display of mating in both sexes and fighting in males. Given the conservation of genes and neuroanatomy across placental mammals, these VMHvl neurons may regulate mating and aggression in many mammals, including humans.

Distributive neural control of sexually dimorphic behaviors

It is curious that ablation of a highly restricted, molecularly defined set of neurons results in deficits in male mating and fighting. These PR+ neurons may integrate social cues relevant

to both behaviors, allowing males to mate or fight appropriately. Such dual control could also reflect further diversity within PR+ VMHvl neurons such that subsets of these neurons regulate one or the other behavior. In fact, in vivo recordings and c-Fos studies (Lin et al., 2011) reveal male VMHvl neurons that are activated during encounters with both sexes as well as neurons that appear responsive to either male or female encounters.

We find that different components of male behaviors require distinct neuronal populations. Males lacking PR+ VMHvl neurons have a male behavioral repertoire: they distinguish between the sexes with vocalizations (Stowers et al., 2002), attack males, and mate with females. Moreover, these males mark territory like WT males, thereby providing an objective indicator that their internal representation of sexual identity is masculine. Nevertheless these males display specific deficits in mating and fighting, indicating that ablation of PR+ VMHvl neurons dissociates the repertoire of masculine behaviors. Such partial behavioral deficits could reflect compensatory mechanisms activated upon the loss of these neurons. However, acute inactivation of the VMH mimics the behavioral deficits we observe (Lin et al., 2011), suggesting a minimal role of compensatory mechanisms. Thus, male mating and fighting are encoded in a distributive or redundant manner in the brain. Similarly, ablation of these neurons reduces female sexual receptivity without overtly disrupting estrous cyclicity or maternal care, indicating that these behaviors and physiology may also be controlled by distinct neuronal groups. Together, our findings show that sex-typical behaviors are represented distributively, and different neuronal populations in the underlying neural circuit control specific components of these behaviors. In fact genes such as *Cckar* also control these behaviors in a modular manner; for instance, $Cckar^{-/-}$ females show reduced sexual receptivity without alterations in other behaviors or physiology (Xu et al., 2012). Thus modular control of sexually dimorphic behaviors across multiple levels, including genes and neurons, may be a general organizational principle of the underlying neural circuits.

Control of sex-typical behaviors by sexually dimorphic VMHvl neurons

Studies in diverse animals have defined the relevance of particular brain regions to sex-typical behaviors (Brenowitz, 1991; Cooke et al., 1998; Ferveur et al., 1995; Kelley, 1997; Konishi, 1989; Morris et al., 2004). However, within a brain region only specific subsets of neurons are sexually dimorphic (Ng et al., 2009; De Vries and Panzica, 2006; Xu et al., 2012), and with rare exceptions in invertebrates (Kohatsu et al., 2011; von Philipsborn et al., 2011), the function of sexually dimorphic neurons is unknown. Ablation of the ~2000 sexually dimorphic PR+ VMHvl neurons, a fraction of the ~10^8 neurons in the mouse brain, results in specific deficits in complex social behaviors. Such specificity likely results from manipulation of a molecularly defined subset of neurons. Indeed PR+ neurons represent only ~50% of VMHvl neurons that, in turn, represent a fraction of VMH neurons.

The mechanisms whereby sexually dimorphic neurons control dimorphic behaviors are poorly understood. It is possible that PR+ VMHvl neurons represent unrelated cell types in the two sexes, as evidenced by the sex differences in cell number and distribution, projection targets, and expression of Cckar. This is unlikely because PR+ VMHvl neurons also share many features, including location, projection targets, gene expression (PR, ERα), and

developmental lineage (Grgurevic et al., 2012). Thus, it appears that a common pool of PR+ VMHvl neurons is present in both sexes, but their sex differences may allow them to transform synaptic inputs in a sex-specific manner or to relay either male or female-specific input to drive sexually dimorphic behavioral output.

Most behaviors are common to the sexes, suggesting that each sex possesses the motor pathways to display dimorphic behaviors of the opposite sex. Most sex differences in the brain represent quantitative and not all-or-none cellular or molecular sex differences. It is unknown whether these shared but dimorphic neurons regulate sex-typical behaviors in both sexes. Alternately, such neurons may regulate a dimorphic output in one sex, and in the other sex, they may be functionally vestigial, subserve a non-dimorphic function, or suppress a function of the opposite sex (De Vries and Boyle, 1998). We show that PR+ VMHvl neurons are functionally bivalent in the sense that they control sex-typical behaviors in both males and females. This dual function may prove adaptive if such neurons can generate a dimorphic behavior of the opposite sex in the appropriate context; in addition, bivalence may permit facile interchange of sex-typical behaviors between the sexes during speciation. Such flexibility may underlie the rapid evolution of sexually dimorphic traits (Darwin, 1871), including behaviors such as allocation of parental care and social dominance hierarchies. Given such evolutionary considerations, it remains to be seen whether all sexually dimorphic neuronal populations control sex-typical behaviors in both sexes.

EXPERIMENTAL PROCEDURES

Viruses

AAV-flex-taCasp3-TEVp—The plasmid encoding AAV-flex-taCasp3-TEVp (Figure S4A) was generated using routine subcloning. High titer virus of serotype 2/1 (3×10^{12} IU/mL) was generated from the plasmid at the UNC, Chapel Hill Vector Core.

Lenti-lxlplap—The plasmid encoding this VSVG pseudo-typed lentivirus was generated using standard subcloning (Figure S3G). High titer virus (~10^8 IU/mL) was generated using standard protocols (Barde et al., 2001).

Stereotaxic surgery

The virus was stereotaxically delivered under anesthesia to the VMHvl (Coordinates: rostrocaudal, −1.48 mm; mediolateral, ±0.78 mm; depth, 5.8 mm; see also Supplemental Procedures) (Paxinos and Franklin, 2003). Injections of taCasp3-encoding AAV were spiked (9:1) with constitutive EGFP-encoding AAV to verify accuracy of the injection placement in control and PR^{Cre} mice.

Behavior

Testing was performed as described previously (Juntti et al., 2010; Wu et al., 2009; Xu et al., 2012) (see also Supplemental Procedures). To test for sexual receptivity, females were castrated and, subsequent to estrus induction with estrogen and progesterone, inserted singly into the home cage of a sexually experienced WT male. Lordosis was defined as the female

holding still with a dorsiflexed neck while being intromitted. Each experimental cohort included a set of control and PR^{Cre} mice.

Supplemental Experimental Procedures

Details regarding animals, histology, data analyses, and the procedures described above can be found in the Supplemental Information. All animal studies were in accordance with IACUC protocols at UCSF.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Widespread adult sex differences in progesterone receptor (PR) expressing neurons
- Ventrolateral compartment of ventromedial hypothalamus (VMHvl) contains PR+ neurons
- PR+ VMHvl neurons exhibit cellular and molecular dimorphisms between the sexes
- PR+ VMHvl neurons essential for normal mating in both sexes and fighting in males

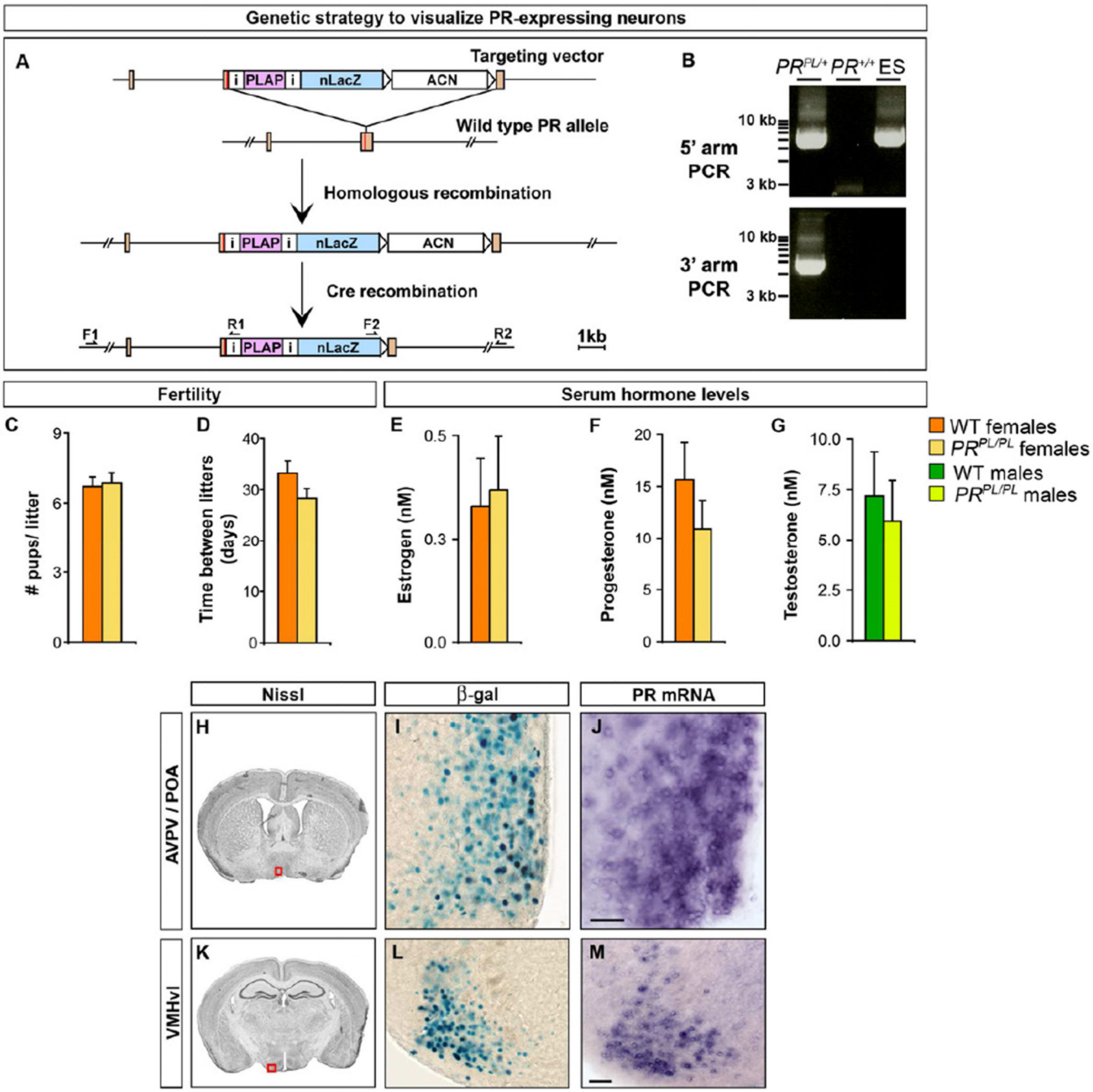

Figure 1. Visualizing PR+ neurons in the mouse brain

(A) Generating the PR^{PL} allele. ACN is a self-excising neomycin selection cassette (Bunting et al., 1999). Orange rectangles are exons and the red line in the 3' exon denotes the stop codon.

(B) PCR to detect homologous recombination at the *PR* locus. Primers used to detect integration of the 5' (F1, R1) and 3' (F2, R2) arms of the targeting vector. ACN precludes detection of the 3' recombination event in ES cells.

(C, D) No difference between WT and $PR^{PL/PL}$ females in litter size and frequency.

(E–G) No difference in titers of sex hormones between WT and $PR^{PL/PL}$ adults.
(H–M) Boxed areas in Nissl-stained coronal sections (Paxinos and Franklin, 2003) through the adult brain depict locations of the regions shown in panels to the right. PR expression in $PR^{PL/+}$female as labeled by β-gal activity mirrors expression of PR mRNA in adjacent sections.
Scale bars = 50 μm.
Mean ± SEM; n ≥ 12/genotype (C–G); n = 3 (H–M).
See also Figure S1 and Table S3.

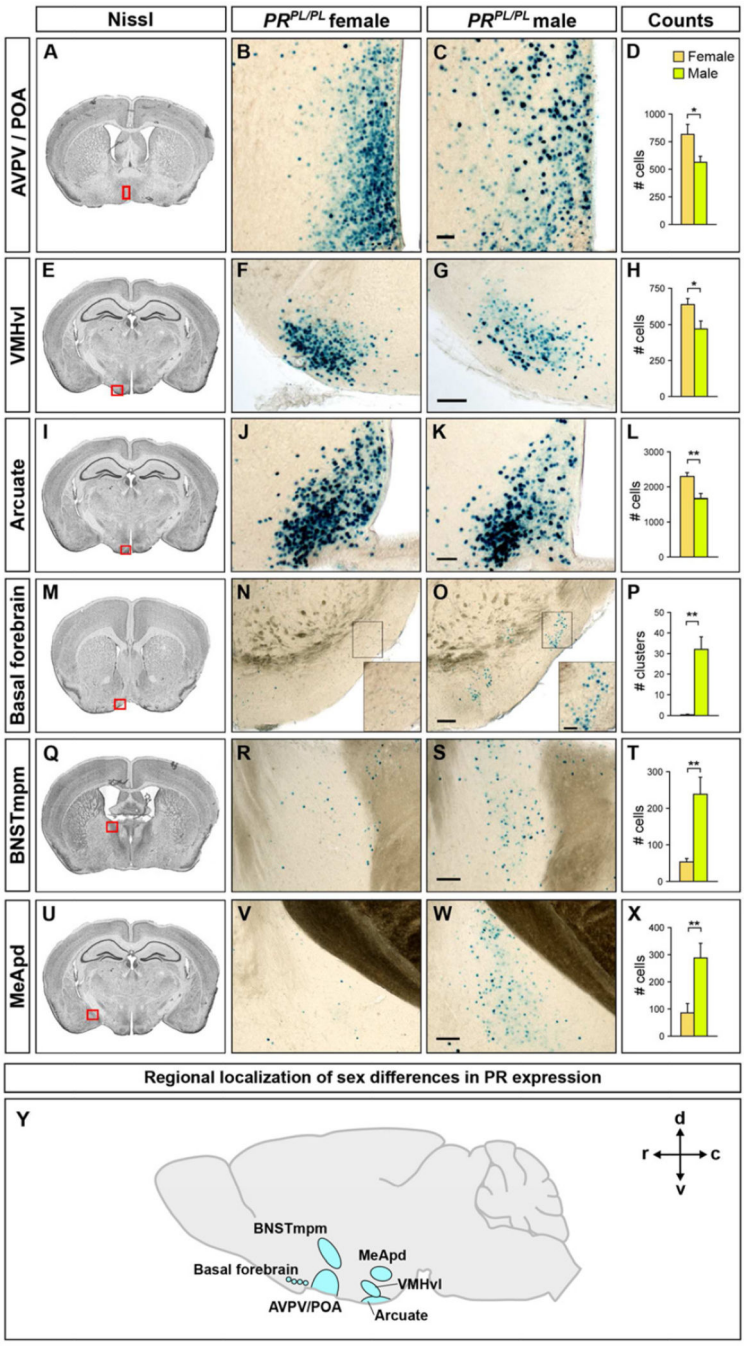

Figure 2. Sexual dimorphism in PR-expression in the adult brain

Boxed areas in Nissl-stained coronal sections through the adult brain depict regions of $PR^{PL/PL}$mice labeled for β-gal activity in the panels to the right.

(A–L) More PR+ cells in the female AVPV/POA, VMHvl, and arcuate nucleus.

(M–X) More PR+ cells in the male basal forebrain, BNSTmpm, and MeApd.

(Y) Representation of sexually dimorphic PR expression in different brain regions as projected on to a mid-sagittal section. c, caudal, d, dorsal, r, rostral, v, ventral.

Scale bars = 50 μm (C, K) and 100 μm (G, O, S, W). Inset scale bars = 25 μm.

Mean ± SEM; n ≥ 4/sex; *p < 0.04, **p < 0.01.
See also Figure S2 and Table S1.

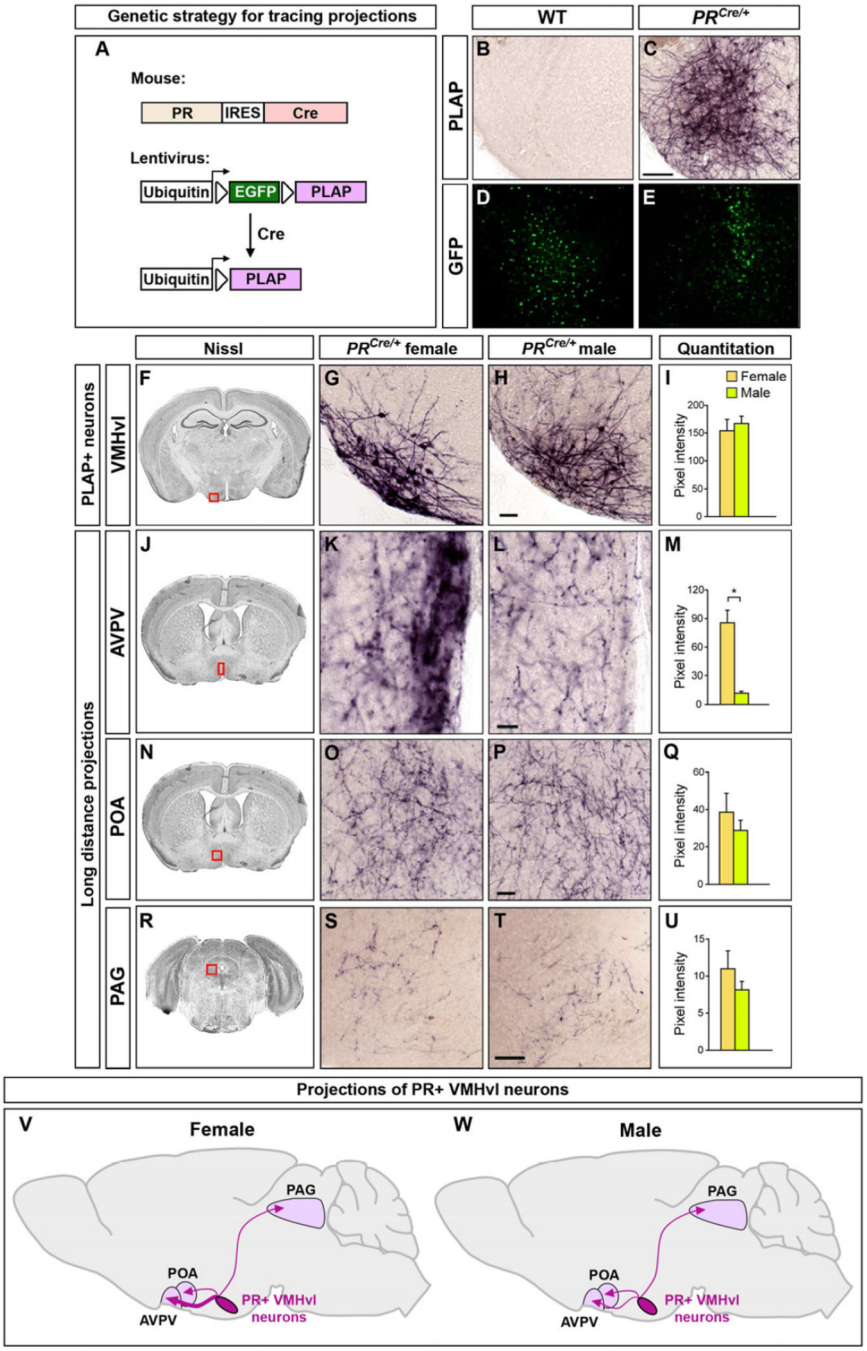

Figure 3. PR+ VMHvl neurons project in a sexually dimorphic manner

(A) Strategy to visualize projections of PR+ neurons.

(B–E) Lenti-lxlplap targeted to the VMH infects cells in $PR^{Cre/+}$ and WT mice as visualized by EGFP+ cells. Only a few cells are PR+ in this region so there is no apparent difference in the number of EGFP+ cells in PR^{Cre} and WT mice. PLAP+ soma and local arbors of VMHvl neurons are only observed in PR^{Cre} mice.

(F–U) Boxed areas in Nissl-stained coronal sections depict regions shown in panels to the right. Lenti-lxlplap targeted to the VMHvl of adult $PR^{Cre/+}$ mice labels PLAP+ soma and

local arbors of VMHvl neurons (F–I). The lentiviral titer limits the number of infected Cre+ neurons and does not highlight the sex difference in the number of these neurons. The variable multiplicity of infection can lead to apparent size differences in PLAP-labeled soma. However, there is no sex difference in the soma size of these neurons (Figure S2G). PR+ VMHvl neurons project to the AVPV, POA, and PAG (J–U). There are more PLAP+ projections to the AVPV in females (J–M).

(V, W) Schematic summarizing projections of PR+ VMHvl neurons. No difference in anatomical extent of projections in different regions, but female AVPV receives more innervation from these neurons.

Scale bars = 100 μm (C), 50 μm (H,P,T), 25 μm (L).

Mean ± SEM; $n \geq 7$/sex; *$p < 0.001$.

See also Figure S3, Tables S2, S3.

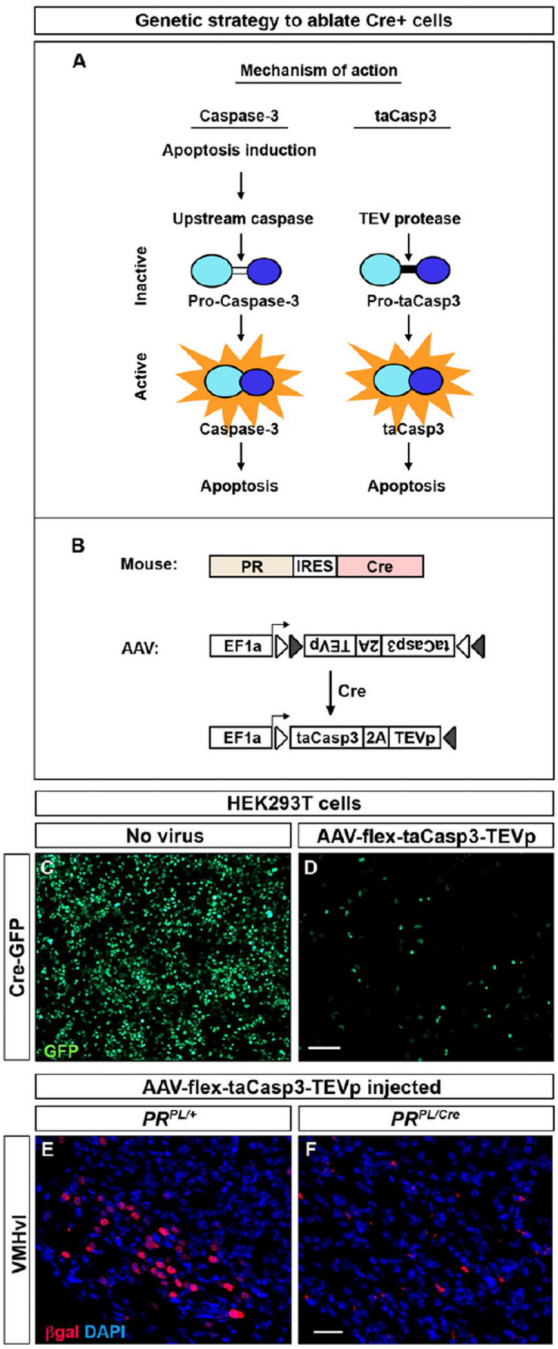

Figure 4. Genetic strategy to ablate neurons in a Cre-dependent manner

(A) Intramolecular cleavage of endogenous pro-caspase-3 by upstream caspases activates caspase-3, which then induces apoptosis. This intramolecular cleavage site has been replaced by a TEV linker domain (black bar) in inactive taCasp3 (pro-taCasp3) such that only TEV protease activates taCasp3, which then induces apoptosis.

(B) Viral strategy to ablate PR+ neurons conditionally.

(C, D) Cell death 1 week following infection of Cre:EGFP+ HEK293T cells with AAV-flex-taCasp3-TEVp. n = 3 experiments.

(E, F) Ablation of PR+ VMHvl neurons in a $PR^{PL/Cre}$ but not $PR^{PL/+}$ female injected with AAV-flex-taCasp3-TEVp. n ≥ 10/experimental group.
Scale bar = 100 μm (C, D) and 25 μm (E, F).
See also Figure S4.

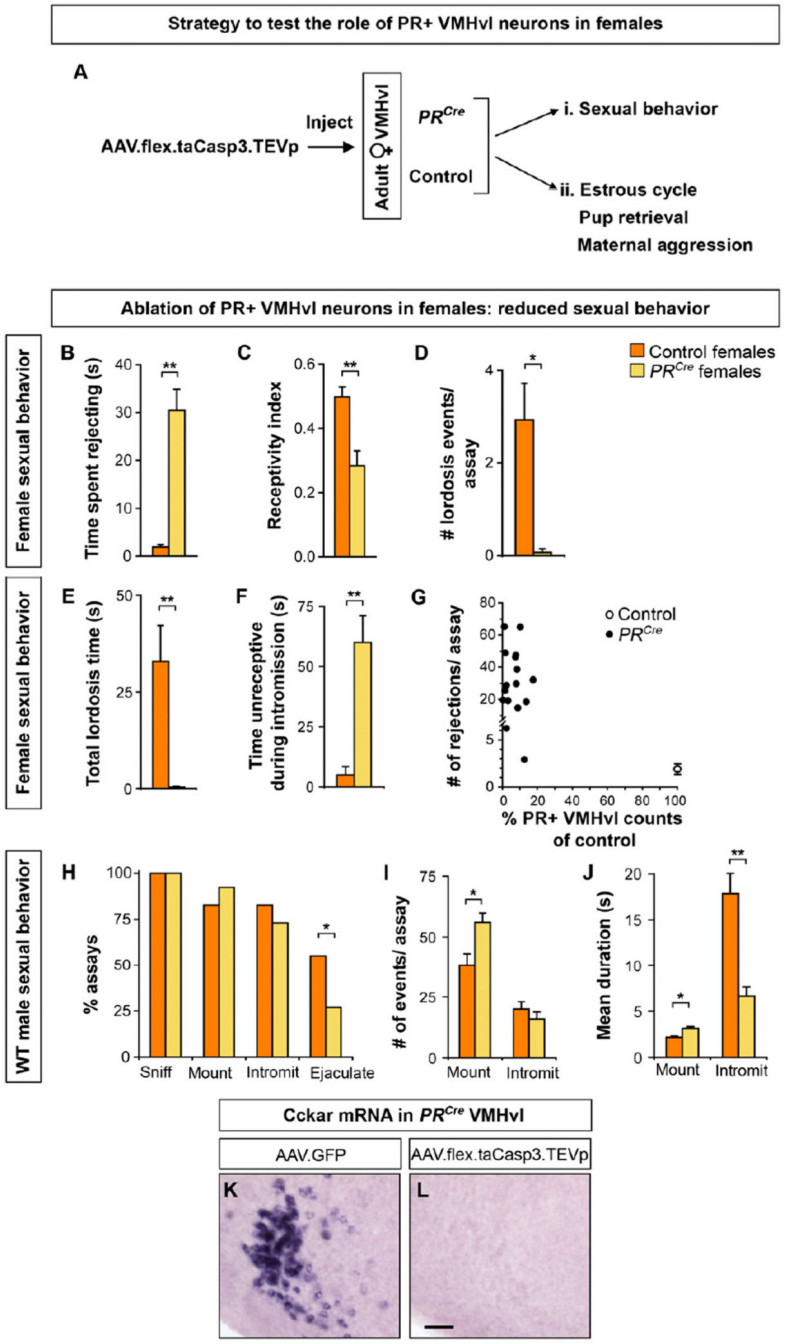

Figure 5. PR+ VMHvl neurons regulate female sexual receptivity

(A) Experimental design to test the role of PR+ VMHvl neurons in female behaviors. Mating was tested with ovariectomized females primed to be in estrus. Other behaviors were tested with gonadally-intact females.

(B–J) *PR*^{Cre} and control females were injected with AAV-flex-taCasp3-TEVp and tested for sexual behavior with WT males.

(B) *PR*^{Cre} females spend more time rejecting male mating attempts, walking away when the male approaches.

(C–E) PR^{Cre} females display lower receptivity index (mounts leading to intromission/total mounts) and reduced number and duration of lordosis events.
(F) PR^{Cre} females spend more time moving about and being unreceptive during intromission.
(G) Fewer than 20% of PR+ neurons remain in the VMHvl of PR^{Cre} females, who reject male mating attempts more than control females.
(H) Males sniff and initiate mating equivalently with PR^{Cre} and WT females but ejaculate in fewer assays with PR^{Cre} females.
(I) Males mount PR^{Cre} females more but without a corresponding increase in intromission.
(J) Males mount PR^{Cre} females longer, but intromit for shorter duration.
(K, L) Ablation of PR+ VMHvl neurons in PR^{Cre} females results in loss of Cckar expression.
Mean ± SEM; n ≥ 10/experimental group (B–J); n = 3 (K, L); *p < 0.02, ** p < 0.005. Scale bar = 50 μm.
See also Figure S5, Table S3, Movies S1, S2.

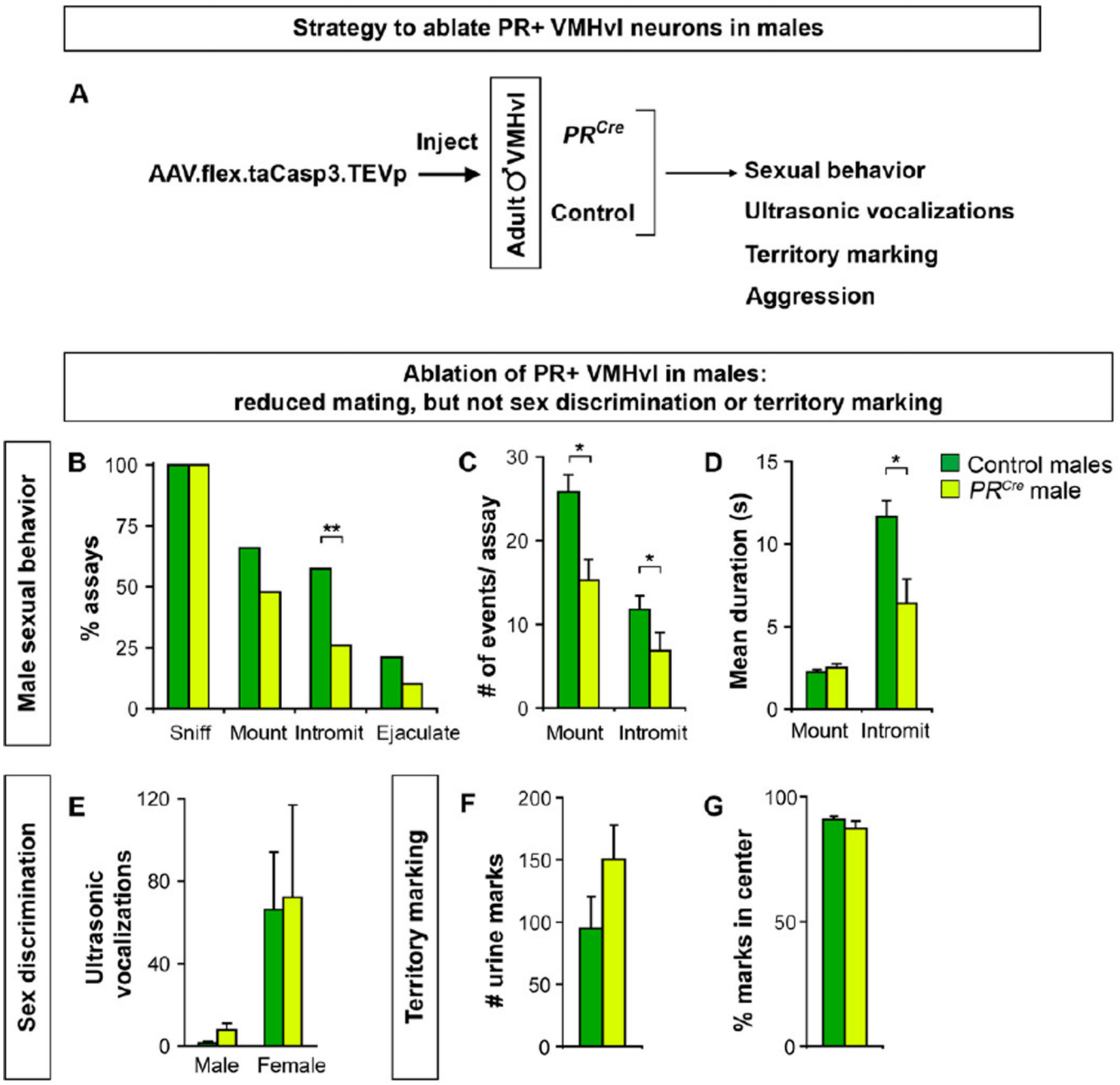

Figure 6. PR+ VMHvl neurons regulate male sexual behavior

(A) Experimental design to test the role of PR+ VMHvl neurons in male behaviors.

(B–G) PR^{Cre} and control males were injected with AAV-flex-taCasp3-TEVp and tested for mating, ultrasonic vocalizations toward male or female intruders, and territory marking.

(B) PR^{Cre} males intromit females in fewer assays.

(C, D) PR^{Cre} males mount and intromit females less and have shorter bouts of intromissions.

(E) Both PR^{Cre} and control males emit more vocalizations to females.

(F, G) No difference between PR^{Cre} and control males in the number and distribution of urine

marks. % marks in center = 100*(# urine marks not abutting cage perimeter/# of all urine marks).

Mean ± SEM; n ≥ 24/experimental group (B–D, F,G), n ≥ 5/experimental group (E); *p < 0.008, **p < 0.001.

See also Figures S6 and S7.

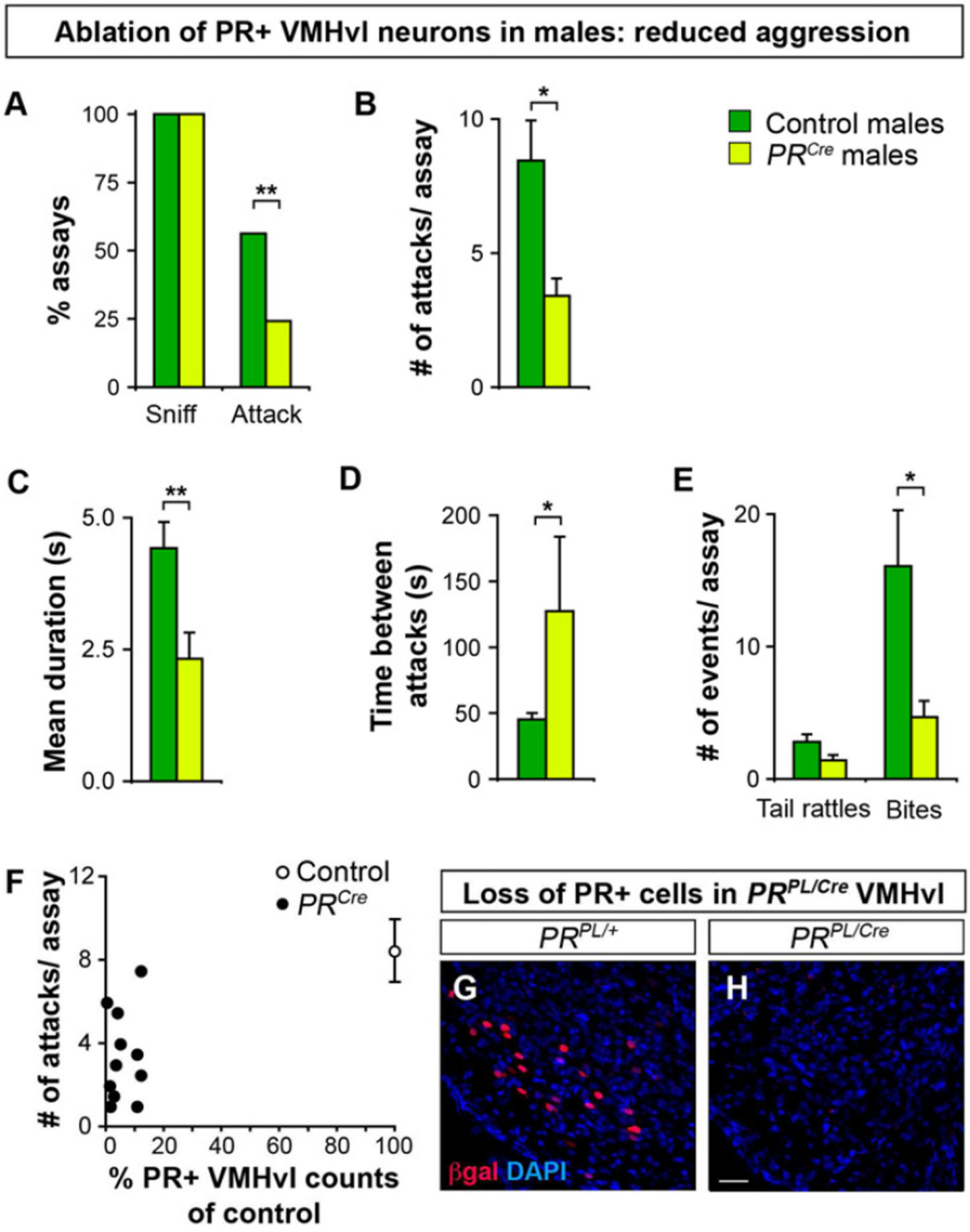

Figure 7. PR+ VMHvl neurons regulate male aggression

(A–F) PR^{Cre} and control resident males were injected with AAV-flex-taCasp3-TEVp targeted to the VMHvl and tested for aggression toward a WT male intruder.

(A) All residents sniff intruders equivalently, but PR^{Cre} males attack less.

(B–D) When PR^{Cre} males fight, they attack less, for a shorter duration, and with longer intervals between attacks.

(E) PR^{Cre} males bite less.

(F) Fewer than 20% of PR+ neurons remain in the VMHvl of PR^{Cre} males, who attack intruders less.

Mean ± SEM; n ≥ 24/experimental group; *p < 0.04, **p ≤ 0.009.

(G, H) Ablation of PR+ VMHvl neurons in a $PR^{PL/Cre}$ male injected with AAV-flex-taCasp3-TEVp. Scale bar = 25 μm.

See also Figure S7.