SUPPLEMENTAL FIGURE LEGENDS

Figure S1: PR expression in the WT and *PR^{PL}* mouse brain

(Related to Figure 1)

(A) Detection of PR by RT-qPCR in microdissected AVPV/POA, VMHvl, arcuate, basal forebrain, BNST, MeA, basal ganglia, cingulate cortex, and dentate gyrus, but not the liver of adult WT C57Bl/6J mice. The data presented is normalized to the expression of *Rpl32*, a housekeeping gene. Mean ± SEM; n = 3.

(B-J) PR is expressed in the basal ganglia, cingulate cortex, and dentate gyrus as visualized by β-gal activity in *PR$^{PL/PL}$* female and male mice. Boxed area in Nissl-stained section indicates the region shown in the panels to the right. n ≥ 3/sex. Scale bar equals 100 μm.

Figure S2: Characterization of sexually dimorphic PR expression in *PR^{PL}* mice

(Related to Figure 2)

(A) Sex differences in density of β-gal+ (PR+) cells in adult *PR$^{PL/PL}$* mice.

(B) A majority of PR+ cells express ERα, and there is no sex difference in co-labeling.

(C-E) Co-labeling VMHvl neurons for PR (anti-β-gal) and Cckar (mRNA) in *PR$^{PL/PL}$* mice. The vast majority (~96%) of Cckar+ cells is PR+, whereas ~67% of PR+ cells are Cckar+. Scale bar equals 25μm.

(F) The vast majority of β-gal+ (PR+) cells in various regions in *PR^{PL}* mice are neurons, as evidenced by co-labeling with NeuroTrace (fluorescent Nissl) in the arcuate and NeuN for the remaining regions.

(G) No sex difference in nuclear or soma size of PR+ VMHvl cells in *PR^{PL}* mice. (>200 cells analyzed for each region).

Mean ± SEM; n = 4/sex (A), n = 3 (B-G); *p < 0.04, **p < 0.008.

Figure S3. Mapping projections of PR+ VMHvl neurons in *PR^{Cre}* knock-in mice

(Related to Figure 3)

(A) Schematic of the *PR* locus with the *IRES-Cre* transgene inserted 3' to the stop codon of the last exon. Primers used to detect integration of the 5' arm (F1, R1) and the 3' arm (F3, R2) only detect homologously recombined insertion events into the genome. Primer sequences listed in Table S3.

(B) PCR genotyping of *PR^{Cre}* allele. PCR primers are shown in panel A. The FRT-flanked neomycin selection cassette was excised in vivo by crossing F1 progeny of *PR^{Cre}* chimeras bearing the targeted allele to mice expressing Flpe recombinase systemically (Rodríguez et al., 2000). Excision of the neomycin selection cassette leaves a single FRT site immediately 3' of *Cre*. We verified this excision event using primers (not shown) to amplify the 3' arm and sequencing through the remaining FRT site.

(C, D) No difference between WT and *PR$^{Cre/Cre}$* females in litter size and frequency. Mean ± SEM; n ≥ 12/genotype. Note that data from WT females shown here are from the same mice shown in Figure 1 C, D.

(E, F) Cre expression mirrors that of PR in the VMHvl of adult *PR$^{Cre/+}$* mice in adjacent sections. n = 3. Scale bar equals 25 μm.

(G) DNA construct used to generate the Lenti-lxlplap virus. The transgene is flanked by long terminal repeats (LTRs). A central polypurine tract (CPPT) resides 5' of a Ubiquitin ligase C promoter (Ub), followed by a loxP-flanked (triangles) *histone 2B:EGFP*. 3' of the 3' loxP site is

the DNA encoding PLAP followed by a woodchuck post-transcriptional regulatory element (WPRE).

(H) No sex difference in the volume of PR+ VMHvl projections in the AVPV, POA, and PAG. The dense distribution of PLAP-labeled projections is only observed in the AVPV of females, but sparsely labeled fibers occupy a similar volume in the male AVPV. Mean ± SEM; n = 3/sex.

Figure S4. Ablation of PR+ VMHvl cells in *PR*^{Cre} mice with AAV-flex-taCasp3-TEVp

(Related to Figure 4)

(A) The DNA construct encoding AAV-flex-taCasp3-TEVp contains inverted terminal repeats (ITRs) flanking the transgene inserted into the viral genome. The transgene consists of an EF1a promoter 5' to an inverted taCasp3-T2A-TEVp sequence that is flanked by heterotypic loxP (open triangles) and lox2722 (closed triangles) sites. 3' of this transgene is a WPRE sequence and an hGH polyA signal sequence (pA).

(B) The vast majority of PR mRNA+ cells is lost upon caspase-mediated ablation of PR+ VMHvl neurons in *PR*$^{Cre/+}$ and *PR*$^{Cre/PL}$ male and female mice. Scale bar = 50 μm. Mean ± SEM; n = 5.

Figure S5. Females lacking PR+ VMHvl neurons do not display pervasive deficits in physiology or behavior

(Related to Figure 5)

Adult *PR*^{Cre} and control females were injected with AAV-flex-taCasp3-TEVp targeted to the VMHvl and tested in different assays.

(A, B) WT males sniffed *PR*^{Cre} and control females equivalently. n ≥ 10/experimental group.

(C, D) No difference between PR^{Cre} and control females in sniffing WT males during assays of female sexual behavior. n ≥ 10/experimental group.

(E-H) No difference between PR^{Cre} and control females in tests of anxiety (E), motivation to find food (F), motor coordination and fatigue in the rotarod test (G), and general locomotor activity (H). n = 8/experimental group.

(I) No difference between PR^{Cre} and control females in percent change in body weight 10 weeks following viral injection. n ≥10/experimental group.

(J) No significant difference between PR^{Cre} and control females in the frequency of estrous cycles in 2 weeks. n ≥ 8/experimental group.

(K-O) The marked diminution in sexual receptivity subsequent to ablation of PR+ VMHvl neurons corresponded with fewer PR^{Cre} females delivering litters. Nevertheless in PR^{Cre} females bearing litters, there was no statistical difference between PR^{Cre} and control females in retrieving their pups (K-M) or attacking unfamiliar intruders in their cage (N, O). Only females who displayed pup retrieval or maternal aggression were included in the analysis of behavioral parameters (L, M, O). n = 5 PR^{Cre} and 8 control females each for tests of pup retrieval and maternal aggression.

Data are represented as Mean ± SEM.

Figure S6. Males lacking PR+ VMHvl neurons investigate females normally but mate less than controls

(Related to Figure 6)

Adult PR^{Cre} and control males were injected with AAV-flex-taCasp3-TEVp targeted to the VMHvl and tested for sexual behavior with WT estrus females.

(A) No difference between PR^{Cre} and control males in the latency to mount, intromit, or ejaculate.

(B) PR^{Cre} males spend less total time mounting and intromitting.

(C-E) No difference between PR^{Cre} and control males in sniffing or grooming females.

Mean ± SEM; n ≥ 24/experimental group, *p < 0.05.

Figure S7. Males lacking PR+ VMHvl neurons do not display pervasive deficits in physiology or behavior

(Related to Figures 6, 7)

Adult PR^{Cre} and control males were injected with AAV-flex-taCasp3-TEVp targeted to the VMHvl and tested in different assays.

(A-C) No difference between PR^{Cre} and control males in sniffing or grooming intruder males. n ≥ 24/experimental group.

(D-G) No difference between PR^{Cre} and control males in tests of anxiety (D), motivation to find food (E), motor coordination and fatigue in the rotarod test (F), and general locomotor activity (G). n ≥ 11/experimental group.

(H) No difference between PR^{Cre} and control males in percent change in body weight 10 weeks following viral injection. n ≥ 11/experimental group.

(I) No difference between PR^{Cre} and control males in weight of testes or seminal vesicles. n ≥ 11/experimental group.

(J) No difference between PR^{Cre} and control males in serum testosterone titer. n = 13 /experimental group.

Data are represented as Mean ± SEM.

SUPPLEMENTAL MOVIE LEGENDS

Movie S1. Control females are sexually receptive to mating attempts by WT males

(Related to Figure 5)

This movie shows the behavior of a control female that was inserted into the cage of a WT, sexually experienced male. This female was primed to be in estrus and previously injected with the taCasp3-encoding AAV bilaterally targeted to the VMHvl. The female is sexually receptive and is still when the male is intromitting.

Movie S2. Ablation of PR+ VMHvl neurons in females diminishes sexual receptivity to mating attempts by WT males

(Related to Figure 5)

This movie shows the behavior of a PR^{Cre} female that was inserted into the cage of a WT, sexually experienced male. This female was primed to be in estrus and previously injected with the taCasp3-encoding AAV bilaterally targeted to the VMHvl. The female rejects male mating attempts by running away when the male approaches and kicks him in the face toward the end of the video clip.

SUPPLEMENTAL TABLES

Table S1. Extent of sexually dimorphic expression of PR in various brain regions in *PR*^{PL} mice

(Related to Figure 2)

Similar location of PR-expressing neurons in *PR*$^{PL/PL}$ mice. Mean ± SEM; n = 3/sex. AP = anteroposterior, DV = dorsoventral, ML = mediolateral, ND, not detected. All coordinates relative to bregma here and in Table S2 (Paxinos and Franklin, 2003).

Female	**Basal Forebrain**	**AVPV/POA**	**BNSTmpm**	**VMHvl**	**Arcuate**	**MeApd**
AP	ND	0.35±0.03 to -0.34±0.00	0.24±0.02 to -0.22±0.00	-1.34±0.07 to -1.96±.07	-1.34±0.00 to -2.51±0.03	-1.75±0.17 to -2.22±0.04
DV	ND	-5.16±0.08 to -5.77±0.02	-3.98±0.13 to -4.58±0.08	-5.43±0.09 to -5.83±0.07	-5.42±0.08 to -5.97±0.03	-4.52±0.16 to -5±0.12
ML	ND	0±0.00 to 0.65±0.08	0.47±0.02 to 0.92±0.22	0.57±0.07 to 1±0.00	0±0.00 to 0.53±0.09	1.87±0.08 to 2.08±0.17
Male	**Basal Forebrain**	**AVPV/POA**	**BNSTmpm**	**VMHvl**	**Arcuate**	**MeApd**
AP	1.43±0.06 to 0.54±0.08	0.34±0.04 to -0.38±0.04	0.34±0.04 to -0.3±0.04	-1.38±0.04 to -1.94±0.00	-1.34±0.07 to -2.06±0.31	-1.62±0.04 to -2.35±0.05
DV	-5.5±0.14 to -5.7±0.10	-4.95±0.10 to -5.83±0.08	-3.8±0.17 to -4.92±0.08	-5.18±0.04 to -5.78±0.02	-5.44±0.06 to -5.93±0.07	-4.6±0.15 to -5.13±0.12
ML	0.5±0.25 to 1.42±0.30	0±0.00 to 0.58±0.08	0.43±0.03 to 1.08±0.08	0.5±0.06 to 1±0.00	0±0.00 to 0.58±0.08	1.92±0.08 to 2.42±0.08

Table S2. Extent of PR+ VMHvl projections to different brain regions in *PR*^{Cre} mice (Related to Figure 3)

Similar regional localization of projections of PR+ VMHvl neurons in *PR*^{Cre} mice injected with Lenti-lxlplap. Mean ± SEM; n = 3/sex.

Female	**AVPV**	**POA**	**PAG**
AP	0.35±0.03 to -0.18±0.04	0.18±0.04 to -0.22±0.07	-2.79±0.13 to -5.00±0.02
DV	-4.92±0.08 to -5.82±0.04	-4.85±0.08 to -5.75±0.00	-1.92±0.08 to -3.17±0.08
ML	0.00±0.00 to 0.18±0.04	0.28±0.02 to 0.83±0.08	0.25±0.00 to 1.00±0.00
Male	**AVPV**	**POA**	**PAG**
AP	0.38±0.07 to -0.18±0.04	0.14±0.00 to -0.34±0.00	-2.77±0.03 to -5.08±0.06
DV	-5.08±0.08 to -5.87±0.03	-4.92±0.08 to -5.75±0.00	-2.0±0.00 to -3.08±0.08
ML	0.00±0.00 to 0.20±0.05	0.26±0.02 to 0.83±0.08	0.25±0.00 to 1.00±0.00

Table S3. List of PCR primers.

(Related to Figures 1, 3, 5)

Primer description	**Primer sequence (5' – 3')**
ISH probe, Cckar, forward	CAGGTTGCATTTGGGAGACT
ISH probe, Cckar, reverse	ATGAGTCCGTAAGCCACCAC
ISH probe, Cre, forward	CCAAGAAGAAGAGGAAGGTGTC
ISH probe, Cre, reverse	ATCCCCAGAAATGCCAGATTAC
ISH probe, PR, forward	CACAGTATGGCTTTGATTCC
ISH probe, PR, reverse	TTTGTGAGTTGGTAGAAGCGC
F1	GGTGTCATCTGTGGCCTCTGGAAGCAG
F2	CTACAGTCAAGAGCAACTGATGG
F3	GACCGCTTCCTCGTGCTTTACGGTATCG
R1	GGCGGAATTCGGCGCGCCTCATCAC
R2	CTACCAGATCCAGTGGGCGGGGAAAG
qPCR, PR, forward	TGTCCGGGATTGGATGAAT
qPCR, PR, reverse	GCTTGCATGATCTTGTGAAACA
qPCR, Rpl32, forward	CGGTTATGGGAGCAACAAGAAAAC
qPCR, Rpl32, reverse	GGACACATTGTGAGCAATCTCAGC

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Animals

Adult mice 10-24 weeks of age were used in all studies. Mice were housed in an UCSF barrier facility with a 12:12 hour light:dark cycle, and food and water were available ad libitum. $PR^{Cre/Cre}$ or $PR^{Cre/+}$ mice and their control WT siblings were used to trace projections of PR+ VMHvl neurons. $PR^{Cre/Cre}$ or $PR^{Cre/PL}$ mice and their control (WT or PR^{PL}) same-sex siblings were used for behavioral studies. Animals were group-housed by sex after weaning at 3 weeks of age.

Generation of PR^{PL} and PR^{Cre} knock-in mice

Genomic clones containing the last exon of *PR* were obtained by screening a 129/SvJ lambda phage library from Stratagene. An ~8.3 kb *BamHI* genomic clone containing the last two exons of *PR* was used to design the targeting vector. An *Asc*I restriction site was inserted 3 bp 3' of the stop codon of the *PR* gene using site-directed mutagenesis (Stratagene). This mutagenized targeting vector has 4.2 kb and 4.1 kb of homology 5' and 3' of the *Asc*I restriction site, respectively. To generate the PR^{PL} mice, we utilized the self-excising neomycin cassette, pACN, which was subcloned 3' of IRES-PLAP-IRES-nuclear LacZ (Bunting et al., 1999; Shah et al., 2004). This IRES-PLAP-IRES-nuclear LacZ-ACN cassette was inserted into the targeting vector as an *Asc*I fragment. The PR-IRES-PLAP-IRES-nuclear LacZ-ACN targeting vector was electroporated into a 129/SvEv mouse ES cell line. We obtained a targeting frequency of 44% for homologous recombinants, which were detected using PCR for the 3' arm for the targeting vector. We used a primer (F3) that was complementary to the ACN cassette and an external primer (R2) that was complementary to genomic sequence located 3' of the 3' homology arm of

the targeting vector (see Table S3 for all primer sequences used in our study). A subset of positive clones was tested by PCR for homologous targeting of the 5' arm using an external primer (F1) and a primer unique to the modified allele (R1). ES clones harboring the homologously recombined *PR* allele were injected into blastocysts to obtain chimeric mice which were crossed to C57Bl/6J females to obtain germline transmission. Chimeric mice that transmitted the PR^{PL} allele were obtained from one positive clone. ACN contains a $neomycin^{R}$ gene that is self-excised upon passage through the male germline, and F1 progeny obtained by crossing the chimeric males to C57Bl/6J females had deleted ACN as determined by PCR using primers F2 and R2 (Figure 1B). The resulting progeny (backcrossed >3 generations in C57Bl/6J) were used for experimental analysis. A similar strategy was used to generate mice bearing the PR^{Cre} allele. We flanked with FRT sites the DNA fragment encoding RNA PolII promoter-Neo^{R}-pA of pACN to generate the pFNF selection cassette. This FNF cassette was subcloned 3' of IRES-Cre to generate the plasmid pCre-FNF. The IRES-Cre-FNF cassette was inserted into the targeting vector as an *Asc*I fragment into the *AscI* site engineered 3 bp 3' of the *PR* stop codon. The targeting vector was electroporated into a 129/SvEv mouse ES cell line, and we obtained a targeting frequency of 14% for homologous recombinants. To detect positive clones, we performed PCR for the 3' arm for the targeting vector. We used a primer (F3) that was complementary to the FNF cassette and an external primer (R2) as above. A subset of positive clones was tested by PCR for homologous targeting of the 5' arm using an external primer (F1) and a primer unique to the modified allele (R1). ES clones harboring the homologously recombined PR^{Cre} allele were injected into blastocysts to obtain chimeric mice that were crossed to C57Bl/6J females to obtain germline transmission. Chimeric mice that transmitted the PR^{Cre} allele were obtained from one positive clone. We bred the F1 progeny of

these chimeras to mice expressing Flpe recombinase ubiquitously (Rodríguez et al., 2000). Deletion of the FNF cassette was verified by PCR and sequencing of the PCR product. The resulting progeny (backcrossed >3 generations in C57Bl/6J) were behaviorally and physiologically WT and used for experimental analysis. Both the PR^{PL} and the PR^{Cre} lines were maintained either by breeding with C57Bl/6J mice or breedings between heterozygotes. Experimental animals were largely derived from such breedings and occasionally from breedings between a mouse homozygote and a mouse heterozygote for these alleles.

Viruses

AAV-flex-taCasp3-TEVp: The *taCasp3-T2A-TEVp* transgene was generated by overlapping PCR of plasmids harboring taCasp3 and TEVp (Gray et al., 2010). This transgene was inserted in reverse orientation into the plasmid pAAV-EF1a-DIO-hChR2(H134R)-EYFP-WPRE-pA such that it replaced hChR2(H134R)-EYFP (Zhang et al., 2006). This yielded the plasmid encoding AAV-flex-taCasp3-TEVp (Figure S4A).

Lenti-lxlplap: The pHIV-CSCG vector (Miyoshi et al., 1998) served as the backbone in the generation of the plasmid encoding this virus. A *histone 2B:EGFP* fusion transgene was flanked by loxP sites such that the 5' loxP site intervened between the ATG and the rest of the transgene, and multiple stop codons in all reading frames were inserted 5' of the 3' loxP site. This histone 2B:EGFP encoding translational stop cassette (lxl) was inserted 3' of the Ubiquitin ligase C promoter in the modified pHIV-CSCG. A PLAP-encoding transgene lacking the first ATG was subcloned 3' of the stop cassette to generate the plasmid pLenti-lxlplap.

Stereotaxic surgery

A mouse was placed in a stereotaxic frame (Kopf Instruments) under anesthesia, the skull was exposed with a midline scalp incision, and the stereotaxic frame was aligned at bregma using visual landmarks. The drill (drill bit #85; ~279 μm diameter) on the stereotaxic frame was placed over the skull at coordinates corresponding to the VMHvl (anteroposterior, -1.48 mm; mediolateral, ±0.78 mm), and a hole was drilled through the skull bone to expose the brain. A 33 gauge steel needle loaded with virus was aligned at bregma (including in the z-axis) and slowly inserted through the hole at 1 mm/min until it penetrated to a depth of 5.8 mm. Virus was delivered (1 μL of AAV; 0.8 μL of lentivirus) at 100 nL/min with a Hamilton syringe by hand or using a syringe pump (Harvard Apparatus). Injections of taCasp3-encoding AAV were spiked (9:1) with constitutive EGFP-encoding AAV of the same serotype to verify accuracy of the injection placement in control and PR^{Cre} mice. The needle was left for an additional 10 min to allow diffusion of the virus, and it was withdrawn at 1 mm/min. Mice were allowed to recover individually over a heating pad in fresh cages and when mobile returned to their home cage.

RT-qPCR

We collected 200 μm thick coronal slices from acutely dissected 10-12 week old brains of C57Bl/6J mice using a vibrating microtome (Leica) into a dish containing ice-cold d-PBS (free of Ca^{++} and Mg^{++}). The basal forebrain, AVPV/POA, BNST, VMHvl, arcuate, cingulate cortex, dentate gyrus, and MeA were dissected from these slices using a stereomicroscope and landmarks from the mouse brain atlas (Paxinos and Franklin, 2003), and the tissue fragments were immediately frozen on dry ice. Total RNA was extracted with Trizol, treated with DNase I and subjected to first strand cDNA synthesis using random hexamers as well as oligo-dT primed

reactions (SuperScript III, Invitrogen). qPCR was performed using the primers for PR mRNA (Table S3) on an Eppendorf Mastercycler EP using 2XSYBR master mix (Fermentas). A separate real time PCR reaction (primers listed in Table S3) to detect expression of the ubiquitous ribosomal message Rpl32 was used to permit normalization of PR expression levels in each of the brain regions.

Histology

Sexually naive, group-housed, age-matched mice were used in all histological studies to quantify sex differences in PR expression or projections of PR+ neurons. PLAP and β-gal histochemistry was performed as described previously on vibratome-collected coronal sections of 80 μm thickness (Shah et al., 2004; Wu et al., 2009). PLAP-labeled projections were imaged in brightfield illumination and analyzed using NIH ImageJ software. For each of the projection targets (AVPV, POA, PAG) of the PR+ VMHvl neurons, we quantitated the projections in the entire region of interest using previously described protocols (Wu et al., 2009; Xu et al., 2012).

Immunolabeling was performed using 65 μm thick vibratome brain sections using previously published protocols (Shah et al., 2004; Wu et al., 2009). The primary antisera used are: rabbit anti-β-gal (ICL, 1:5000), chicken anti-β-gal (Abcam, 1:6000), mouse anti-NeuN (Chemicon, 1:300), rabbit anti-ERα (Millipore, 1:10000), rabbit anti-GFP (Invitrogen, 1:2000). The fluorophore conjugated secondary antisera are: Cy3 donkey anti-rabbit, Cy3 donkey anti-chicken (Jackson ImmunoResearch, 1:800), Cy5 donkey anti-mouse (Jackson ImmunoResearch, 1:300), and AlexaFluor 488 donkey anti-rabbit (Invitrogen, 1:300). To quantitate sex differences in PR expression, we enumerated β-gal+ cells from $PR^{PL/PL}$ mice on both sides of the brain (left

and right) individually for each region of interest and obtained the mean for each animal. These cells were imaged with confocal microscopy (Zeiss) as described earlier, using methods validated with unbiased stereology (Wu et al., 2009). An identical approach was used to enumerate β-gal+ cells in the VMH subsequent to delivery of the taCasp3-encoding AAV. To enumerate β-gal+ cells along the entire rostrocaudal extent of the hypothalamus in these studies, we imaged every third section in this region (starting from the AVPV to the mamillary bodies) and quantitated the cells as described earlier, using methods validated with unbiased stereology (Wu et al., 2009).

Cckar, Cre, and PR probes for in situ hybridization (ISH) were generated from subcloned RT-PCR products (primers listed in Table S3). The ISH was performed as described previously (Xu et al., 2012). Briefly, mice were perfused with 4% paraformaldehyde (PFA), and the brains were dissected, post-fixed, and sectioned at 100 μm with a vibrating microtome (Leica). Sections were treated with proteinase K (10 μg/mL, Roche) and fixed at room temperature. Sections were then acetylated and equilibrated to hybridization solution for 2-5 hours at 65°C, followed by incubation at 65°C overnight in hybridization buffer containing 0.5 μg/mL digoxigenin-labeled RNA probe. The sections were then washed in high stringency buffers and incubated overnight at 4°C in buffer containing alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (Roche, 1:2000). The sections were then incubated for 4-6 hours at 37°C in staining solution containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (NBT and BCIP, respectively; Roche). Finally, sections were washed, post-fixed, and mounted on glass slides. mRNA expression was quantified as described previously (Xu et al., 2012).

For dual colorimetric in situ hybridization and fluorescent immunolabeling, adult brains were fresh frozen in embedding medium (M1 Embedding Matrix, Thermo Scientific) and cryosectioned at 16 μm on to glass slides (Superfrost, Fisher). Sections were fixed in 4% PFA and then acetylated as described previously (Juntti et al., 2010). After permeabilization with 1% TritonX-100, sections were incubated with prehybridization solution in a humidifying chamber for 2-4 hours at room temperature. Sections were hybridized with digoxigenin-UTP-labeled Cckar riboprobe (0.3 μg /ml) overnight at 65°C. After washes, brain sections were incubated with sheep anti-digoxigenin conjugated to alkaline phosphatase (Roche, 1:5000) and chicken anti-β-gal (Abcam, 1:3000) in 5% heat inactivated serum from sheep and donkey overnight at 4°C. The sections were washed and stained using a colorimetric reaction with NBT/BCIP (Roche) at 37°C overnight. The reaction was stopped with PBS containing 1 mM EDTA, and the sections were washed and incubated with a secondary antibody, donkey anti-chicken Cy3 antibody (Jackson ImmunoResearch, 1:800), and DAPI for 2 hours at room temperature. After washes and a 10 min post-fix in 4% PFA, slides were coverslipped with Aquamount mounting medium (Polysciences). We imaged these sections on an upright microscope (Zeiss) using a 20X objective, switching between brightfield illumination (Cckar) and epifluorescence (β-gal). These images were overlaid in Adobe Photoshop and enumerated for the region of interest (VMHvl). Detailed protocols are available upon request.

NeuroTrace 640/660 (Invitrogen, 1:200) was used per instructions from the manufacturer in sections immunolabeled for β-gal. To quantify nuclear and soma size of PR+ VMHvl neurons, β-gal and NeuN immunolabeled sections were imaged with a confocal microscope

using a 63X objective lens. The center 3 optical slices for each z-stack were flattened (maximal projection) in ImageJ. The nuclear and soma profiles were outlined and measured in ImageJ.

Behavior and Physiology

All behavioral testing was initiated ≥1 hour after onset of the dark cycle, and recorded and analyzed as described previously (Juntti et al., 2010; Wu et al., 2009; Xu et al., 2012). Mice were rested for ≥ 2 days between behavioral tests, and residents were always exposed to a novel intruder. For tests of sexual receptivity, females were rested for 7-10 days after an assay to allow hormone levels to subside to baseline levels prior to estrus induction for the next assay.

For male mice, singly housed residents were tested 2 times each for sexual behavior for 30 min with a WT female intruder hormonally primed to be in estrus. These residents were then tested 2 times each for aggression with a WT group-housed male intruder for 15 min. Performance in urine marking was tested once for 60 min in a fresh cage following social experience. Males were tested for ultrasonic vocalizations once each for 3 min to a WT male and female intruder introduced separately into the cage. Following all behavioral testing, the males were sacrificed and blood was collected to determine serum hormone levels as described previously (Juntti et al., 2010; Wu et al., 2009; Xu et al., 2012).

To test females in assays of sexual receptivity, the ovaries were removed, and the mice were allowed to recover from surgery for 4 weeks. Prior to behavioral testing, the females were hormonally primed to be in estrus using previously published protocols (Ogawa et al., 2000;

Ring, 1944; Xu et al., 2012). Briefly, we administered subcutaneously 17 β-estradiol benzoate on day -2 (10 μg in 100 μL sesame oil), day -1 (5 μg in 50 μL sesame oil), and progesterone (50 μg in 50 μL sesame oil) on the day of testing. The females were tested with resident males 4-6 hours after receiving progesterone for 30 min each in 3 assays.

For the rotarod test, we followed standard procedures described previously (Jones and Roberts, 1968; Juntti et al., 2010; Xu et al., 2012). In brief, the mice were tested on an accelerated rotarod set-up (Ugo Basile) for 5 min each. We monitored the amount of time each mouse could successfully remain on the rotarod. For the cookie finding test, we followed a previously described protocol (Xu et al., 2012). Briefly, mice were food deprived overnight and then placed into a fresh cage containing a cookie buried under the bedding. Their behavior was recorded for 3 min following which the assay was terminated. Each mouse was tested twice in this cookie finding assay, and performance was assessed by the average of these two tests. For the elevated plus maze test, mice were placed in the center of an elevated maze facing the open arm at the start of the assay (Walf and Frye, 2007). Time spent in the closed or open arms during a 5 min interval was recorded.

Pup retrieval and maternal aggression were tested in females impregnated by a WT male that were singly housed 3–5 days prior to parturition. At 2, 4, and 6 days after parturition, the dam was removed briefly from the cage, and 4 pups were scattered across the cage floor away from the nest. The dam was returned to the cage, and her ability to retrieve these pups to the nest was tested for 10 min. To test for maternal aggression, pups of postnatal age 8, 10, and 12 days

were removed, and a group-housed adult WT male intruder was inserted into the cage for 15 min. The pups were returned to the mother at the end of each assay.

For assessing any change in body weight subsequent to ablation of PR+ VMHvl neurons, the mice were weighed immediately prior to stereotaxic injection of the taCasp3-encoding virus. The mice were then weighed at the time of sacrifice (~10 weeks) and analysis of histology through the hypothalamus.

Daily vaginal smears were obtained from group-housed females for 2 weeks, and the cytological characteristics of the smear were examined with brightfield optics as described previously (Xu et al., 2012). An experimenter blind to the genotypes independently scored the stage of the estrous cycle.

Hormone assays

Hormone titers were assayed with kits from Cayman Chemicals (estradiol, progesterone) and DRG International (testosterone) according to the manufacturer's protocols. Trunk blood was collected at the time of sacrifice 2-3 hours after the onset of the dark cycle.

Data analysis

Behavioral and histological studies were performed and analyzed while blinded to the relevant variables, including sex, genotype, virus injected, and hormone treatment. For analysis of non-categorical parameters of mating, aggression, and maternal care, we only included data from the animals that performed the behavior. Linear regression analysis was performed using MATLAB.

We used Fisher's exact test to analyze categorical data. For all other comparisons, we first analyzed the data distribution with the Lillefors' goodness-of-fit test of normality. Datasets not violating this test of normality were analyzed with Student's t-test; otherwise we used the non-parametric Wilcoxon rank sum test. We always processed in parallel ≥1 mouse/sex for quantitating sex differences in PR+ neurons and ≥1 mouse/genotype (control and PR^{Cre}) for analyzing ablations.

REFERENCES

Bunting, M., Bernstein, K.E., Greer, J.M., Capecchi, M.R., and Thomas, K.R. (1999). Targeting genes for self-excision in the germ line. Genes Dev. *13*, 1524–1528.

Gray, D.C., Mahrus, S., and Wells, J.A. (2010). Activation of specific apoptotic caspases with an engineered small-molecule-activated protease. Cell *142*, 637–646.

Jones, B.J., and Roberts, D.J. (1968). The quantitative measurement of motor inco-ordination in naive mice using an accelerating rotarod. Journal of Pharmacy and Pharmacology *20*, 302–304.

Juntti, S.A., Tollkuhn, J., Wu, M.V., Fraser, E.J., Soderborg, T., Tan, S., Honda, S.-I., Harada, N., and Shah, N.M. (2010). The androgen receptor governs the execution, but not programming, of male sexual and territorial behaviors. Neuron *66*, 260–272.

Miyoshi, H., Blömer, U., Takahashi, M., Gage, F.H., and Verma, I.M. (1998). Development of a Self-Inactivating Lentivirus Vector. J. Virol. *72*, 8150–8157.

Ogawa, S., Chester, A.E., Hewitt, S.C., Walker, V.R., Gustafsson, J.A., Smithies, O., Korach, K.S., and Pfaff, D.W. (2000). Abolition of male sexual behaviors in mice lacking estrogen receptors alpha and beta (alpha beta ERKO). Proc. Natl. Acad. Sci. U.S.A *97*, 14737–14741.

Paxinos, G., and Franklin, K.B.J. (2003). The Mouse Brain in Stereotaxic Coordinates: Compact Second Edition, Second Edition (Academic Press).

Ring, J.R. (1944). The estrogen-progesterone induction of sexual receptivity in the spayed female mouse. Endocrinology *34*, 269–275.

Rodríguez, C.I., Buchholz, F., Galloway, J., Sequerra, R., Kasper, J., Ayala, R., Stewart, A.F., and Dymecki, S.M. (2000). High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. Nat. Genet. *25*, 139–140.

Shah, N.M., Pisapia, D.J., Maniatis, S., Mendelsohn, M.M., Nemes, A., and Axel, R. (2004). Visualizing sexual dimorphism in the brain. Neuron *43*, 313–319.

Walf, A.A., and Frye, C.A. (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nat Protoc *2*, 322–328.

Wu, M.V., Manoli, D.S., Fraser, E.J., Coats, J.K., Tollkuhn, J., Honda, S.-I., Harada, N., and Shah, N.M. (2009). Estrogen masculinizes neural pathways and sex-specific behaviors. Cell *139*, 61–72.

Xu, X., Coats, J.K., Yang, C.F., Wang, A., Ahmed, O.M., Alvarado, M., Izumi, T., and Shah, N.M. (2012). Modular genetic control of sexually dimorphic behaviors. Cell *148*, 596–607.

Zhang, F., Wang, L.-P., Boyden, E.S., and Deisseroth, K. (2006). Channelrhodopsin-2 and optical control of excitable cells. Nat. Methods *3*, 785–792.