Supplemental Data

Lhx6 Delineates a Pathway Mediating Innate Reproductive Behaviors from the Amygdala to the Hypothalamus

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Supplemental Experimental Procedures

Generation of PLAP Mice

VelociGene technology was used to generate a deletion and exchange of Lhx6 and Lhx9 coding regions with the placental alkaline phosphotase (PLAP) reporter gene as well as a neomycin-selection cassette (Valenzuela et al., 2003). The resultant chimeric male mice were then bred to C57BL/6 females to generate F1 mice or embryos.

In Situ Hybridization and PLAP Staining

Nonradioactive in situ hybridization was performed as previously described (Zhou et al., 2001). The following mouse genes were used: *Lhx6*, *Lhx9*, *Lhx5*, *Gad65*, *Vglut2*, and *c-fos*.

For fluorescent in situ hybridization, digoxygenin- or fluorescein-labeled probes were detected with horseradish peroxidase-conjugated antibodies. The signal was amplified with the avidin-biotin complex system (ABC Elite Kit; Vector Laboratories, Burlingame, CA) and subsequently developed with fluorochrome-conjugated tyramide (Cy3- or FL-Tyramide; NEN, Boston, MA). For double fluorescent in situ hybridization, the first peroxidase-conjugated antibody was inactivated at 85°C. The second probe was detected in essentially the same way as the first.

PLAP staining was performed as previously described (Shah et al., 2004).

Immunohistochemistry

The following primary antibodies were used: rabbit anti-Lhx6 (1:1000; kind gift of Dr. Vassilis Pachnis), rabbit anti-Lhx2/Lhx9 (1:1000; kind gift of Dr. Thomas M. Jessell), rabbit anti-Lhx5 (1:2000; kind gift of Dr. Thomas M. Jessell), goat anti-CTB (1:1000; List), rabbit anti-FG (1:1000; Chemicon), and goat anti-c-fos (1:1000; Santa Cruz Biotechnology), and chicken anti-GFP (1:1000; Aves Labs). The fluorophore-conjugated secondary antisera used were: Cy5 donkey anti-rabbit (1:30;, Jackson ImmunoResearch), Alexa 488 donkey anti-rabbit (1:250; Molecular Probes), Alexa 488 donkey anti-goat (1:250; Molecular Probes), Alexa 568 donkey anti-goat (1:250; Molecular Probes), and Alexa 568 Goat anti-rabbit (1:250; Molecular Probes).

Retrograde Tracing Experiments
Sexually naive 8-week-old C57BL/6 male mice from (The Jackson Laboratory) received bilateral injections of 0.5% cholera toxin B subunit (CTB, low salt; List, Campbell, CA) and/or 2% Fluoro-Gold (FG; Fluorochrome, Englewood, CO) in the following brain areas: MPN, AHN, VMHdm, VMHvl, PMv, and BST. The tracers were delivered with a positive-pulsed current of 5 μA for 2 min. After surviving for 7 days, the animals were anesthetized with a mixture of ketamine and xylazine and perfused transcardially with 5 ml of PBS followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were carefully dissected out, cryoprotected in 15% sucrose, and sectioned at 20 μm. The sections were immunostained with anti-CTB (see below) and/or anti-FG (see below) antibody and subsequently incubated with a fluorescent Nissl staining reagent, Neurotrace Green (1:100; Molecular Probes).

Behavioral Assays
Retired C57BL/6 male mice (Harlan Sprague-Dawley) were used for all behavioral assays.

For the behavioral assays following retrograde tracing experiments, animals were allowed to recover for a minimum of 1week after the surgery. For cat-odor exposure experiments, animals were exposed for 30 min to pieces of a cat collar, which had been worn for 2 weeks by a domestic cat. The control odor group was exposed to equivalent pieces from a collar that had not been worn by a cat. For urine exposure experiments, males were exposed to distilled water, urine from C57BL/6 males, or urine from C57BL/6 females of various ages. For resident-intruder assays, C57 males were allowed to interact with no animal (left alone), with DBA2 females, or with DBA2 males for 10 min. Thirty minutes (for c-fos mRNA detection) or 1 hour (for c-fos protein detection) after the introduction of the stimulus, animals were processed for in situ hybridization and/or immunohistochemistry as described above.

Quantification
Slices (~1 μm optical thickness) of the MEApd, MEApv, or MEA from each animal were imaged to quantify the number of c-fos$^+$ cells, c-fos$^+$Lhx6$^+$ cells, or c-fos$^+$CTB$^+$ cells in those regions. The total number of such cells was obtained by analyzing slices taken at 120 μm intervals over all MEA sections. The number of cells per section was obtained by averaging the total number by the number of the sections analyzed for each animal. For CTB and FG double injection experiments, the number of CTB$^+$FG$^-$ or the number of total CTB$^+$ cells were counted in the same manner.

Figure 1A. Supplemental Abbreviations
AHN = anterior hypothalamic nucleus; BLAa,p = basolateral amygdala, anterior or posterior part; BMAa,p = basomedial amygdala, anterior or posterior part; CEAc,l,m = central amygdala, capsular, lateral, or medial part; LA = lateral amygdala; MEApd,pv = medial amygdala, posterior dorsal or posterior ventral part; MPNI = medial preoptic nucleus; PMd = dorsal premammillary nucleus; PMv = ventral premammillary nucleus; VMHdm,vl = ventromedial hypothalamic nucleus, dorsomedial or ventrolateral part.

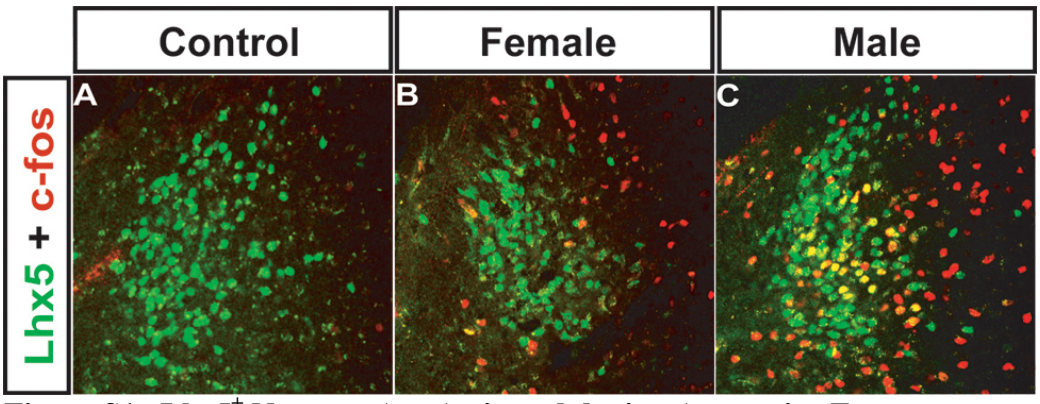

Figure S1. Lhx5^{+} Neurons Are Activated during Aggressive Encounters

(A–C) Transverse sections through the MEAa of C57 males interacting with no other animals (A), DBA2 females (B), or DBA2 males (C), immunostained with anti-Lhx5 and anti-c-fos antibodies.

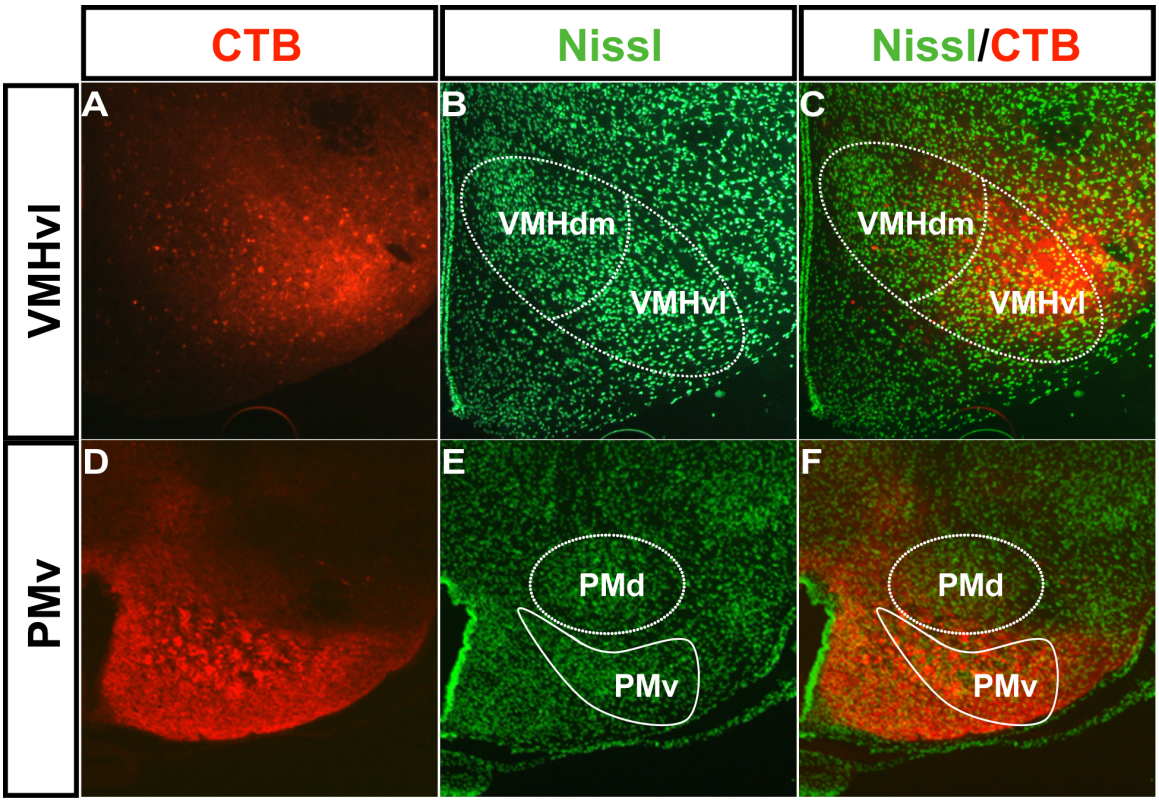

Figure S2. Injection Sites into the PMv and VMHvl

(A–C) Transverse section through the VMH from the mouse injected with CTB in the VMHvl, immunostained with anti-CTB antibody and followed by fluorescent Nissl staining.

(D–F) Transverse section through the PM from the mouse injected with CTB in the PMv, immunostained with anti-CTB antibody and followed by fluorescent Nissl staining.

Table S1. Abbreviations

AOB	Accessory Olfactory Bulb
AHN	Anterior Hypothalamic Nucleus
BSTif	Bed Nucleus of Stria Terminalis, Posterior Division, Interfascicular Nucleus
BSTpr	Bed Nucleus of Stria Terminalis, Posterior Division, Principal Nucleus
MEAa	Medial Amygdala, anterior division
MEApd	Medial Amygdala, posterior dorsal division
MEApv	Medial Amygdala, posterior ventral division
MPN	Medial Preoptic Nucleus
PMv	Premammillary nucleus, ventral portion
PMd	Premammillary nucleus, dorsal portion
PVT	Paraventricular thalamic nucleus
TU	Tuberal hypothalamic nucleus
VMHdm	Ventromedial Hypothalamic Nucleus, dorsomedial
VMHvl	Ventromedial Hypothalamic Nucleus, ventrolateral
VNO	Vomeronasal Organ

Supplemental References

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