Supporting Information

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SI Materials and Methods

The experimental procedures were approved by the local ethical committee (Stockholm's Norra Djurförsöksetiska Nämnd) and were in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1996 revision). During the investigation, every effort was made to minimize animal suffering and to reduce the number of animals used.

Anatomy. The animals were deeply anesthetized using tricane methane sulfonate (MS-222; 100 mg/L; Sigma) diluted in fresh water. They were then transected caudally at the seventh gill, and the dorsal skin and cartilage were removed to expose the brain. During the dissection and the injections, the head was pinned down and submerged in ice-cooled oxygenated Hepes-buffered physiological solution [138 mM NaCl, 2.1 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 4 mM glucose, and 2 mM Hepes (pH 7.4)].

Anatomical Tracing. All injections were made with glass (borosilicate, outer diameter = 1.5 mm, inner diameter = 1.17 mm) micropipettes, with a tip diameter of 10–20 μm. The micropipettes were fixed in a holder, which was attached to an air supply and a Narishige micromanipulator. A total of 50–200 nL of Neurobiotin [20% (wt/vol) in distilled water containing Fast Green to aid visualization of the injected tracer; Vector] was pressure-injected unilaterally into the habenula, lfHb, right habenula, rdHb, ntp, lateral hypothalamus, periventricular hypothalamus, dMAM, vMAM, IPN, mOB, striatum, subhippocampal lobe, and pretectum.

Dissection and Histology. Following injections, the heads were kept submerged in Hepes in the dark at 4 °C for 24 h to allow retrograde transport of the tracers. The brains were then dissected out of the surrounding tissue and fixed by immersion in 4% (vol/vol) formalin and 14% (vol/vol) saturated picric acid in 0.1 M phosphate buffer (PB), pH 7.4, for 12–24 h, after which they were cryoprotected in 20% (wt/vol) sucrose in PB for 3–12 h. In the case of histamine immunohistochemistry, brains were fixed by immersion in 4% (wt/vol) 1-ethyl-3-(3-diimethylaminopropyl)-carbodiimide hydrochloride (Sigma–Aldrich) in 0.1 M PB. Transverse sections (20 μm thick) were made using a cryostat, collected on gelatin-coated slides, and stored at −20 °C until further processing. For GABA immunohistochemistry, animals were perfused through the ascending aorta with 4% (vol/vol) formalin, 2% (vol/vol) glutaraldehyde, and 14% (vol/vol) of a saturated solution of picric acid in PB. The brain was postfixed for 24–48 h and cryoprotected as described above.

Immunohistochemistry. For immunohistochemical detection, brains were dissected out and processed as described above. Sections were then incubated overnight with a mouse monoclonal anti-GABA antibody (1:1,000, mAb 3A12; kindly donated by Peter Streit, Brain Research Institute, Zurich, Switzerland) (1), rabbit anti-histamine antiserum (1:10,000, 19C; kindly donated by Pertti Panula, University of Helsinki, Helsinki, Finland) (2), mouse monoclonal anti-calbindin-D28K antibody (1:2,000, C9848; Sigma–Aldrich), mouse monoclonal anti-tyrosine hydroxylase antibody (1:200; MAB318; Millipore), and rabbit polyclonal anti–5-HT antiserum (1:1,000; ID 20080; Immuno Star, Inc.). Sections were subsequently incubated with appropriate secondary antibodies (1:500; Jackson ImmunoResearch Laboratories) for 2 h and cover-slipped with glycerol containing 2.5% (wt/vol) DABCO (Sigma–Aldrich).

1. Matute C, Streit P (1986) Monoclonal antibodies demonstrating GABA-like immunoreactivity. *Histochemistry* 86(2):147–157.

2. Panula P, Airaksinen MS, Pirvola U, Kotilainen E (1990) A histamine-containing neuronal system in human brain. *Neuroscience* 34:127–132.

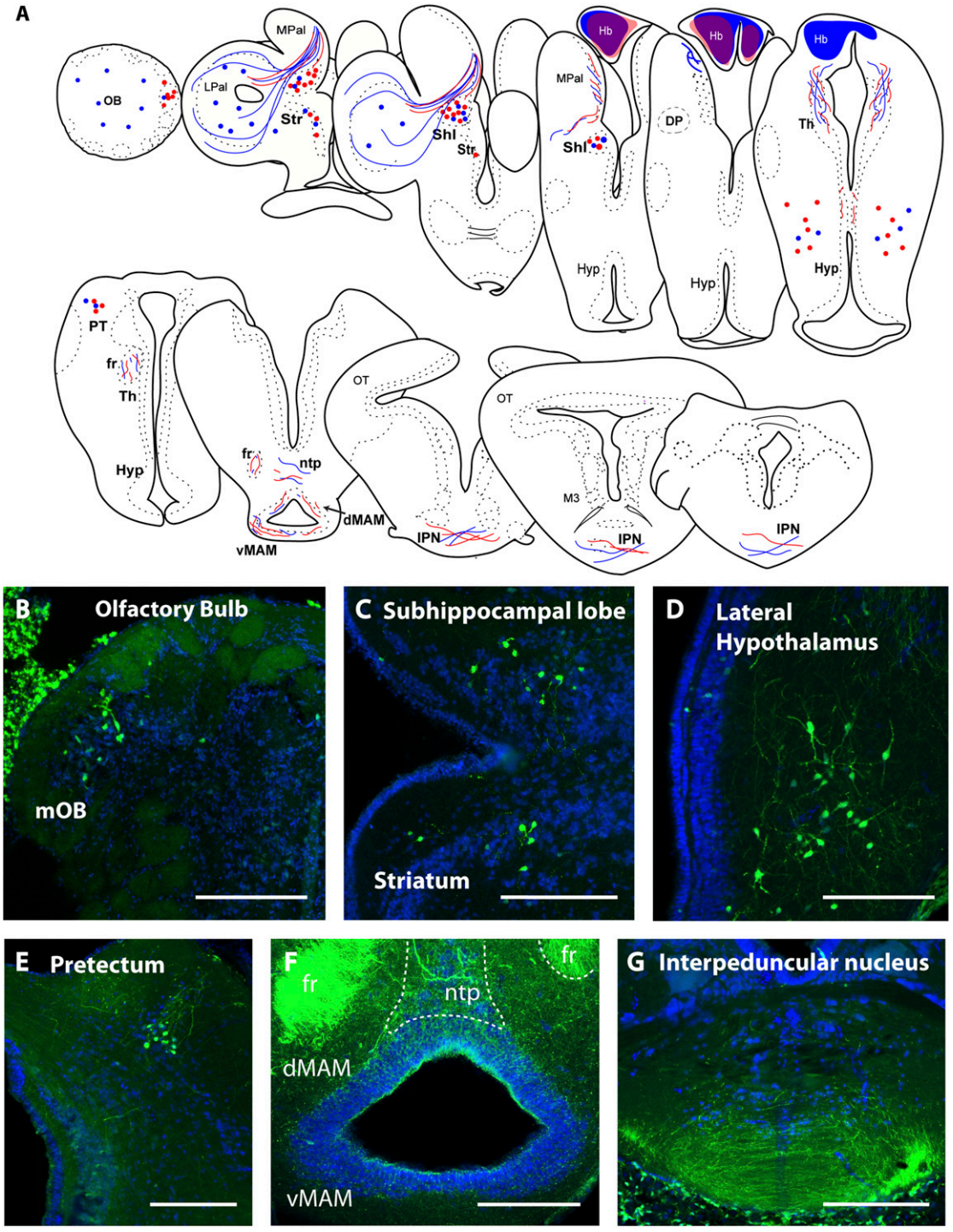

Fig. S1. Afferent and efferent connections of the lamprey habenula. (*A*) Schematic transverse sections through the lamprey brain showing the location of retrogradely labeled cells (red and blue dots) and anterogradely labeled fibers (red and blue lines) from two habenula injection sites (Neurobiotin), including (blue) and excluding (red) the habenular commissure. Photomicrographs show the location of retrogradely labeled cells and anterogradely labeled fibers in the mOB (*B*); subhippocampal lobe (Shl) and striatum (Str) (*C*); lateral hypothalamus (*D*); pretectum (PT; *E*); ntp, dMAM, and vMAM (*F*); and IPN (*G*). fr, fasciculus retroflexus; Hb, habenula; Hyp, hypothalamus; M3, mesencephalic Muller cell 3; MPal, medial pallium; LPal, lateral pallium; OB, olfactory bulb; OT, optic tectum; PT, pretectum; Th, thalamus. (Scale bars: 200 μM.)

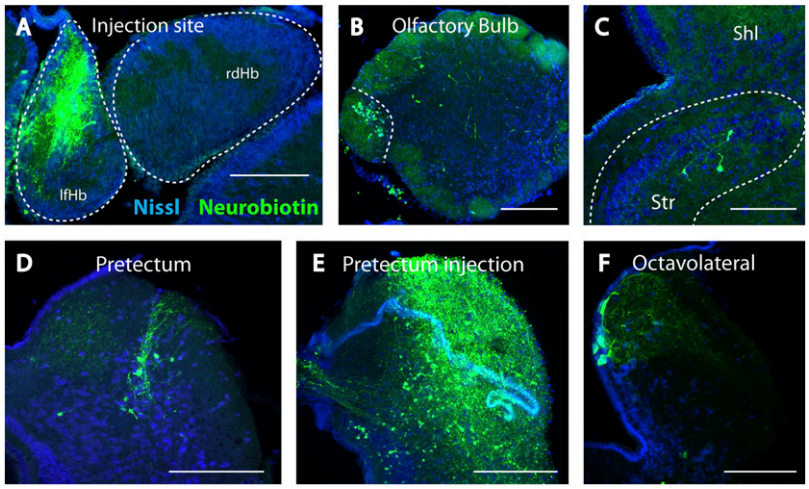

Fig. S2. Afferent projections to the lfHb. (*A*) Neurobiotin injection (green) into the lfHb resulting in retrogradely labeled cells in the mOB (*B*), striatum (Str; *C*), and pretectum (*D*). Neurobiotin injection (green) into the pretectum (*E*) resulting in retrogradely labeled cells in the octavolateral area (*F*). Shl, subhippocampal lobe. (Scale bars: 200 μM.)

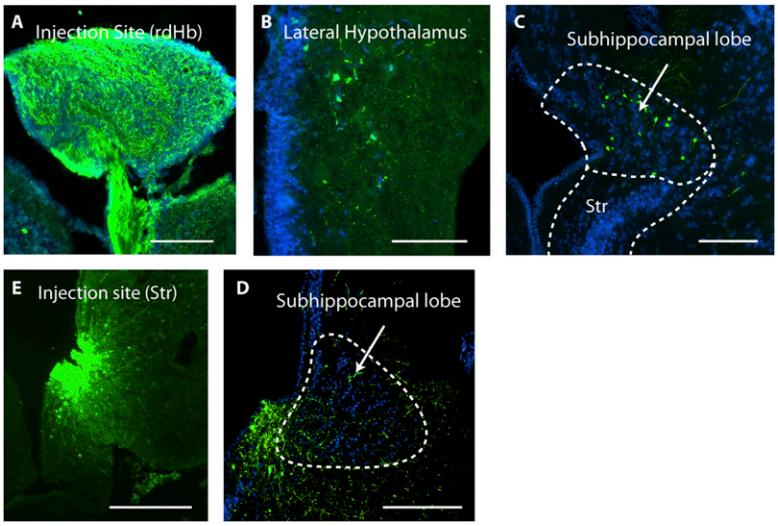

Fig. S3. Afferent projections to the rdHb. (*A*) Neurobiotin injection (green) into the rdHb resulting in retrogradely labeled cells in the lateral hypothalamus (*B*) and subhippocampal lobe (*C*). Neurobiotin injection (green) into the striatum (Str; *D*) resulting in anterogradely labeled fibers in the subhippocampal lobe (*E*). (Scale bars: 200 μM.)