Supporting Information

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SI Materials and Methods

Fish Strains and Maintenance. Zebrafish were maintained at 27 °C in a 14:10-h light/dark cycle. The WT AB strain (1) and *Tg*(*slc17a6b:DsRed*)^{nns9} (2), *TgBAC*(*gng8:nfsB-CAAX-GFP*)^{c375} (3), *Tg*(*brn3a-hsp70:GFP*)^{rw0110b} (4), and *Tg*(*UAS:ChR2-mCherry*)$^{s1985t/+}$ (5) lines were used. To construct *TgBAC*(*gng8:GAL4*)^{c426}, sequences encoding Gal4FF, a modified form of Gal4 (6), were inserted into a Tol2-Amp^{r}-Tol2 cassette that contained homology arms to exon 2 of the *guanine nucleotide binding protein* (*G protein*), *gamma 8* (*gng8*) gene. The bacterial artificial chromosome DKEY-182I3 BAC (GenBank accession no. CR450711) was used for recombineering (3, 7) into the *gng8* locus. Maintenance of zebrafish stocks and experiments on larvae and adults were carried out in accordance with the Carnegie Institutional Animal Care and Use Committee.

RNA-Sequencing and Identification of *chatb*. To enrich for *choline acetyltransferase b (chatb)* transcript, brains were collected from 80 *TgBAC*(*gng8:nfsB-CAAX-GFP*)^{c375} adults. Habenular nuclei (Hb) were microdissected in cold PBS and stored in RNA-later (Invitrogen). RNA was extracted using TRIzol (Ambion) and prepared using the Illumina TruSeq RNA Library Prep kit with Poly-A selection. Paired-end DNA sequencing was performed on an Illumina HiSeq2000. Paired-end reads were estimated to have a median length of 153 nt using CollectInsertSizeMetrics (version 1.84; Picard). We screened for reads that aligned to the partial *chatb* sequence at one end, expecting the unaligned end to contain additional coding sequence. Using SAMtools (filter parameters: -f 4 -F 264 and -f 8 -F 260), 2,022 fragments were extracted and converted back to the FastQ format using bam2fastq (version 1.1.0; HudsonAlpha Institute). Velvet 1.2.08 and Oases version 0.2.08 were used to assemble reads, yielding a transcript of 1,880 nt in length. For verification, we carried out Sanger DNA sequencing and identified the translation initiation site and stop codons by comparisons to zebrafish ChATb with ChATa and ChAT-related proteins from other species.

RNA in Situ Hybridization. Techniques for colorimetric (8) and fluorescent (3) RNA in situ hybridization (ISH) were performed as described. To synthesize RNA probes, PCR fragments for *chata* (ENSDARG00000015854), *chatb*, *vesicular acetylcholine transporter a* (*vachta*) (ENSDARG00000006356), *vachtb (*ENSDARG00000090189), *high affinity choline transporter a* (*hacta*) (ENSDARG00000074860), *high affinity choline transporter b* (*hactb*) (ENSDARG00000060912), *tachykinin 1* (*tac1*), cholinergic receptor, nicotine *(chrn)3*, and *chrnb4* were subcloned into pCRII-TOPO vector using the TOPO TA Cloning kit (Invitrogen). Restriction enzymes and RNA polymerases used to synthesize antisense probes were as follows: *vachta* (*EcoRV*/SP6), *vachtb* (*Xho*/T7), *chata* (*Bam*H1/T7), *chatb* (*Not*1/Sp6), *hacta* (*Not*1/SP6), *hactb* (*Not*1/SP6), *tac1* (*Sal*1/T7), *chrna3* (*Bam*H1/T7), and *chrnb4* (*Bam*H1/T7). Probes for the *amine oxidase, copper containing 1* (*aoc1*) (9), *chrna2a*, and *chrna7* (10) genes were prepared as indicated. Antisense RNA probes for the zebrafish *chrna2b* and *chrna5* genes were generated by direct PCR amplification from genomic DNA using forward and reverse primers (*chrna2b*: GTGACCCACATGACCAAAG and GGATCCATTAACCCTCACTAAAGGGAAAGCATCTTCGGCTCTCAG; *chrna5*: ATG GCACAATCTCCTGGACT and ATTCTCCTTCACCACATGGC) and transcribed using the T3 and T7 RNA polymerases, respectively. A 753-bp fragment was amplified from the zebrafish *v-fos FBJ murine osteosarcoma viral oncogene homolog* (*fos*) gene (ENSDARG00000031683) using forward (GAAACTGACCAGCTTGAGGATG) and reverse (GCAGGCATGTATGGTTCAGA) primers and subcloned into the pCR2.1-TOPO vector (Invitrogen). Plasmid was linearized by HindIII and antisense RNA probe synthesized using T7 RNA polymerase.

Sectioning and Microscopy. Larvae or adult brains were embedded in 4% low melt agarose (Lonza) in PBS (100 gm/ml) and sectioned to 50 μm using a vibratome (Leica VT1000S; Leica Microsystems, Inc.). Sections were covered in 50% glycerol (1:1 vol/vol with H_2O) or Aqua-Poly/Mount (Polysciences, Inc.) under coverslips. Bright-field images were captured with an Axiocam HRc digital camera mounted on an Axioskop (Carl Zeiss). Fluorescent images were collected using a Leica SP5 confocal microscope and processed via ImageJ (National Institutes of Health).

Nicotine Treatment. Adult fish were acclimated in breeding tanks containing system water for a minimum of 3 h. System water or a nicotine solution (N3876; Sigma) was added for a final concentration of 5 μM. After 30–45 min, fish were killed and brains were dissected in cold PBS. To detect *fos* expression, ISH was carried out as above.

Electrophysiology. Larvae (5 d postfertilization) were anesthetized in Tricaine (0.01%), and their brains were dissected in artificial cerebrospinal fluid (ACSF) containing 126 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.25 mM $NaHPO_4$, and 10 mM glucose and saturated with 95% O_2/5% CO_2 at room temperature (20–25 °C). Each brain was affixed to a glass coverslip ventral side up using the plasma thrombin embedding procedure of Legendre and Korn (11). The coverslip was transferred to a recording chamber, and the brain was perfused with ACSF at a rate of 1–2 mL/min. Patch electrodes were filled with an intracellular solution containing 130 mM K-gluconate, 8 mM NaCl, 4 mM $MgCl_2$, 4 mM Na-ATP, 0.3 mM Na-GTP, 10 mM Hepes, and 10 mM EGTA, adjusted to pH 7.2, 290 mOsm, for resistances between 5 and 8 MΩ. Whole-cell recordings were obtained using a Multiclamp 700B amplifier (Molecular Devices) and monitored via pClamp 10 (Molecular Devices). Analyses were performed using Clampfit 10 (Molecular Devices). For all experiments, data were filtered at 4 kHz during recording and the traces were digitized at 20 kHz. To isolate excitatory cationic currents (E_{cation} = 0 mV) from inhibitory chloride currents, interpeduncular nucleus neurons were recorded in voltage-clamp configuration at a holding potential of −60 mV, close to the theoretical reversal potential of chloride (E_{Cl} = −60 mV) under the described conditions.

Optogenetic Stimulation. The entire brain was exposed to blue light. Optogenetic stimulation was performed using a 200-μm optic fiber connected to a 470-nm light-emitting diode (LED) source (M470F1; Thorlabs SAS). LED intensity and the duration of illumination were controlled via a dedicated LED Driver (DC2100; Thorlabs SAS) using pClamp10.

Electrical Stimulation. The right Hb was stimulated via a low-resistance (<1 MΩ) glass pipette filled with ACSF containing an AgCl electrode. Pulse sequences (20 stimulations at 50 Hz for 400 ms) were generated using pClamp 10 and applied with a constant current stimulus isolator (DS3; Digitimer Ltd.).

Stimuli varied between 10 and 500 μA in intensity and were 100 μs in duration.

Pharmacology. Drugs were obtained from Tocris Bioscience, dissolved in ACSF, and applied through bath application with an exchange rate of 2–3 min. (+)-D-tubocurarine chloride (10 μM), mecamylamine (100 μM), 6-cyano-7-nitroquinoxaline-2,3-dione (20 μM), DL-2-amino-5-phosphonopentanoic acid (100 μM), and gabazine (SR-95531, 10 μM) were used.

Statistical Analyses. Values are expressed as the mean ± SD. Statistical significance for the effect of cholinergic antagonists was assessed by a Wilcoxon matched pair test (GraphPad Software).

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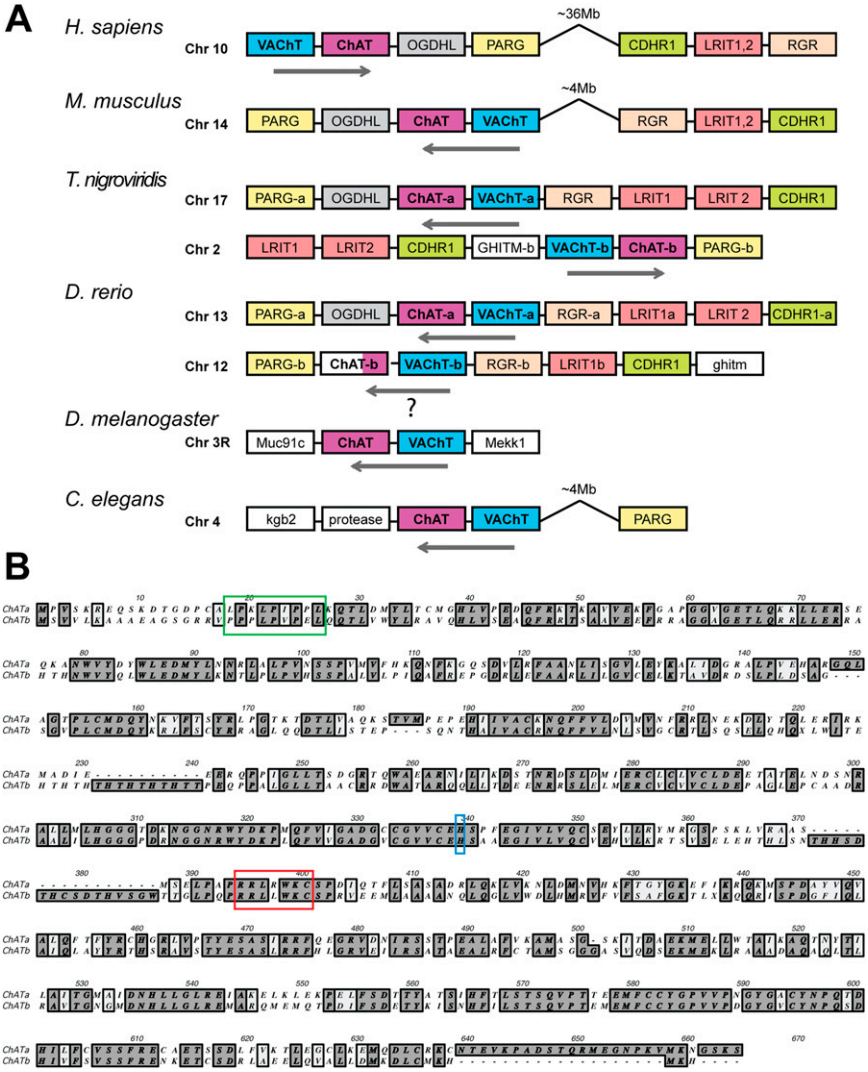

Fig. S1. Conservation of cholinergic gene locus (CGL) among species. (*A*) Schematic of chromosomal regions containing the CGL of *Homo sapiens*, *Mus musculus*, *Tetraodon nigroviridis*, *Danio rerio*, *Drosophila melanogaster*, and *Caenorhabditis elegans*. Arrows beneath the CGL indicate transcriptional orientation. Available contigs for zebrafish CGLb are not continuous, so the direction of transcription is unconfirmed. Syntenic loci are shown in color. Chr, chromosome. (*B*) Alignment of zebrafish ChATa and ChATb protein sequences indicates ~50% homology in amino acid identity. The green box demarcates the acyltransferase site, the blue box demarcates the conserved histidine domain, and the red box demarcates the putative enzyme catalytical domain.

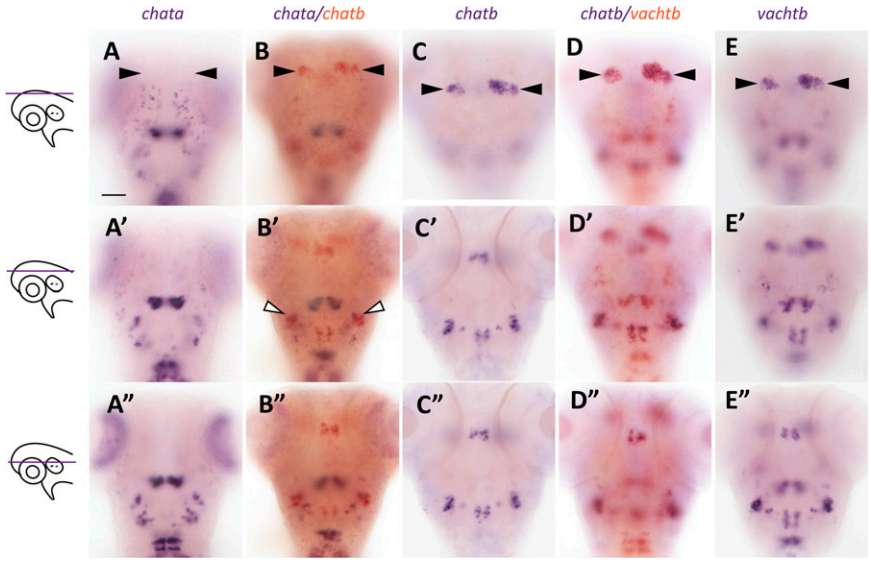

Fig. S2. Coexpression of *chatb* with *chata* and *vachtb*. Single and double ISH for *chata* (*A*–*A*′′), *chata/chatb* (*B*–*B*′′), *chatb* (*C*–*C*′′), *chatb/vachtb* (*D*–*D*′′), and *vachtb* (*E*–*E*′′) in 4-d postfertilization (dpf) larvae, imaged at the designated focal plane (at left). (*A*–*E*) Arrowheads mark the Hb. (*B*′) White arrowheads indicate *chata* and *chatb* coexpression in hindbrain neurons. (Scale bar: 50 μm.)

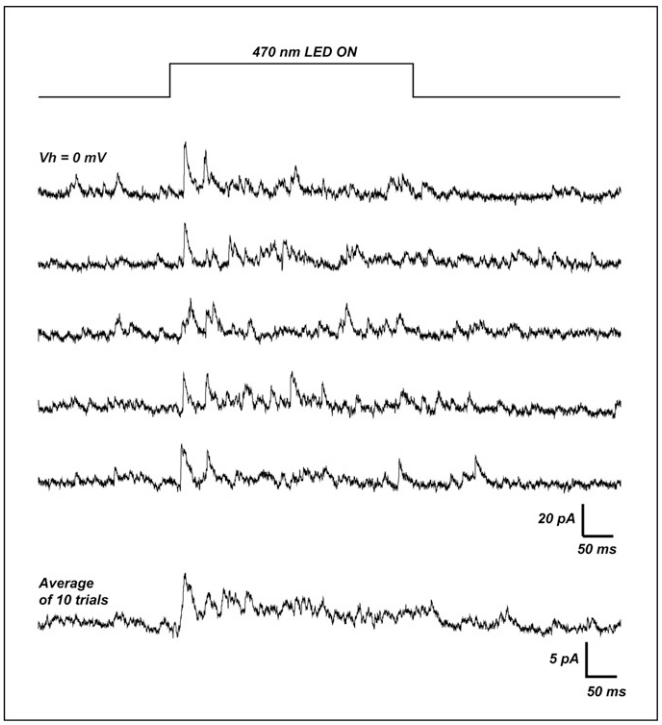

Fig. S3. Sustained optogenetic stimulation of Hb induces GABAergic currents in ventral interpeduncular nucleus (vIPN) neurons. (*Upper*) Representative examples of GABAergic currents evoked in a vIPN neuron (Vh = 0 mV) in response to optogenetic stimulation (400 ms) of Hb neurons in a channel rhodopsin 2 (ChR2)-mCherry–expressing 5-dpf larval brain. (*Lower*) Average of 10 responses.