Supporting Information

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SI Methods

Animals and Housing. A total of 40 violet-eared waxbills (29 male; 11 female) and 44 male zebra finches were used in the present experiments. Animals were housed on a 14 h light:10 h dark photoperiod and provided finch seed mix, fortified gravel, cuttlebone, dried greens, and water ad libitum. For the waxbills, finch seed was mixed with a high-protein crumb for insectivorous birds. We additionally used Angolan blue waxbills as stimuli in our experiments, which were maintained on the same waxbill diet. All cages were 43 cm high × 36 cm deep; for standard housing these were 1.0 or 1.3 m wide; experimental cages for waxbills were 61 cm wide.

Fos Immunocytochemistry and Analysis. Birds were perfused intracardially using 0.1 M phosphate buffered saline (PBS) followed by 4% (wt/vol) paraformaldehyde. Brains were sunk in 30% (wt/vol) sucrose and collected into three series of 40-μm sections. Fos labeling followed a standard protocol (1, 2), using rabbit anti-Fos (Santa Cruz Biotechnology) and an Alexa Fluor 594 secondary (Invitrogen). Tissue was rinsed five times for 10 min in 0.1 M PBS (pH 7.4), incubated for 1 h in block (PBS + 10% normal donkey serum + 0.3% Triton X-100; both vol/vol), and then incubated for ∼40 h at 4 °C in PBS + 5% normal donkey serum + 0.3% Triton X-100 containing rabbit anti-Fos (1:1,000). The primary incubation was followed by two 30-min rinses in PBS and 2 h in Alexa Fluor 594 secondary (5 μL/mL) at room temperature. Following two 30-min rinses in PBS, the sections were mounted on slides subbed with gelatin and chrome alum and were coverslipped with ProLong Gold antifade reagent with DAPI nuclear stain (Invitrogen). Images were acquired at 10× using a Zeiss AxioImager microscope outfitted with a *Z*-drive and an Apotome optical dissector (Carl Zeiss), and cell counts were conducted blindly by an observer using Photoshop CS5 (Adobe Systems) and ImageJ (National Institutes of Health, Bethesda, MD).

Photomicrographs of the anterior hyperthalamus (AH) were obtained at two rostrocaudal levels, lateral/ventrolateral to the caudal paraventricular nucleus, under the anterior commissure (first level; Fig. 1*D*) and ∼120 μm caudal to the commissure (second level). Shots were taken at 10× using a Zeiss Axioimager microscope outfitted with *Z*-drive, an optical dissector (Apotome), and an Axiocam digital camera. Cell counts were conducted as previously described (2, 3) using a grid of eight boxes (∼83 μm^2 each; 2 across × 4 down) at each level to examine spatial heterogeneity in function. Although initial analyses were conducted separately for both rostrocaudal levels and each of the eight grid boxes at each level, the data strongly suggest a dorsal–ventral distinction. We therefore report data for the dorsal and ventral halves of the AH.

VIP Sequencing and in Situ Hybridization. The entire coding region of the vasoactive intestinal polypeptide (VIP) gene was sequenced for zebra finches using standard PCR and 3′ and 5′ RACE techniques (see GenBank accession XM002187768.1), and a riboprobe was generated for a 405-bp sequence at the 3′ end. Labeling of VIP mRNA was conducted in both male and female zebra finches to confirm the pattern and specificity of VIP immunolabeling and to help us assess the quality of our colchicine approach (e.g., based on the number of immunolabeled cells observed). Sections are rinsed in PBS + Triton (PBST) two times for 5 min, incubated in 0.1 M TEA for 5 min, 0.1 M TEA + 0.25% acetic anhydride for 10 min, rinsed in PBST two times for 5 min, and incubated in hybridization buffer containing salmon sperm DNA for 30 min at 37 °C to minimize background staining. During hybridization, VIP probe is denatured at 75 °C for 5 min and placed on ice. Probe is diluted to ∼750 ng/mL in the hybridization buffer and incubated 24–48 h in a humidified chamber at 52 °C. At posthybridization, sections are rinsed with agitation 2× for 10 min in 2× SSC, two times for 10 min in 1× SSC, and two times for 10 min in 0.1× SSC, all at 37 °C. To visualize mRNA, sections are washed for 10 min in a buffer containing 0.1 M Tris·HCl and 0.15 M NaCl, pH 7.5 (TN buffer) at room temperature (RT) and blocked in TN buffer containing 0.5% Roche Blocking Reagent (TNB buffer) for 30 min at RT in a humidified chamber; incubated with Fab fragments from an anti-digoxigenin antibody from sheep, conjugated with horseradish peroxidase (Anti-Digoxigenin-POD, Fab fragments; Roche) at a concentration of 2 μL antibody to 1 mL TNB for 1 h in a humidified chamber at RT; and washed two times for 10 min in TN buffer containing 0.05% Tween-20 (TNT) at RT with agitation. For fluorescent amplification of mRNA signal, streptavidin-tyramide is diluted 1:200 from the TSA Plus kit (Invitrogen) and sections are incubated in TSA solution for 10 min with agitation. Sections are washed three times for 10 min in TNT at RT and coverslipped with ProLong Gold (Invitrogen).

Antisense Production and Validation. Using the sequence data from zebra finches and additional sequence data from violet-eared waxbills (a 300-bp 5′ fragment, including the start codon, and entire second exon) we identified a 16-nucleotide sequence spanning the start codon for antisense targeting. This sequence exhibits 100% identity with java finch (or java "sparrow"; *Padda oryzivora*, a basal estrildid) and only a single nucleotide difference with the domestic chicken (*Gallus domesticus*). Locked nucleic-acid–modified antisense oligonucleotides (Exiqon) were validated as shown in Fig. S1. Tissue was immunolabeled using the protocol described above. Sections from the validation experiment were labeled for aromatase using a custom sheep antibody (2) (1:500; Bethyl Laboratories) and anti-sheep 488 secondary (3:1,000) to verify that other cell types in the vicinity of the infusions were not affected by antisense infusions, and some tissue from the other experiments was additionally labeled using guinea pig anti-vasopressin (1:500; Bachem) and Alexa Fluor 488 secondary (3:1,000), providing local landmarks. All tissue was labeled using rabbit anti-VIP (1:1,000; Bachem) and Alexa Fluor 594 secondary. Finally, we compared hemispheres infused with saline or scrambled oligonucleotides in three males to verify that the scrambled oligonucleotides had no unanticipated effects ($P = 0.35$).

Surgery. Subjects were stereotaxically fitted with bilateral guide cannulae (Plastics One) under isoflurane vapor anesthesia, which were adhered to the skull using dental acrylic and veterinary-grade cyanoacrylate glue. At least 5 d of recovery were allowed before infusions and testing. Cannulae were kept free of debris with use of wire obturators and screw-on dust caps. Before antagonist testing in waxbills, we used obturators that extended 0.5 mm beyond the cannulae tip into the lateral ventricle. These were replaced before oligonucleotide infusions with obturators that extended 2.7 mm to the dorsal margin of the AH, the intended infusion site. AH infusions were targeted to drop just behind the anterior commissure, and medial to the ventral amygdalofugal pathway ("occipitomesencephalic tract"). This was achieved in the vast majority of subjects, although a few animals exhibited minor damage to the caudal aspect of the commissure. We discarded data from four zebra finches that exhibited major damage to the anterior commissure and the AH VIP neurons. An additional zebra finch and waxbill were discarded due to placements that were too caudal. Overall, placements were remarkably consistent.

1. Goodson JL, Rinaldi J, Kelly AM (2009) Vasotocin neurons in the bed nucleus of the stria terminalis preferentially process social information and exhibit properties that dichotomize courting and non-courting phenotypes. *Horm Behav* 55:197–202.
2. Kabelik D, Kelly AM, Goodson JL (2010) Dopaminergic regulation of mate competition aggression and aromatase-Fos colocalization in vasotocin neurons. *Neuropharmacology* 58:117–125.
3. Goodson JL, Kabelik D, Kelly AM, Rinaldi J, Klatt JD (2009) Midbrain dopamine neurons reflect affiliation phenotypes in finches and are tightly coupled to courtship. *Proc Natl Acad Sci USA* 106:8737–8742.

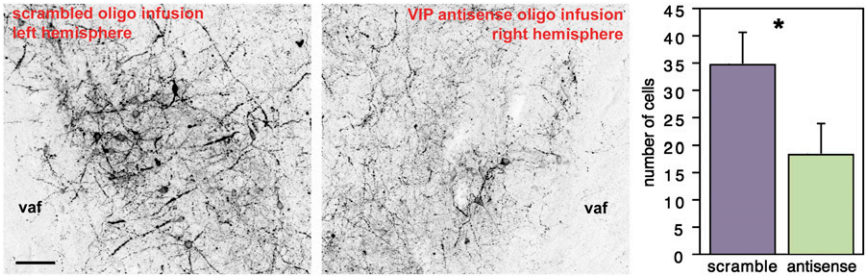

Fig. S1. Validation of VIP antisense oligonucleotides. Ten male zebra finches were fitted with bilateral cannulae directed just above the AH and were infused unilaterally with antisense oligonucleotides (1 μg in 0.25 μL) and contralaterally with scrambled oligonucleotides (screened for nonalignment on National Center for Biotechnology Information databases) at 12-h intervals for 3.5 d (sides counterbalanced). The sixth infusion was combined with 3% colchicine and subjects were perfused 20–24 h later. Antisense significantly reduced VIP-immunoreactive (ir) cell number ($P = 0.02$, paired t test; three sections counted). vaf, ventral amygdalofugal pathway (occipitomesencephalic tract).

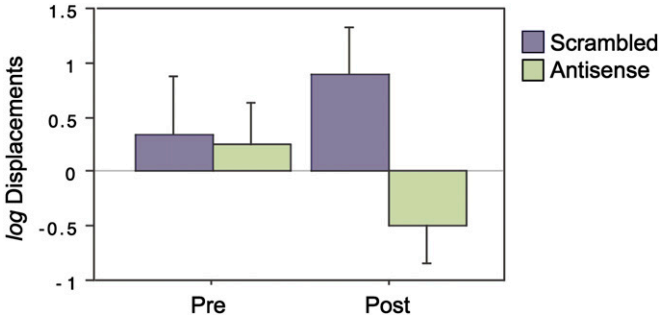

Fig. S2. Aggressive displacements are reduced by infusions of VIP antisense oligonucleotides into the AH of violet-eared waxbills. Pretreatment aggression was measured following infusions of saline into the lateral ventricle. Repeated-measures ANOVA demonstrates a significant test × treatment interaction ($P = 0.02$).

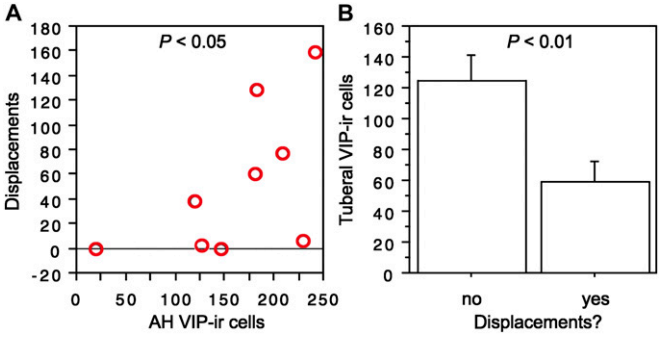

Fig. S3. VIP cell numbers in the anterior and tuberal hypothalamus are differentially related to aggression in violet-eared waxbills. (*A*) Spearman rank correlation of displacements and VIP-ir cell numbers in the AH of control subjects infused with scrambled oligonucleotides (three sections summed). (*B*) Although the number of VIP-ir neurons in the tuberal hypothalamus of control subjects (two sections summed) does not correlate with aggression, less aggressive subjects have significantly more neurons than do subjects that exhibit displacements during the 3-min test ($n = 2$ and 7, respectively, unpaired t test).