Supporting Information

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SI Materials and Methods

Animals. A total of 38 female and 35 male zebra finches exhibited accurate cannula placement and were retained for analyses. An additional seven females exhibiting accurate cannula placement were used for oxytocin (OT) antisense validation; vasopressin (VP) antisense validation has been described previously (1). Before the experiments, subjects were housed in groups of 12–16 same-sex individuals on a 14-h light:10-h dark photoperiod with full-spectrum lighting. Zebra finches are opportunistic breeders capable of continuous reproduction under standard laboratory conditions. Subjects were fitted with leg bands of unique color combinations for identification purposes. All experiments were conducted in a humane manner and in compliance with all federal and institutional regulations.

Antisense Production. As previously described for the production of antisense oligonucleotides targeting zebra finch Ile^3-VP (vasotocin) (1), RNA was collected from zebra finch brains, and 5′ rapid amplification of cDNA ends (RACE; 5′ RACE System for Rapid Amplification of cDNA Ends; Life Technologies) was used to generate cDNA. The 5′ end of the VP gene was PCR-amplified from multiple brains using gene-specific downstream primers to determine if there were any polymorphisms surrounding the start codon. An antisense, locked nuclei acid (LNA)-modified 15-mer antisense oligonucleotide was synthesized for consensus sequence starting nine nucleotides upstream from the start codon, as well as an LNA-modified scrambled control using the same 15 nucleotides (Exiqon). Sequences were searched on BLAST (National Center for Biotechnology Information) to ensure no significant alignment with other known transcripts. The sequence for the zebra finch VP LNA antisense was CTCTGCCATGGCTCA, and that for the zebra finch VP LNA scrambled oligonucleotide was AGCGTATCTTGCCCC.

An identical approach was taken for the sequencing and targeting of the Ile^8-OT (mesotocin) gene, and an LNA-modified 16-mer antisense oligonucleotide was synthesized for a consensus sequence spanning the start codon, along with an LNA-modified scrambled control using the same 16 nucleotides. The sequence for the OT LNA antisense was GATGTAGCAAGCGGAG, and that for the OT scrambled oligonucleotide was CACGTCTATACACCAC.

Antisense Validation. The efficacy of the VP antisense construct was previously established in male and female zebra finches using a within-subjects design, in which subjects were infused with antisense oligonucleotides into the dorsolateral aspect of the medial bed nucleus of the stria terminalis (BSTm) of one hemisphere and with scrambled oligonucleotides into the contralateral hemisphere (each 1 μg in 0.25 μL of isotonic saline at 12-h intervals for 3 d) (1, 2). In brief, eight subjects demonstrated accurate bilateral cannula placement and were retained for analysis. Antisense infusions reduced VP-immunoreactive (ir) cell numbers by ∼54% relative to the scrambled oligonucleotides infused into the contralateral hemisphere (Wilcoxon tied $P = 0.03$), whereas similar reductions were not observed for the VP population in the paraventricular nucleus (PVN), the closest hypothalamic cell group to the BSTm (Wilcoxon tied $P = 0.60$).

Because all major OT-ir cell groups are close to the midline, a similar hemispheric comparison for validation was not possible. Consequently, we conducted a between-subjects comparison of antisense and scrambled oligonucleotides using 12 female zebra finches (targeted as described in the next section; same infusion schedule as for VP), of which 4 antisense subjects and 3 scrambled subjects exhibited accurate placement. OT-ir cell numbers (summed across two sections) were reduced by ∼61% in subjects infused with antisense oligonucleotides relative to the scrambled control (44.3 ± 17.8 cells vs. 112.3 ± 23.1 cells; $P < 0.01$, unpaired t test) (Fig. S6). Immunohistochemistry and quantification were performed as described previously (1, 3).

General Targeting Considerations. VP neurons are most numerous in the finch PVN at the level of the anterior commissure and just caudal to the commissure. This produces a difficulty in targeting, in that these neurons lie ventral to the VP population in the BSTm. Thus, to avoid damage to the BSTm cell group, cannulae were inserted at an angle from posterior to rostral, with the cannulae tips placed underneath the caudal pole of the PVN. This placement also maximizes the distance between the infusion site and the BSTm, diminishing concerns about diffusion. Note that previous experiments have shown that VP antisense infusions in the dorsolateral BSTm do not affect VP production in the PVN, even at shorter distances (1).

OT neurons are most numerous at the level of the anterior commissure and just rostral to the commissure. Thus, most placements were adjacent to the PVN just rostral to the anterior commissure, although placements just caudal to the commissure were also accepted, contingent on immunohistochemical verification that the caudal aspect of the VP-ir cell group of the BSTm did not sustain damage.

Surgeries, Infusions, and Histology. Subjects were fitted stereotaxically with a bilateral 26-gauge cannula device (1-mm tip separation; Plastics One). Cannulae were referenced to the anterior pole of the cerebellum, and for the PVN VP experiments were moved 1.6 mm rostral, and advanced 4.8 mm at an 18.5° angle (posterior to rostral) into the brain. For the PVN OT experiments, cannula were moved 2.6 mm rostral and advanced 4.6 mm at a 10° angle into the brain. Cannulae were mounted to the skull using dental acrylic and veterinary-grade cyanoacrylate glue. The skin was closed with cyanoacrylate glue, and at least 5 d of recovery was allowed before infusions. Injectors extended 1 mm beyond the tip of the guide cannula. Subjects were infused bilaterally with 1 μg of VP or OT antisense oligonucleotides (or scrambled oligonucleotides) in 0.25 μL of isotonic saline at 12-h intervals. Behavioral testing was initiated after the fifth infusion.

At the completion of testing, subjects were euthanized by an overdose of isoflurane vapor and transcardially perfused with 0.1 M PBS and then 0.4% paraformaldehyde. Brains were postfixed overnight, transferred to 30% sucrose for 2 d, and then sectioned at 40 μm on a cryostat for verification of accurate cannula placement.

Behavioral Testing. ***Group size and novel-familiar choice tests.*** Subjects were placed in a testing cage (1 m wide × 0.43 m high × 0.36 m deep) that was divided into seven zones by perches (Fig. S1*A*). The perches at each end of the cage were placed ∼4 cm from the cage wall, which adjoined cages (0.50 m wide × 0.43 m high × 0.36 m deep) containing 10 stimulus birds at one end and 2 stimulus birds at the other end (sides counterbalanced across subjects). The location of the subject was recorded every 15 s for 4 min, with stimulus cage positions changed at 2 min to reduce the impact of potential side biases. "Social contact" was operationally defined as the percentage of test time that the subject spent in the zones closest to the stimulus cages combined, and

"gregariousness" was defined as the percentage of contact time spent next to the larger group.

After testing for group size preference, subjects were tested in the same paradigm for novel-familiar preferences (Fig. S1*B*). One stimulus cage contained five novel same-sex conspecifics, and the other cage contained five familiar same-sex cagemates. Stimulus animals were not used as subjects.

Anxiety and stress-coping assays. The two antisense experiments were conducted independently. The later experiment (OT antisense) included assays that were more recently developed in addition to the novelty suppression of feeding test, which was also used in the VP experiment.

For the VP antisense experiment, anxiety-like behavior was measured in a novelty suppression of feeding test and in an exploration test conducted in a novel environment. Because zebra finches typically eat soon after lights on in the morning (i.e., after fasting all night), we conducted novelty suppression of feeding tests at lights on in the morning. Food was removed from the subject's cage before lights on, and after 10 min the subject was transferred into a novel testing cage (31 cm wide × 20 cm high × 36 cm deep) containing a novel object (purple nitrile glove) hanging over a food cup. The 45-min trials were videorecorded, and the latencies to move, approach the food cup, and feed were quantified.

For the exploration test, subjects were placed in a novel cage (1.3 m wide × 0.43 m high × 0.36 m deep) containing three tree branch clusters, and the latency to move, number of hops and cluster visits were recorded during a 3-min trial.

For the PVN OT experiments, anxiety-like behavior was quantified in the same novelty suppressed feeding test, along with another test of novelty response. In this additional test, subjects were placed in a novel cage (0.61 m wide × 0.43 m high × 0.36 m deep) containing perches at three heights, and birds were videorecorded for 3 min to determine how much time they spent perched on the highest perch (typically preferred). The lights were then turned off, and a purple paper triangle was hung from the top of the cage above the top perch. The lights were turned back on, and subjects were videorecorded for another 3 min. The latencies to move and visit the top perch (closest to the triangle) were recorded, and each subject's location was recorded at 15-s intervals. Data were analyzed as a difference measure (i.e., relative to the baseline phase). This test is a modification of an assay recently developed by Kluen et al. (4).

We also developed an assay to quantify active and passive stress coping in response to gentle restraint. Subjects were captured with the room lights off and restrained by hand in a standardized manner with the head uncovered (Fig. S7). The lights were then turned on, and the number of struggles (occurring as distinct bouts of shuddering) in a 30-s period were counted. This assay was conducted before the first infusion and again at the completion of testing. Data were analyzed as a pre–post difference measure.

Colony testing. The colony cages were 1.3 m wide × 0.43 m high × 0.36 m deep (Fig. S2). The duration of focal observations for the VP antisense experiment was 5 min for session 1 and 10 min for sessions 2–5. For the OT experiment, focal observations were 6 min in the first session and 8 min in sessions 2–4. Observations for the first session were shorter to allow for quantification of the initial burst of courtship and competitive aggression for all subjects. Behavioral quantification followed standard laboratory protocols (5–7); a complete list of the behaviors quantified is provided in *Results*. Only animals of the focal sex were cannulated, with a mix of antisense and control subjects in each colony cage.

Because colony tests are not amenable to videorecording, behaviors must be recorded for quantification in real time. Thus, various behaviors that are not quantified independently are considered when evaluating pairing status. Determination of pairing status is based on selective patterns of association, including maintaining close proximity, feeding together, perching side by side, and co-occupying a nest cup. Of these, only side-by-side perching is quantified independently. Nest cup ownership is also quantified independently, but this measure is focused on exclusion of same-sex birds, not co-occupation with a partner. In addition, subjects will often displace same-sex birds that are near their partner (mate guarding) or near their nest cup (nest defense). This is simply recorded as a displacement, but the context of the displacement contributes to the determination of pair bonding. Importantly, side-by-side perching (which is significantly altered by OT knockdown) is typically considered the primary indicator of pairing (8, 9). The methodology used here is standard for zebra finch experiments in numerous laboratories (5, 8, 10, 11), and researchers in our group are trained to a high degree of interrater reliability. Importantly, because zebra finches are highly gregarious and will actively interact with novel individuals (12), preference assays, such as those used in prairie voles (13), cannot be used.

Because zebra finches are highly colonial, testing subjects in groups is an ecologically valid (and arguably necessary) approach to the assessment of pair bonding. However, it is possible that an individual will fail to pair bond because it cannot compete with other birds. For instance, the bird may exhibit deficits in courtship, competitive aggression, or attractiveness. Thus, to dissociate the impact of those factors from the motivation to pair bond, an excess of opposite-sex birds is placed into the colony cages, such that any focal subject should be able to obtain a partner.

Testing order. Group size and novel-familiar choice tests were conducted in the afternoon after the fifth infusion. All subjects were first tested for group size preferences, and then tested for novel-familiar preferences in the same subject order. Thus, there was a ∼2-h interval between social choice tests for each subject. Subjects received a sixth infusion that evening. At lights on the next day, subjects were first tested for novelty suppression of feeding and were then returned to their home cage with fresh seed. Approximately 20 min later, subjects were administered an exploration test (in the VP antisense experiment) or novelty suppression of perching test (in the OT antisense experiment), followed by the seventh infusion.

Approximately 4 h later, in the afternoon of the second day of testing, the subjects were introduced to colony cages. All subjects in each cage had previously been cagemates; all opposite-sex stimuli were novel. Observations were initiated 8 min after establishment of colonies. Subjects received an eighth infusion that evening, and a ninth infusion the next morning. Colony observations were again conducted in the morning and afternoon of the third day of testing, and subjects received a final infusion in the evening. The order of testing was the same for all subjects. Finally, in the OT antisense experiment, subjects underwent a stress-coping test before the first infusion and on the morning after the conclusion of colony testing.

Statistical Analysis. Data that were not normally distributed were analyzed using Mann–Whitney tests with sexes pooled and separate. Normally distributed data were analyzed by ANOVA, with sex and treatment as between-subject variables. We previously found that aggressive behavior is modulated differentially in the contexts of mate competition (as in the first session) and nest defense (as in later sessions) (6); however, we noted no significant treatment × session interactions in this study, and thus data were subsequently pooled across sessions.

SI Discussion

PVN OT Neurons Inhibit Feeding. In addition to the OT antisense effects on social behaviors and stress coping, we also observed effects on feeding behavior that are consistent with findings in rodents showing that PVN OT neurons exert potent anorectic effects (14). Similarly, a recent study found that central OT infusions decrease food intake in chicks (15). We found antisense effects only in males, as quantified in the colony environment.

Certainly further work is warranted to clarify a potential role of PVN OT neurons in female feeding, but the observed consistencies with mammals suggest that the relationship between these neurons and ingestion is evolutionarily deep.

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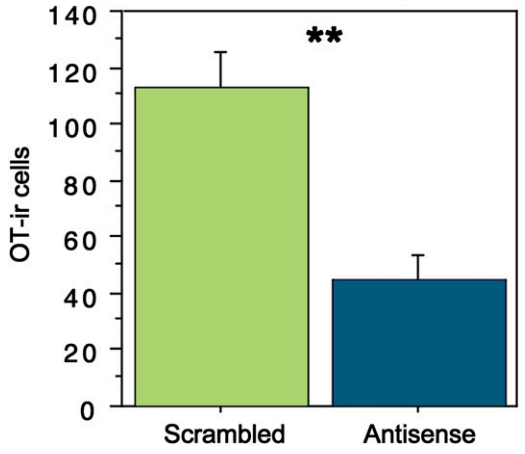

Fig. S1. Validation of the OT antisense oligonucleotides. Females infused with antisense oligonucleotides exhibit significantly fewer OT-ir neurons in the PVN (two sections summed) compared with females infused with scrambled oligonucleotides. $^{**}P < 0.01$, unpaired *t* test; $n = 4$ control females, $n = 3$ antisense females.

Fig. S2. Choice apparatus design. A 1-m–wide testing cage was subdivided into zones by seven perches (indicated by thin lines). Subjects were considered to be within close proximity when they were within 4 cm of a stimulus cage (corresponding to the perches closest to the sides). The location of the subject was recorded every 15 s for 4 min, with sides changed at 2 min. (*A*) For sociality tests, the stimulus cages contained either 2 or 10 same-sex conspecifics. "Social contact" was operationally defined as the percent of test time spent in close proximity to the stimulus cages, and "gregariousness" was operationally defined as the percent of social contact time spent with the larger group. (*B*) For novel-familiar choice tests, the two stimulus cages contained either five familiar cagemates or five unfamiliar individuals (all same sex). Sides were counterbalanced across subjects. Modified from Kelly et al. (1).

Fig. S3. Two 1-m–wide colony cages, showing the standard arrangement for testing, with a single nest cup in each corner and food, water and nesting material placed in the center of the floor. A finch pair on the bottom left is exhibiting side-by-side perching and allopreening, a behavior in which one member of the pair preens the other.

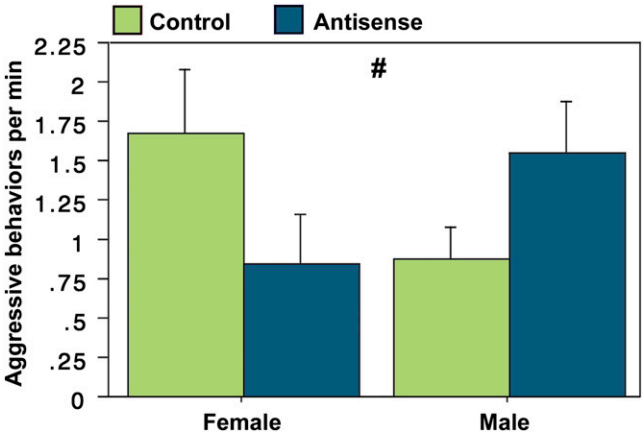

Fig. S4. Knockdown of VP production in the PVN exerts sex-specific effects on aggression directed toward opposite-sex birds. #Sex × treatment $P = 0.03$; $n = 7$ control females, $n = 8$ antisense females, $n = 9$ control males, and $n = 11$ antisense males.

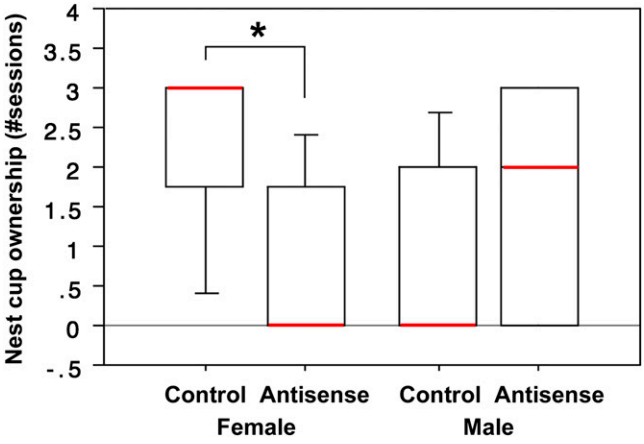

Fig. S5. Knockdown of OT synthesis in the PVN produces a female-specific reduction in nest cup ownership. Antisense-treated females own a nest cup for significantly fewer sessions than control females, whereas treatment has no significant effect on nest cup ownership in males. Box plots show the median (red line), 75th and 25th percentiles (box), and 95% CI (whiskers). *$P < 0.05$, Mann–Whitney U test; $n = 9$ control females, $n = 11$ antisense females, $n = 8$ control males, and $n = 10$ antisense males.

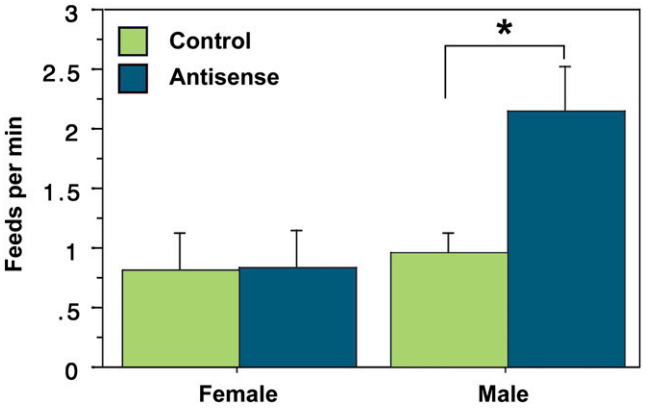

Fig. S6. Knockdown of OT synthesis in the PVN induces hyperphagia, but only in males. ANOVA reveals a trend toward main effect of treatment ($P < 0.07$), although this appears to be driven predominantly by males (sex × treatment $P = 0.10$). Based on this pattern, we conducted post hoc *t* tests and find a significant effect in males only. *$P < 0.05$, unpaired *t* test; $n = 9$ control females, $n = 11$ antisense females, $n = 8$ control males, and $n = 10$ antisense males.

Fig. S7. A novel assay of stress coping behavior. Subjects are cupped gently in the hand with the head free, and the number of struggling bouts is quantified for 30 s.