Hypothalamic oxytocin and vasopressin neurons exert sex-specific effects on pair bonding, gregariousness, and aggression in finches

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Antagonism of oxytocin (OT) receptors (OTRs) impairs the formation of pair bonds in prairie voles (*Microtus ochrogaster*) and zebra finches (*Taenioypygia guttata*), and also reduces the preference for the larger of two groups ("gregariousness") in finches. These effects tend to be stronger in females. The contributions of specific peptide cell groups to these processes remain unknown, however. This issue is complicated by the fact that OTRs in finches and voles bind not only forms of OT, but also vasopressin (VP), and >10 cell groups produce each peptide in any given species. Using RNA interference, we found that knockdown of VP and OT production in the paraventricular nucleus of the hypothalamus exerts diverse behavioral effects in zebra finches, most of which are sexually differentiated. Our data show that knockdown of VP production significantly reduces gregariousness in both sexes and exerts sex-specific effects on aggression directed toward opposite-sex birds (increases in males; decreases in females), whereas OT knockdown produces female-specific deficits in gregariousness, pair bonding, and nest cup ownership; reduces side-by-side perching in both sexes; modulates stress coping; and induces hyperphagia in males. These findings demonstrate that paraventricular neurons are major contributors to the effects of VP-OT peptides on pair bonding and gregariousness; reveal previously unknown effects of sex-specific peptide on opposite-sex aggression; and demonstrate a surprising lack of effects on same-sex aggression. Finally, the observed effects of OT knockdown on feeding and stress coping parallel findings in mammals, suggesting that OT modulation of these processes is evolutionarily conserved across the amniote vertebrate classes.

vasotocin | mesotocin | social behavior

Numerous pharmacological studies, such as those using site-specific infusions of agonists and antagonists, have demonstrated that the vasopressin (VP)-oxytocin (OT) nonapeptides modulate diverse behaviors, including parental care, aggression, communication, sexual behavior, affiliation, anxiety, pair bonding, and stress response (1–3). Such manipulations do not allow us to infer the functions of specific VP-OT neuronal populations, however, because the peptides are produced in numerous cell groups, at least some of which give rise to widespread and long-distance paracrine modulation, including release from axons, dendrites, and soma (4–6). The various VP-OT cell groups also exhibit overlapping axonal projections, and thus any given brain area potentially receives peptides from many different sources (4). Indeed, there is an extremely high potential for overlapping modulation from the various cell groups, because numerous brain areas produce OT and VP in any given species. For instance, 20 different forebrain areas produce OT in the mustached bat (*Pteronotus parnellii*) (7), and sites of VP production are comparably numerous (8). Importantly, even the smallest of these populations, such as the accessory VP populations of the anterior hypothalamus (AH), may exert profound effects on behavior (9).

Compounding this complexity is the fact that behavioral effects mediated via OT receptors (OTRs) and VP 1a receptors (V1aRs) are not necessarily related to OT and VP release, respectively, given the promiscuity of OTRs and V1aRs in both mammalian and nonmammalian species. For instance, in male prairie voles (*Microtus ochrogaster*), septal VP promotes partner preference via OTRs (10), OT induces analgesia in mice via V1aRs (11), and Ile^3-VP (vasotocin, the nonmammalian form of VP) promotes oviposition in birds via OTRs (12).

All jawed vertebrates produce single forms of OT and VP. The canonical forms in mammals are Leu^8-OT and Arg^8-VP, although some mammals produce other OT and VP variants. Homologous forms in birds are Ile^8-OT (mesotocin) and Ile^3-VP (13). No fewer than 14 terms are currently used to identify the numerous VP and OT variants, and thus for clarity, we here use "VP" and "OT" to refer to all known homologs of the canonical mammalian forms. We likewise use mammalian nomenclature for their receptors, consistent with recent definitive works on the vertebrate nonapeptide receptors (14).

VP-OT peptides are produced in magnocellular and parvocellular neurons of the preoptic area and hypothalamus of all vertebrates. In amniotes, these cells lie in the supraoptic nucleus (SON) of the hypothalamus (magnocellular) and paraventricular nucleus (PVN) of the hypothalamus (both magnocellular and parvocellular), although there are many other sites of production, as noted above (8). As in mammals, magnocellular neurons in birds also project to the posterior pituitary, whereas parvocellular PVN neurons project to the median eminence (15).

Central effects of VP and OT are mediated primarily via OTRs and V1aRs. These receptors play important roles in social behavior across a wide range of vertebrate species, including the

Significance

Vasopressin (VP) and oxytocin (OT) modulate numerous social behaviors, including aggression, grouping, and pair bonding, and many sites of action are known. However, the sources of peptide at those sites remains largely unknown. All sites of action likely receive VP and OT from multiple cell groups (of 20 or more), through either direct innervation or paracrine signaling; thus, experimental manipulations are required to determine functions of individual cell groups. Relevant data are scarce, however. Using RNA interference, we found that OT and VP neurons of the paraventricular hypothalamus modulate various diverse behaviors, including grouping, opposite-sex aggression, pair bonding, intrapair affiliation, and stress coping. Hence paraventricular neurons are major contributors to the effects of VP-OT on behavior.

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highly gregarious and socially monogamous zebra finch, which is the focus of the present experiments. Central V1aR antagonism in male zebra finches reduces the preference for the larger of two groups and modulates aggression in a context-specific manner (16, 17). OTR antagonism also reduces the preference for the larger of two groups, impairs pair bonding, and reduces the preference for familiar same-sex birds. These effects are significantly stronger in females (18, 19). We initially hypothesized that the antagonist effects on gregariousness and pair bonding reflect endogenous release from VP neurons in the medial bed nucleus of the stria terminalis (BSTm), given that those neurons increase their transcriptional (Fos) activity selectively in response to positive, affiliation-related stimuli (1). However, antisense knockdown of BSTm VP production has no effect on pair bonding in either sex and no effects on other social behaviors in females. Nonetheless, antisense knockdown of BSTm VP production in males increases aggression and decreases gregariousness and courtship singing (20). Hence multiple VP-OT functions cannot be attributed to the BSTm VP cell group, and consequently the source(s) of numerous nonapeptide effects in finches remain to be elucidated, particularly in females.

A similar case exists in mammals, in which the majority of data on the social functions of individual VP-OT cell groups comes from correlational approaches, such as studies of mRNA expression and neuronal Fos response (2, 21). Importantly, however, because the major VP-OT cell groups modulate diverse hormonal and autonomic variables (1, 2), correlations with behavior may often reflect influences on context-appropriate aspects of physiology rather than effects on behavior. In fact, very few experiments have actually demonstrated the roles of specific VP-OT cell groups in behavior. In mammals, PVN OT production and intra-PVN OT signaling promote maternal care and modulate maternal aggression (22, 23), and accessory VP neurons of the AH promote male aggression in an experience-dependent manner (9). The behavioral roles of other cell groups have not been determined. Thus, it remains unknown which cell groups are relevant to such behaviors as pair bonding, nonsexual affiliation, social recognition, and male parental care.

Using antisense oligonucleotides to knock down peptide production, we here examined the behavioral functions of VP and OT neurons in the PVN. We focused on the PVN because it is larger than many of the other VP-OT cell groups, and because VP-OT neurons of the PVN project to diverse brain areas, including the amygdala, striatum, diencephalon, midbrain, hindbrain, and spinal cord, where VP and OT are known to exert various behavioral effects (1, 24).

Results

General Approach. To directly assess the behavioral functions of PVN OT and VP neurons, we surgically fitted subjects with bilateral cannulae positioned immediately adjacent to the caudal PVN, where peptide neurons are most numerous. Subjects exhibiting damage to the VP population of the BSTm were excluded from the study. After recovery, subjects were given twice-daily infusions of antisense oligonucleotides or scrambled oligonucleotides. The VP antisense construct reduces immunoreactive cell numbers by ~54%, as reported previously (17), and comparable knockdown (~61%) was obtained with the OT antisense construct (*SI Materials and Methods* and Fig. S1). Testing was initiated after the fifth infusion.

Subjects were administered tests of social contact and group size preference ("gregariousness") (Fig. S2*A*), novel-familiar social preference (Fig. S2*B*), and multiple tests of anxiety and stress coping (*Materials and Methods*). The novel-familiar and group-size choice tests were conducted before and after treatment, and thus postinfusion data are presented as percent change from baseline. In these tests, subjects were placed into a 1-m–wide testing cage subdivided into zones by seven perches, with stimulus cages adjoining at each end that contained either 2 or 10 same-sex birds (for assays of group size preference) and either 5 novel or 5 familiar same-sex birds (for assays of novel-familiar preference). Subjects were considered within close proximity when they were within 4 cm of a stimulus cage.

Postsurgical testing was conducted in the following order: group size and novel-familiar choice tests (day 1), anxiety testing (day 2 AM, beginning with the novelty suppression of feeding test), and colony tests (day 2 PM to day 3 PM). For the OT antisense experiment, a stress coping assay was conducted on the morning of day 4.

Colony testing allows for the quantification of numerous behaviors. Colony cages contained four randomly selected individuals of the focal sex, five opposite-sex individuals, four nest cups, and nesting material (Fig. S3). Focal observations were made four or five times over 2 d (for OT and VP experiments, respectively). Aggressive behaviors quantified were displacements, threats, beak fences, and pecks. Aggressive behaviors directed toward same-sex and opposite-sex animals were analyzed separately, and we also quantified displacements received from other birds. Other behaviors quantified were greet, allopreen, follow, side-by-side perching, directed song, undirected song, pick up nest item, carry nest item to nest, time spent on the nest (or in a nest cup), beak wipe, feed, drink, and preen. For statistical analysis, these behaviors were converted to units per minute of time not spent on the nest. Pair bond status was assessed based on selective affiliation, exhibited through patterns of following, side-by-side perching, co-occupation of a nest cup, and allopreening. Finally, nest cup ownership was determined based on occupancy to the exclusion of other same-sex birds.

Behavioral Effects of PVN VP Knockdown. Antisense knockdown of PVN VP synthesis had no effect on social contact in the group size preference test, operationally defined as the total amount of time spent immediately adjacent to the stimulus cages containing 2 and 10 same-sex birds. However, relative to control treatments, antisense treatments significantly impaired gregariousness, defined as the percent of social contact time spent with the larger group. This was observed in both males and females (i.e., with sexes pooled; Mann–Whitney tied $P = 0.04$; Fig. 1). Knockdown of VP production also produced sex-specific effects

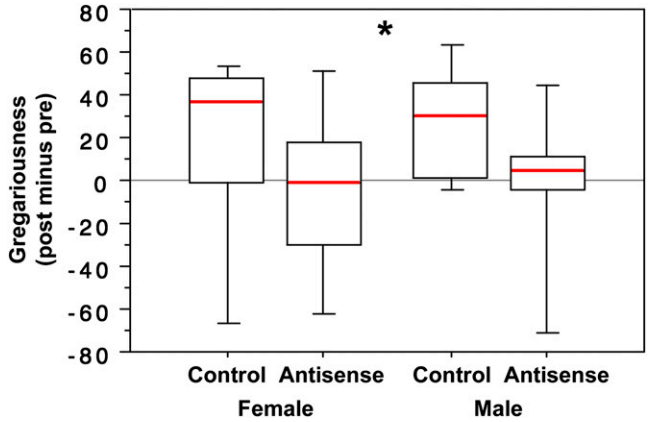

Fig. 1. Knockdown of VP production in the PVN reduces gregariousness, defined as the percentage of social contact time spent with the larger of two groups ($n = 2$ vs. $n = 10$). Data are shown as the posttreatment change from presurgical baseline. Boxplots show the median (red line), 75th and 25th percentiles (box), and 95% CI (whiskers). *$P = 0.04$, Mann–Whitney U test, sexes pooled. Sexes are shown separately for visualization purposes only. $n = 7$ control females, $n = 8$ antisense females, $n = 9$ control males, $n = 10$ antisense males.

on aggression, as assessed in the colony environment (decreased in females, increased in males), but only toward opposite-sex individuals (ANOVA, sex × treatment $P = 0.03$; Fig. S4). We found no effect of PVN VP knockdown on other behaviors quantified in the colonies (all $P > 0.08$), novel-familiar social preference ($P > 0.15$), or anxiety-like behaviors (all $P > 0.20$).

Behavioral Effects of PVN OT Knockdown. In contrast to VP knockdown, OT knockdown produced sex-specific effects on gregariousness. Females infused with antisense oligonucleotides exhibited significantly lower gregariousness relative to control females infused with scrambled oligonucleotides, whereas this treatment had no effect on males (Mann–Whitney tied $P = 0.02$ for females, $P = 0.92$ for males; Fig. 2). We observed no effect of treatment on social contact or novel-familiar preference in either sex (all $P > 0.35$).

Numerous other behavioral effects were evident in the colony setting. Most striking was a profound and female-specific reduction in pair bonding after OT antisense infusions. As shown in Fig. 3*A*, antisense-treated females were pair-bonded for fewer sessions than control females, whereas no such effect was observed in males (Mann–Whitney tied $P < 0.01$ for females, $P = 0.84$ for males). Similarly, antisense-treated females were less likely than control females to be paired at the end of testing ($\chi^2 = 9.01$; $P < 0.01$), with no comparable effect in males ($\chi^2 = 0.01$; $P = 0.91$). Finally, the latency to pair bond was significantly greater in antisense-treated females compared with control females (Mann–Whitney tied $P < 0.01$), and again, this effect was not observed in males (Mann–Whitney tied $P = 0.84$). As shown in Fig. S5, OT knockdown also reduced the number of sessions in which females, but not males, owned nest cups (Mann–Whitney tied $P = 0.01$ for females, $P = 0.15$ for males).

Despite the lack of effects on pair bond status in males, we found that knockdown of OT synthesis in the PVN reduced side-by-side perching in both sexes (Mann–Whitney tied $P = 0.03$; Fig. 3*B*). This behavior, termed "clumping," is characteristically exhibited between paired individuals. Therefore, despite the ability of antisense-treated males to form selective attachments, the quality of their pair bonds may have been otherwise impaired.

No other behaviors quantified in the colonies were affected by knockdown of OT synthesis in the PVN (all $P > 0.09$), with the exception of feeding. ANOVA revealed a trend toward a main effect of treatment ($P < 0.07$), with antisense-treated subjects feeding more, although this trend appears to be driven primarily by males (sex × treatment $P = 0.10$; Fig. S6). Based on this pattern, we conducted post hoc *t* tests and found a significant effect only in males ($P = 0.02$ in males, $P = 0.97$ in females). However, social behavior is extremely intense during the first 2 d after colony establishment, and thus the quantitation of non-social behaviors likely is not optimal at this time (*SI Discussion*).

Anxiety-like behaviors, as measured by novelty suppressed feeding and exploration tests, did not differ between antisense and scrambled subjects of either sex (all $P > 0.50$). Given that OT knockdown tends to increase food intake, the use of a novelty suppressed feeding test as a measure of anxiety might not be valid, and thus the lack of exploration effects in the novel environment is an important piece of confirmatory evidence. In contrast to these anxiety-like behaviors, we did find effects on stress coping, as determined using a novel assay of struggling behavior during gentle restraint by hand (*SI Materials and Methods* and Fig. S7). As shown in Fig. 4, knockdown of PVN OT production significantly increased struggling behavior (i.e., active coping) in both sexes [ANOVA, main effect of treatment $F(1,26) = 5.09$; $P = 0.03$].

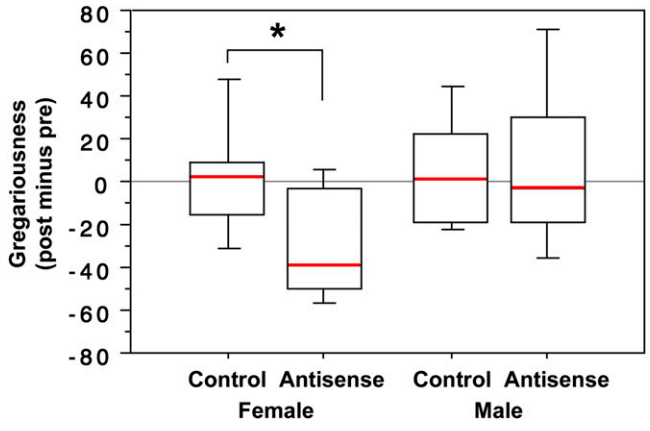

Fig. 2. Knockdown of OT production in the PVN reduces gregariousness in a female-specific manner. Data are shown as the posttreatment change from the presurgical baseline. Boxplots show the median (red line), 75th and 25th percentiles (box), and 95% CI (whiskers). *$P = 0.02$, Mann–Whitney *U* test; $n = 8$ control females, $n = 10$ antisense females, $n = 8$ control males, $n = 10$ antisense males.

Discussion

VP and OT have been extensively studied in relation to numerous social behaviors, including aggression, parental care, grouping, and pair bonding. Relevant sites of action are also well known (1, 5). What has remained largely unaddressed, however, is the question of where the peptide in those sites derives from, and unfortunately, anatomy cannot provide clear answers to this question. This is because all sites of VP-OT action likely receive peptide from multiple cell groups, through either direct innervation or paracrine signaling, and peptide release from any given cell group is apt to be context-specific (and thus may or may not be relevant to the behavior in question). Nonetheless, very few experiments have addressed the social behavior functions of individual VP-OT cell groups. Perhaps most remarkably, after more than 20 y of research on the contributions of VP and OT to pair bonding, the cell groups that promote pair bonding have not been experimentally identified, although postmating male aggression has recently been linked to accessory VP neurons of the AH (25).

Using RNA interference, we now show that OT neurons in the PVN promote pair bonding and gregariousness in females and reduce side-by-side perching in both sexes. Our experiments also provide much-needed information on the functions of PVN VP neurons. As discussed below, the potential roles of these neurons in aggression and affiliation have been a matter of much speculation, with often-opposing hypotheses, and thus direct experimental evidence has been sorely needed. We now show that these neurons promote gregariousness in both males and females, but exert context- and sex-specific effects on aggression. The effects in males are particularly noteworthy because they demonstrate that PVN VP neurons do not modulate male–male aggression, contrary to expectations based on findings for other hypothalamic VP cell groups. However, these cells do exert significant effects on aggression toward opposite-sex birds.

PVN OT Neurons Promote Pair Bonding and Intrapair Affiliation. Numerous experiments have investigated the role of VP and OT in pair bonding and have identified sites of action as well as sex differences. For example, activation of OTRs, but not V1aRs, is necessary for the establishment of pair bonds in zebra finches, with much stronger effects in females (16, 19, 26). Similarly, pair bonding in female prairie voles is more strongly dependent on OTR activation than on V1aR activation, whereas the converse is true for males (27). The basis for these sex differences is not clear, but recent experiments in zebra finches have demonstrated that socially induced Fos activity of VP and OT neurons varies in relation to sex, social context, and personality (28).

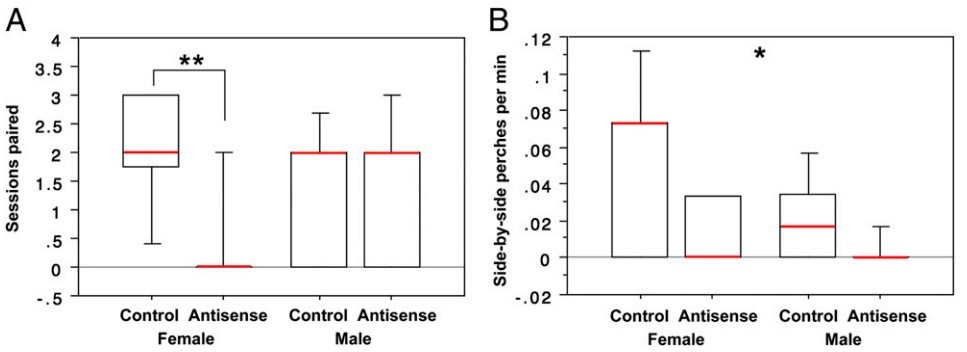

Fig. 3. Knockdown of OT synthesis in the PVN produces female-specific deficits in pair bonding and virtually abolishes side-by-side perching in both males and females. (*A*) Females treated with antisense oligonucleotides were paired for significantly fewer sessions than control females. $*P < 0.01$, Mann–Whitney *U* test; $n = 9$ control females, $n = 11$ antisense females, $n = 8$ control males, $n = 10$ antisense males. (*B*) Antisense-treated males and females exhibit a profound reduction in side-by-side perching, shown as the number of behaviors exhibited per minute not on the nest. $*P = 0.03$, Mann–Whitney *U* test, sexes pooled. Sexes are shown separately for visualization purposes only. $n = 9$ control females, $n = 11$ antisense females, $n = 8$ control males, $n = 10$ antisense males. Boxplots show the median (red line), 75th and 25th percentiles (box), and 95% CI (whiskers).

Thus, the sex differences in peptide function may derive from sexually differentiated patterns of neuronal activation.

To date, experiments in voles have focused most extensively on nonapeptide receptors in the nucleus accumbens, ventral pallidum, and lateral septum (LS) (27). These brain regions receive direct input from several cell groups, including the SON, PVN, and medial extended amygdala (including the BSTm) (29, 30), yet it is unclear which of these cell groups are relevant to pair bonding, and whether paracrine modulation from other cell groups plays a role.

Interestingly, unlike voles, zebra finches and other socially monogamous songbirds do not express OTR or V1aR mRNA at detectable levels in either the ventromedial striatum (including the nucleus accumbens) or ventral pallidum (31). In contrast, they do express high densities of OTRs in the LS (18, 31), an area where both OTRs and V1aRs are required for VP-mediated effects on pair bonding in male prairie voles (10). Comparable experiments have not been conducted in female voles. In rodents, the LS receives direct innervation from VP neurons in the BSTm and OT neurons in the PVN (although light) (32), in addition to apparent paracrine modulation from VP-OT neurons in the SON and PVN (4–6). VP neurons in the suprachiasmatic nucleus also project heavily to areas that border the ventral LS and may therefore exert paracrine effects (29).

Given that BSTm VP neurons directly innervate the LS (32), exhibit strong responses to affiliation-related stimuli (1), and increase their mRNA in male prairie voles after overnight cohabitation with a female (27), we hypothesized that these neurons would play a primary role in pair bonding. However, contrary to our predictions, antisense knockdown of VP production in the BSTm had no effect on pair bonding in either male or female zebra finches (20). Thus, if OTRs in the LS mediate effects on pair bonding in zebra finches, then the relevant peptide must be derived from another cell group.

We now demonstrate that PVN OT neurons, but not PVN VP neurons, potently promote pair bonding, and do so in a female-specific manner. This sex-specific effect was evident in multiple measures, including latency to pair bond, number of sessions in which birds were paired, and probability of being paired at the end of testing. Paired birds typically own a nest cup with their partners, and thus it is notable that nest cup ownership was also reduced by OT knockdown in a female-specific manner. However, antisense knockdown of PVN OT production strongly reduced side-by-side perching ("clumping") in birds of both sexes. This behavior is most commonly observed between pair bond partners, and thus the reduction of clumping in males suggests that PVN OT production contributes to male affiliation behaviors related to pair bonding, and may contribute to the long-term ability and/or motivation of both males and females to retain partners.

In summary, zebra finches exhibit both similarities and differences with respect to prairie voles. OT and the OTR are required for pair bonding in both species, although at least some sites of action are clearly different, given that zebra finches do not express OTRs in the striatopallidum. This underscores an important point—that the mechanisms underlying evolutionarily labile behaviors such as pair bonding may evolve in different ways in different species, although there are apt to be commonalities as well. We hypothesize that the pair bonding functions of PVN OT neurons (as shown in finches) and LS OTRs (as shown in voles) are two such commonalities, although this remains to be fully tested.

VP-OT Modulation of Nonsexual Affiliation. In addition to effects on pair bonding, VP and OT are known to modulate a diversity of other affiliation behaviors, even in nonmonogamous species. For instance, VP and OT promote maternal care and play critical roles in social recognition (1, 5). These functions may represent precursors to the evolutionarily derived roles of the nonapeptides in pair bonding.

In zebra finches, both septal V1aRs and septal OTRs promote gregariousness (the preference for the larger of two same-sex groups) without impacting the overall amount of time spent in social contact (17, 18). These V1aR and OTR experiments were conducted in males and females, respectively, and thus whether the findings extend to both sexes is not clear. However, peripheral injections of an OTR antagonist reduced gregariousness in females only, whereas intraventricular infusions produced comparable results in both sexes. Thus, males appear to be slightly less sensitive to OTR antagonism than females.

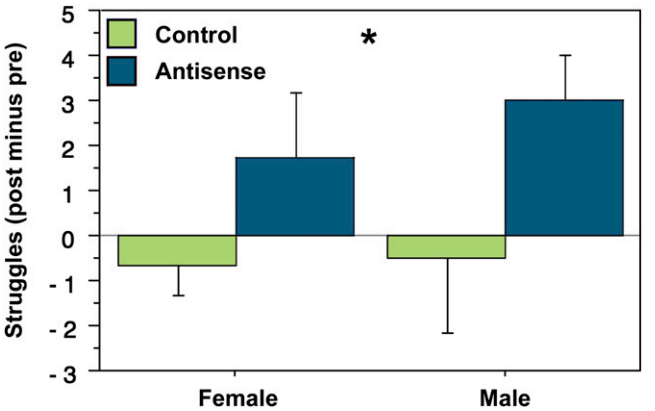

Fig. 4. Knockdown of OT production in the PVN increases active coping (struggling) in a restraint test. Data are shown as the posttreatment change from presurgical baseline. $*P = 0.01$, ANOVA; $n = 9$ control females, $n = 10$ antisense females, $n = 4$ control males, $n = 7$ antisense males.

We here show that knockdown of PVN OT synthesis reduces gregariousness only in females, whereas VP knockdown produces weaker effects that are not sexually differentiated. Previous findings show that knockdown of VP production in the BSTm reduces gregariousness in males but not in females, and thus gregariousness is related to nonapeptide production in a sexually differentiated pattern across the cell groups of the PVN and BSTm. This observation is consistent with other recent data showing that interactions of BSTm and PVN VP-OT cell groups (as established through a principal components analysis of cell numbers and socially induced Fos expression) are related to zebra finch gregariousness in a manner that differs strongly between males and females (28). Although collected only in males, other correlational data from sparrows and mice are likewise consistent with the present findings. Sparrows that flock during winter exhibit significantly more VP neurons in the PVN compared with species that do not flock (this is not observed in the spring), and also exhibit a winter increase in the direct OT innervation of the dorsal LS (33). This innervation likely originates in the PVN, as suggested by findings in rats (32). Similarly, social interaction is positively correlated with VP and OT mRNA in the PVN of mice (21).

Because VP and OT are major modulators of the hypothalamopituitary-adrenal (HPA) axis, and also modulate autonomic functions, it remains to be determined whether the correlations described above exist because (*i*) PVN neurons directly modulate central behavioral processes; (*ii*) behavioral state influences autonomic and hormonal systems via the PVN; and/or (*iii*) PVN neurons influence peripheral physiology in a manner that feeds back on the brain to influence behavior. This latter scenario is consistent with data in goldfish (*Carassius auratus*) showing that VP modulates social approach via effects on hindbrain autonomic processes and feedback from the periphery (34). Although such peripheral mechanisms cannot be ruled out as contributors to the behavioral effects observed here, it is important to note that whereas OT and VP exert opposing influences on the HPA axis, PVN VP and OT neurons exert consistent effects on gregariousness. Hence we hypothesize that the effects observed here are largely independent of the HPA axis.

PVN VP Neurons Modulate Opposite-Sex, but Not Same-Sex, Aggression. VP is commonly presented as a neuropeptide that promotes aggression (35). This portrayal is accurate, but only to a certain extent. VP acts via V1aRs in the AH to promote aggression in male Syrian hamsters (*Mesocricetus auratus*) that have been socially isolated and trained as fighters, and in male prairie voles after mating (i.e., mating-induced aggression, which is associated with pair bonding) (9). Aggression in these contexts is associated with increased Fos expression in an intrinsic population of VP neurons in the AH, and also with experience-dependent plasticity in V1aR densities (9). No effects of AH VP manipulations are observed in male hamsters that are socially housed (9), however, and in fact, intra-AH infusions of a V1aR antagonist increase aggression in female hamsters, opposite of the effects in males (36). Additional complexity is observed in the amygdala, where VP both promotes and inhibits aggression, effectively magnifying the dose-dependent effects of corticotropin-releasing hormone (37).

A different pattern of results is seen for the VP neurons of the BSTm. Antisense knockdown of BSTm VP synthesis dramatically increases aggression in male zebra finches, but has no effect in females (20). Numerous observations suggest that BSTm VP neurons exert similar effects in mammals. For instance, in both birds and mice, Fos expression in BSTm VP neurons is induced by positive, affiliative interactions but not by fighting or the presentation of negative social stimuli (38–40), and VP immunoreactivity in the BSTm is negatively correlated with aggression (33, 41).

Given this complexity, it is clear that the assumption that "VP promotes aggression" is not entirely accurate. Indeed, we hypothesized that PVN VP neurons actually inhibit same-sex aggression, at least in males, based on the findings that VP-Fos colocalization correlates negatively with aggression in male song sparrows (*Melospiza melodia*) (4) and is greater in subordinate male mice than in dominant mice (39). However, contrary to our expectations, we found no effect of PVN VP knockdown on same-sex aggression. Thus, the correlations between VP-Fos colocalization and behavior likely reflect activation of the HPA axis, not direct modulation of behavior. This conclusion is consistent with findings in birds and mammals showing that aggressive males are hyporesponsive to stress (35). Thus, our results strongly underscore the need to move beyond correlational evidence when making inferences about the behavioral functions of PVN peptide neurons.

Despite the negative findings for same-sex aggression, we did find that aggression directed toward opposite-sex birds is altered by VP knockdown in a sexually differentiated manner. Thus, whereas antisense-treated females tend to exhibit less aggression than control females, antisense-treated males exhibit more aggression than control males. The functional significance of this difference is not clear, but it is notable that VP manipulations in the PVN, BSTm, and AH all produce results that are sexually differentiated, as described above. Effects in each of these sites also differ from effects in other sites, suggesting that gross generalizations cannot be made about the relationship(s) between VP and aggression.

Modulation of Anxiety and Stress Coping. VP and OT have been studied extensively in relation to stress-coping and anxiety-like behaviors in mammals. Whereas central VP in rodents is often (although not always) associated with anxiogenesis, central OT tends to be anxiolytic, at least in contexts of heightened OT activity (5) (also see 42). Central administration of OT also attenuates stress-induced effects on the brain and reverses stress-induced social avoidance (5, 43). Interestingly, although intra-PVN release of OT inhibits the HPA axis (44), intraventricular infusion of an OT antagonist reduces anxiety only in pregnant and lactating female rats, and not in virgin female or male rats (44, 45). Consistent with these latter findings, we observed no effects of PVN OT knockdown on anxiety-like responses to novelty. However, the novelty was not presented in a context that we would expect to produce OT release (e.g., during parental care), and thus further tests in social contexts could potentially yield different results.

Notably, the findings for OT described above suggest that effects on the HPA axis are not necessarily coupled with modulation of anxiety. In this light, it perhaps is not surprising to find that knockdown of PVN VP synthesis also produced no effects on anxiety measures. In contrast to these effects for the PVN, knockdown of VP production in the BSTm produced profound increases in anxiety, as measured in the novelty suppression of feeding paradigm (identical to the test used here). This has been observed in both males and females (17, 20), indicating that the various VP cell groups have very different relationships to anxiety.

Finally, we found that knockdown of PVN OT production increases struggling behavior (i.e., active coping) in a mild restraint paradigm. This suggests that PVN OT neurons endogenously promote passive coping in zebra finches, consistent with findings for OT in the central amygdala of rats (46), although OT promotes active coping in other paradigms as well (2). Overall, this pattern of results suggests that PVN nonapeptide neurons are more intimately involved in stress-related processes than in anxiety-like processes, which are likely less strongly coupled to activation of the HPA axis.

Conclusions

In summary, we now provide direct evidence for the involvement of PVN VP-OT neurons in diverse behavioral functions, including pair bonding, gregariousness, physical contact, nest site ownership, intersexual aggression, feeding, and stress coping. Some of the functions elucidated are quite surprising relative to expectations

(particularly for PVN VP neurons), as is the lack of effect on same-sex aggression, anxiety, and novel-familiar social preferences. Overall, the present findings substantially expand our knowledge about the behavioral contributions of individual VP-OT cell groups, and set the stage for the circuit analysis of multiple behavioral processes.

Materials and Methods

Detailed descriptions of the methodology are provided in *SI Materials and Methods*. All experiments were approved by the Institutional Animal Care and Use Committee of Indiana University. Antisense production, surgeries, infusions, histology analyses, and most behavioral assays were conducted as described previously (17, 18, 20). In addition, two novel assays of anxiety and stress coping were used in the OT antisense experiment. One of these assays was an additional test of novelty suppression, in which a novel object is used to suppress perching on the highest possible perch, which is typically preferred. The second was a short (30-s) test of struggling behavior during gentle restraint. All observations were conducted by investigators blinded to treatment assignment. Pretesting was performed for the social preference assays only.

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