Hormones and Behavior 60 (2011) 12–21

Contents lists available at ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh

Vasotocin neurons and septal V_{1a}-like receptors potently modulate songbird flocking and responses to novelty

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ARTICLE INFO

Article history:
Received 8 December 2010
Revised 24 January 2011
Accepted 27 January 2011
Available online 3 February 2011

Keywords:
Vasotocin
Vasopressin
Sociality
Affiliation
Anxiety
Bold-shy
Exploration
Lateral septum
Zebra finch
Songbird

ABSTRACT

Previous comparisons of territorial and gregarious finches (family Estrildidae) suggest the hypothesis that arginine vasotocin (VT) neurons in the medial bed nucleus of the stria terminalis (BSTm) and V_{1a}-like receptors in the lateral septum (LS) promote flocking behavior. Consistent with this hypothesis, we now show that intraseptal infusions of a V_{1a} antagonist in male zebra finches (*Taeniopygia guttata*) reduce gregariousness (preference for a group of 10 versus 2 conspecific males), but have no effect on the amount of time that subjects spend in close proximity to other birds ("contact time"). The antagonist also produces a profound increase in anxiety-like behavior, as exhibited by an increased latency to feed in a novelty-suppressed feeding test. Bilateral knockdown of VT production in the BSTm using LNA-modified antisense oligonucleotides likewise produces increases in anxiety-like behavior and a potent reduction in gregariousness, relative to subjects receiving scrambled oligonucleotides. The antisense oligonucleotides also produced a modest increase in contact time, irrespective of group size. Together, these combined experiments provide clear evidence that endogenous VT promotes preferences for larger flock sizes, and does so in a manner that is coupled to general anxiolysis. Given that homologous peptide circuitry of the BSTm-LS is found across all tetrapod vertebrate classes, these findings may be predictive for other highly gregarious species.

Central nonapeptide circuits play phylogenetically widespread roles in the modulation of social behaviors and stress responses, and often exert their effects in a species-specific manner (Engelmann et al., 2004; De Vries and Panzica, 2006; Donaldson and Young, 2008; Veenema and Neumann, 2008; Choleris et al., 2009; Goodson and Thompson, 2010). These circuits arise primarily from magnocellular and parvocellular neurons in the preoptic area and hypothalamus that produce either arginine vasotocin (VT; in nonmammalian vertebrates) or arginine vasopressin (VP; in mammals), plus a single oxytocin-like peptide in any given species. In addition to these cell groups, virtually all tetrapods, including humans, exhibit VT/VP neurons in the medial extended amygdala, which lie primarily within the medial bed nucleus of the stria terminalis (BSTm) (De Vries and Panzica, 2006; Goodson and Thompson, 2010). Unlike VT/VP cells of the hypothalamus, the BSTm neurons and their projections to basal forebrain sites such as the lateral septum (LS), medial preoptic area and habenula (De Vries et al., 1983; Absil et al., 2002) are typically sexually dimorphic, with males expressing more cells and a higher density of projections than females, are strongly regulated by sex steroids in most seasonally breeding species, and virtually disappear in animals that are in non-reproductive condition (De Vries and Panzica, 2006). Interestingly, these latter two features are not exhibited by several finch species that breed opportunistically in response to unpredictable rainfall, including the zebra finch (Estrildidae: *Taeniopygia guttata*) (Kabelik et al., 2010).

Another notable characteristic of the BSTm VT/VP neurons is their sensitivity to the valence of social stimuli, such that these neurons increase their transcriptional (Fos) activity in response to positive, affiliation-related stimuli, and show no response or even a decreased response to aversive stimuli (Goodson and Wang, 2006; Goodson et al., 2009b). This functional profile has now been demonstrated in C57BL/6J mice (Ho et al., 2010) and also in five estrildid finch species that are all monogamous and biparental, but that differ selectively in their species-typical group sizes (ranging from territorial pairs to flocks of hundreds) (Goodson and Wang, 2006). Simple exposure to a same-sex conspecific through a wire partition significantly increases Fos expression in the BSTm VT cells of highly gregarious finches that naturally affiliate with same-sex individuals, but decreases VT-Fos colocalization (i.e., the percent of VT neurons that colocalize Fos) in territorial finch species that typically attack or avoid same-sex individuals. In addition, VT-Fos colocalization in the BSTm is increased in the territorial finches following exposure to the subject's

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0018-506X/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.yhbeh.2011.01.012

pair bond partner (a presumably positive stimulus), whereas in the highly gregarious zebra finch, courtship-induced increases in VT-Fos colocalization are blocked by intense subjugation (a presumably negative stimulus) (Goodson and Wang, 2006). Similarly, in male C57BL/6J mice, BSTm VP neurons exhibit a robust Fos response to copulation and a modest Fos response to nonaggressive same-sex interaction, but no specific response to male–male fights (Ho et al., 2010).

In addition to the species differences in socially induced VT-Fos colocalization, signaling from the BSTm VT neurons may be effectively magnified in the gregarious finch species, relative to the territorial species, in a few different ways. First, the flocking species exhibit significantly higher levels of constitutive VT-Fos colocalization than do the two territorial species. If this constitutive transcriptional activity is associated with actual VT release, which remains to be demonstrated, VT derived from the BSTm may play a relatively greater role in tonic brain modulation in gregarious finches as compared to territorial finches. Tonic modulation may be further compounded in the two most gregarious species given that they exhibit approximately 10 times the number of VT neurons in the BSTm than do the less social species (Goodson and Wang, 2006). Finally, all three flocking species exhibit significantly higher densities of V_{1a}-like VT binding sites in the LS than do the territorial species (Goodson et al., 2006).

These findings strongly suggest the hypothesis that VT circuitry of the BSTm and LS promotes gregariousness, which we explicitly test in the present experiments, initially using intra-LS infusions of a V_{1a} receptor antagonist in male zebra finches and a recently developed behavioral assay of group size preference (Goodson et al., 2009c). However, the specific contributions of the BSTm VT neurons to behavior are difficult to infer from pharmacological manipulations given that 1) VT/VP projections from the BSTm appear to partially overlap with those from hypothalamic populations, 2) magnocellular hypothalamic neurons may bathe much of the brain in peptide released volumetrically from dendrites and soma, and 3) paracrine modes of action appear to be widespread (Landgraf and Neumann, 2004; Ludwig and Leng, 2006; Goodson and Kabelik, 2009). Thus, a major goal of the second experiment was to determine the direct contribution of the BSTm VT cells to gregariousness, which we achieved through the use of VT antisense oligonucleotide infusions into the BSTm.

Given the importance of septal VP for anxiety modulation in rodents (Landgraf et al., 1995; Everts and Koolhaas, 1999; Bielsky et al., 2005), we additionally examined the effects of antisense and antagonist infusions on two measures of anxiety-like behavior—exploration of a novel environment and novelty-suppressed feeding. All together, the present experiments provide clear evidence that endogenous VT titrates gregariousness, and does so in a manner that is coupled with unexpected *anxiolytic* behavioral effects.

Methods

Subjects

A total of 97 adult male zebra finches were used as subjects in the behavioral experiments, 68 of which exhibited accurate cannula placement and were retained for analyses. Of those 97 males, 15 were used exclusively for antisense validation tests, as were 5 females. Validation tests were also conducted using tissue from experimental subjects, as described below. Subjects were housed in groups of 6–10 same-sex individuals on a 14L:10D photoperiod with full spectrum lighting and were provided finch seed mix, cuttlebone, grit, and water *ad libitum*. Experiments were conducted in a humane manner and in full compliance with all federal and institutional regulations.

Vasotocin antisense production and validation

Antisense production

RNA was collected from zebra finch brains, and 5' RACE techniques were used to generate cDNA (5' RACE System for Rapid Amplification of cDNA Ends; Invitrogen, Carlsbad, CA). The 5′ end of the VT 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, LNA-modified 15-mer antisense oligonucleotide was synthesized for consensus sequence beginning 9 nucleotides upstream from the start codon, as well as an LNA-modified scrambled control using the same 15 nucleotides (Exiqon, Woburn, MA). Sequences were searched on BLAST (National Center of Biotechnology, Bethesda, MD) to ensure no significant alignment with other known transcripts. The sequence for the zebra finch VT LNA antisense was CT + CTGC CAT GG + CT + CA, and the sequence for the zebra finch VT LNA scrambled oligonucleotide was AG + C GTA TCT TG + CC + CC.

Antisense validation

Zebra finches exhibit very large individual variation in the number of BSTm VT-ir cells, with the standard deviation in cell number being only slightly less than the mean (e.g., present validation data from hemispheres infused with scrambled oligonucleotides, and data from normal zebra finches; Goodson et al., 2009b), suggesting that between-subjects comparisons of birds infused with scrambled and antisense oligonucleotides would produce a very imprecise estimate of knockdown. Thus, in order to accurately quantify antisense efficacy, 15 bilaterally cannulated male birds (8 of which exhibited accurate bilateral cannula placement and were retained for analysis) were infused with VT antisense oligonucleotides into one side of the BSTm and scrambled oligonucleotides into the contralateral side (each 1 μg in 0.25 μl of isotonic saline) at 12 h intervals for 3 days. An additional 5 animals were used for a similar assay of saline versus scrambled oligonucleotides in order to determine whether the scrambled oligonucleotides had unanticipated effects on transcription. Finally, in order to determine whether the antisense may have produced nonspecific knockdown of other proteins, we also labeled BSTm tissue for calbindin and aromatase using tissue from 7 randomly selected antisense subjects and tissue from 7 scrambled control subjects.

Similar to mammals (Moore and Lowry, 1998), birds exhibit many VT cell groups, including populations in the preoptic area; and suprachiasmatic, paraventricular (PVN), and supraoptic nuclei of the hypothalamus; plus numerous accessory cell clusters in the anterior and lateral hypothalamus (Aste et al., 1996; Panzica et al., 1999). Given that our present hypotheses are focused on the BSTm and LS, we did not target these many other cell groups, although they may also be involved in similar functions (Goodson and Kabelik, 2009). However, in order to ensure that our infusions were selectively targeting the BSTm and not diffusing to other structures, we additionally conducted VT cell counts in the PVN, the closest hypothalamic population to the BSTm.

Surgeries and infusions

In order to determine the functions of the VT circuitry in the BSTm and LS, we first infused a V_{1a} receptor antagonist into the LS, which is a primary target of VT projections from the BSTm, but not a site of VT production. In a second experiment, we then knocked down VT production in the BSTm using antisense oligonucleotides.

For the antagonist experiments, subjects were stereotaxically fitted with a unilateral 26-ga cannula directed at the right LS (Fig. 1A; small animal cannulae from Plastics One, Akron, OH). Subjects were anesthetized with isoflurane vapor delivered at 1.5–3.5% of a compressed air flow. Cannulae were referenced to the anterior pole of the cerebellum, and then moved 2.9 mm rostral to the reference point, 1.7 mm right of the midline, and inserted at a 26° angle to avoid midline vasculature. Guide cannulae were advanced 2.3 mm into the brain. The cannula was mounted to the skull using dental acrylic and