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Neurotensin neural mRNA expression correlates with vocal communication and other highly-motivated social behaviors in male European starlings

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HIGHLIGHTS

- Neurotensin mRNA expression in Area X positively related to courtship song.
- Neurotensin mRNA expression in POM positively linked to non-vocal courtship behavior.
- Neurotensin mRNA expression in POM positively related to agonistic behavior.
- Data implicate neurotensin synthesis in POM and Area X in highly-motivated behaviors.
- Study suggests a role for neurotensin in vocal communication and social behaviors.

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ABSTRACT

Vocalizations coordinate social interactions in many species and often are important for behaviors such as mate attraction or territorial defense. Although the neural circuitry underlying vocal communication is well-known for some animal groups, such as songbirds, the motivational processes that regulate vocal signals are not as clearly understood. Neurotensin (NT) is a neuropeptide implicated in motivation that can modulate the activity of dopaminergic neurons. Dopaminergic projections from the ventral tegmental area (VTA) are key to mediating highly motivated, goal-directed behaviors, including sexually-motivated birdsong. However, the role of NT in modifying vocal communication or other social behaviors has not been well-studied. Here in European starlings (*Sturnus vulgaris*) we analyzed relationships between sexually-motivated song and NT and NT1 receptor (NTSR1) expression in VTA. Additionally, we examined NT and NTSR1 expression in four regions that receive dopaminergic projections from VTA and are involved in courtship song: the medial preoptic nucleus (POM), the lateral septum (LS), Area X, and HVC. Relationships between NT and NTSR1 expression and non-vocal courtship and agonistic behaviors were also examined. NT expression in Area X positively related to sexually-motivated song production. NT expression in POM positively correlated with non-vocal courtship behavior and agonistic behavior. NT expression in POM was greatest in males owning nesting sites, and the opposite pattern was observed for NTSR1 expression in LS. These results are the first to implicate NT in Area X in birdsong, and further highlight NT as a potential neuromodulator for the control of vocal communication and other social behaviors.

1. Introduction

Vocalizations coordinate social interactions in many species and often are important for behaviors such as mate attraction or territorial defense [1]. The neural circuitry underlying vocal communication is well-known for some animal groups, such as songbirds [2], but the motivational processes that regulate vocal signals are not as clearly understood. Neurotensin (NT) is a neuropeptide implicated in aspects of motivation that also is noted for its strong interactions with dopamine [3,4]. Dopaminergic projections from the ventral tegmental area (VTA) are key to mediating highly motivated, goal-directed behaviors [5,6], including sexually-motivated birdsong [7–9]. NT and NT1 receptors (NTSR1; the only known NT receptor in birds [10]) co-localize with dopamine neurons in VTA [11–13]. NT neurons are also present in regions containing dopaminergic projections from VTA [14] that are strongly implicated in sexually-motivated birdsong (reviewed below). NT immunolabeling in VTA positively relates to courtship song [15], suggesting that NT may modulate dopaminergic circuits to influence vocal communication and other motivated behaviors, but this has not been well-studied.

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VTA connects reciprocally to multiple brain regions involved in social behavior, including the medial preoptic area (often abbreviated as POM in birds) and lateral septum (LS). POM is an area that is an important regulator of male sexual behavior and sexual motivation [16]. Dopamine in the medial preoptic area plays a complex modulatory role in male sexually-motivated behaviors (see [17] for review), including courtship song. There is some evidence for positive associations between D1 dopamine receptor activation and D1 receptor measures in POM and song production [18,19]; however, in other studies dopamine markers relate negatively to singing [20,21]. Though seemingly inconsistent, these results may reflect an inverted U-shaped effect of D1 receptor stimulation in POM on song. In support of this hypothesis, sexually-motivated song in male starlings is facilitated at intermediate levels of D1 receptor stimulation, but reduced at lower and higher levels of receptor stimulation [19]. Furthermore, both male starlings with the highest and lowest D1 expression in the POM sing significantly less than males with intermediate levels of expression [18]. Dopamine in LS has not been previously implicated in courtship song, but the only study to date on NT and vocal communication did show a relationship between NT immunolabeling in LS and courtship song [15]. No associations were seen for NT labeling in POM and song, but given the complex association between dopamine in this region and song, other studies with additional measures of NT are needed to fully understand the role of NT in POM in singing behavior.

The songbird brain contains a specialized group of regions that is necessary for song development and production, known as the song control system [22] (reviewed in [23]). VTA directly projects to the song control nucleus Area X [24], which is part of the avian striatum and is involved in song learning [25,26] and modifying structural elements of song [27,28]. Area X has dense concentrations of dopamine and dopamine receptors [29,30], and dopaminergic activity in Area X is related positively to sexually-motivated song production [21,31–34]. Additionally, the song control nucleus HVC (acronym used as the proper name) also expresses NT [35,36] and receives dopaminergic projections from VTA [37]. The role of NT in brain regions involved in vocal control and sensorimotor processing is not known, but given the relationship between singing behavior and dopamine in Area X, and the fact that HVC receives dopaminergic projections from VTA, these regions are potential targets for NT to modify vocal communication.

VTA, POM, and LS are components of the “social behavior network,” a group of reciprocally connected nuclei in which neuropeptides have been hypothesized to act to influence behavior [38–40]. In addition to vocal behavior in starlings (reviewed above), NT in these regions has been implicated in the mediation of other social behaviors in rodents. In mice, NT expression in the medial preoptic area is associated inversely with maternal defense of offspring [41], intracerebroventricular injections of NT reduce this agonistic behavior and increase expression of the immediate early gene cFOS in the medial preoptic area and LS [42], and maternal mice demonstrate altered NT mRNA expression profiles in POM and LS [43]. These findings highlight that NT may be a neuromodulator for multiple social behaviors, including song.

To further explore the association between NT and vocal communication, and to provide insight into the role of NT in social behavior more broadly, we examined NT and NTSR1 relative mRNA expression using quantitative PCR (qPCR) in VTA, POM, LS, Area X, and HVC, and related these measures to singing and other social behaviors in male European starlings (*Sturnus vulgaris*).

2. Materials and methods

2.1. Animals

Twenty male and 4 female starlings were captured in the winter of 2009–10 on a farm in Madison, WI with baited fly-in traps and brought to the University of Wisconsin-Madison. Birds were housed indoors in stainless steel cages (91 cm × 47 cm × 47 cm) in single sex groups of 5. Food and water were provided ad libitum. All procedures and protocols followed the guidelines of the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* and a protocol approved by the University of Wisconsin Institutional Animal Care and Use Committee.

2.2. Housing conditions

Birds were placed indoors on photoperiods of 18 h light (L):6 dark (D) for 6 weeks, and then 8L:16D for 6 weeks. This sequence of photoperiods induces photosensitivity in starlings, which means that males exposed to day lengths of more than 11 h of light respond with increased gonad volume and plasma T concentrations indicative of the spring breeding season [44]. Males were randomly assigned to outdoor aviaries (2.13 m × 2.4 m × 1.98 m) containing 5 birds each; aviaries contained nest boxes, perches, nesting material, a bird bath, and ad libitum food and water. The natural day length was approximately 13L:11D. Birds habituated to the aviaries for 12 days prior to observations.

2.3. Behavioral observations

Behavioral observations were conducted by a single observer for 4 consecutive days prior to tissue collection. To observe male courtship behavior, a female was released into an aviary, and fresh nesting material (grass clippings and green leaves) was also placed in the aviary. The same female was used across all aviaries on a given day, but a novel female was used for each new observation day. Aviaries were observed in a rotating order across days for 20 min each day. Singing behaviors observed included the number of complete songs and total time singing (s). Agonistic behavior was measured by recording displacements, which were defined as the number of times an individual approached within 5 cm of another individual who then departed. Non-vocal courtship behavior was defined as the sum of the number of times males entered a nest box, landed on a nest box, gathered nesting material, and wing waved. These behaviors are indicators of sexual motivation because males only perform them during the spring breeding season as a component of courtship behavior [45,46]. Non-specific behaviors recorded included bouts of feeding and drinking, with bouts separated by at least 2 s from a previous behavior, and calling, which is a non-song vocalization. Only males with nesting sites respond with high rates of courtship song when presented with females [47], indicating that these males are the most highly sexually motivated. In the present study, males were classified as nest box owners or non-owners based on their propensity to remain on nest box perches and to enter the box, in order to identify the most highly sexually-motivated individuals.

2.4. Tissue preparation for qPCR

Following final observations, males were rapidly decapitated and brains were removed and placed in isopentane (Cat. #277258; Sigma, St. Louis, MO) over dry ice for 30 s, and then stored at −80 °C. Brains were sectioned coronally at 250 μm on a cryostat (−15 °C) on slides over dry ice. Samples from VTA, POM, LS, Area X, and HVC were punched using a Stoelting brain punch set (Cat. #57401; Stoelting, Wood Dale, IL) as in [48]. Punches were transferred to centrifuge tubes on dry ice and stored at −80 °C.

RNA was extracted with the Bio-Rad Aurum Total RNA Fatty and Fibrous Tissue Kit (Cat. #732-6830; Bio-Rad, Hercules, CA). Tissue was homogenized with a Dremel tool, RNA was isolated with PureZOL, and then treated with DNase. RNA was eluted with 30 μl nucleotide free water and the RNA concentration was measured with a NanoDrop system (Thermo Scientific, Wilmington, DE). RNA integrity was validated with Agilent 2100 BioAnalyzer and Agilent RNA 6000 Pico Kit (Agilent Technologies, Santa Clara, CA).