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Research report

Neurotensin immunolabeling relates to sexually-motivated song and other social behaviors in male European starlings (*Sturnus vulgaris*)

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HIGHLIGHTS

- Neurotensin in VTA, LS and BNST related to sexually-motivated song.
- Neurotensin in VTA, LS, PAG, and POM related to agonistic behavior.
- Unique patterns of neurotensin labeling explained sexual and agonistic behaviors.
- Results suggest a role for neurotensin in birdsong and other social behaviors.
- Study supports idea that neuropeptides in social nuclei modulate behavior.

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ABSTRACT

The brain regions involved in vocal communication are well described for some species, including songbirds, but less is known about the neural mechanisms underlying motivational aspects of communication. Mesolimbic dopaminergic projections from the ventral tegmental area (VTA) are central to mediating motivated behaviors. In songbirds, VTA provides dopaminergic innervation to brain regions associated with motivation and social behavior that are also involved in sexually-motivated song production. Neurotensin (NT) is a neuropeptide that strongly modulates dopamine activity, co-localizes with dopamine in VTA, and is found in regions where dopaminergic cells project from VTA. Yet, little is known about how NT contributes to vocal communication or other motivated behaviors. We examined the relationships between sexually-motivated song produced by male European starlings (*Sturnus vulgaris*) and NT immunolabeling in brain regions involved in social behavior and motivation. Additionally, we observed relationships between NT labeling, non-vocal courtship behaviors (another measure of sexual motivation), and agonistic behavior to begin to understand NT's role in socially-motivated behaviors. NT labeling in VTA, lateral septum, and bed nucleus of the stria terminalis correlated with sexually-motivated singing and non-vocal courtship behaviors. NT labeling in VTA, lateral septum, medial preoptic nucleus, and periaqueductal gray was associated with agonistic behavior. This study is the first to suggest NT's involvement in song, and one of the few to implicate NT in social behaviors more generally. Additionally, our results are consistent with the idea that distinct patterns of neuropeptide activity in brain areas involved in social behavior and motivation underlie differentially motivated behaviors.

1. Introduction

Vocal communication is necessary for appropriate social functioning in many vertebrates. Vocal signals are context-specific behaviors that convey information about motivational states and intents, e.g. sexual or agonistic [1]. The brain regions involved in the learning, production, and processing of vocalizations are well described for some species, including songbirds [2], but less is known about the neural mechanisms that underlie motivational aspects of communication.

Across vertebrates, mesolimbic dopaminergic projections from the ventral tegmental area (VTA) are involved in motivated, goal-directed behaviors [3,4], including sexually-motivated singing behavior used by male songbirds to attract females [5–7]. Dopamine, its metabolites, or enzymes involved in dopamine synthesis in VTA are tightly linked to sexually-motivated song [8–11], suggesting that dopaminergic projections from VTA may be crucial for controlling this type of communication. Neurotensin (NT)

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is a neuropeptide that strongly modulates activity in the mesolimbic dopamine system (reviewed in [12]). NT and NT1 receptors (the only known avian NT receptor [13]) are highly co-localized with dopamine neurons in VTA [14–16]. Regions with dopaminergic projections are enriched with NT-containing neurons and NT1 receptors [17–20], including areas involved in sexually-motivated song in songbirds (reviewed below). Despite the close relationship between NT and dopamine in multiple brain regions central to motivation and vocal production, NT's role in vocal communication or other social behaviors is unclear.

VTA projects directly to brain areas involved in vocal production [21,22], and is reciprocally connected to additional NT- and dopamine-rich areas implicated in sexually-motivated song [23–28]. These areas include the lateral septum (LS), the bed nucleus of the stria terminalis (BNST), the medial preoptic area (POM), and the periaqueductal gray (PAG) [10,29–37]. These regions, in addition to others, are considered nodes in a "social behavior network," a group of reciprocally connected brain regions in which distinct patterns of activity underlie specific social behaviors [38,39]. Some authors suggest that input from the mesolimbic dopamine system to these social behavior regions constitutes a larger "social decision-making" network that facilitates appropriate social behaviors in a given context, and that LS and BNST specifically may serve as relay nodes between these circuits for social behavior and motivation [40]. It has been proposed that neuropeptides act within these social behavior/social decision-making regions to generate wide variation in social behaviors [38–40]. The dense NT presence in these areas along with the strong interactions between NT and dopamine suggest that NT may be an important modulator of activity within the social behavior network.

Little is known about how NT contributes to social behaviors generally, but evidence suggests it inversely relates to at least one form of agonistic behavior. In mice, higher NT gene expression in the preoptic area (analogous to POM in birds) as measured via real-time polymerase chain reaction is associated with lower levels of maternal defense of offspring, an agonistic behavior [41]. Intracerebroventricular injections of NT decrease maternal defense of offspring in mice, and several social brain regions, including LS, BNST, POM, and dorsomedial PAG, exhibit increased c-Fos expression in response to injections of NT [42], suggesting these regions as sites in which NT may act to inhibit maternal defense. Since NT is seen so robustly in multiple social brain regions that are implicated in the motivation to sing, highly interacts with DA, and is involved in aspects of social behavior, NT is a strong candidate neuropeptide to contribute to the regulation of sexually-motivated vocal communication.

To gain insight into a possible role for NT in sexually-motivated song production, we determined the extent to which NT immunolabeling in VTA, LS, BNST, POM, and PAG related to sexually-motivated singing behavior in male European starlings, *Sturnus vulgaris*. These areas were selected as starting points because prior work has implicated dopamine in VTA, POM, and PAG in sexually-motivated song [11,32]. Furthermore, activity in LS and BNST is related to sexually-motivated song production [31,32], and these are also sites in which neuropeptides, including NT, act to influence social behaviors [42–47]. Additionally, we examined other non-vocal social behaviors to explore the possibility that distinct patterns of NT activity in the social behavior network relate to separate social behaviors. We examined relationships between NT labeling and non-vocal courtship behaviors indicative of sexual motivation, agonistic behavior, and, as controls, non-socially motivated behaviors. This is the first study to our knowledge to examine the relationship between NT and birdsong, and one of the few studies to explore the relationship between NT and social behavior.

2. Materials & methods

2.1. Animals

20 male and 4 female starlings were captured in the winter of 2009–10 on a farm in Madison, WI with baited fly-in traps. Birds were then housed indoors in single sex groups of 5 in stainless steel cages (91 cm × 47 cm × 47 cm) at the University of Wisconsin-Madison. Food and water were available ad libitum. All procedures and protocols were in accordance with 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 housed in captivity for at least a year after capture. Before the study began, birds were placed on photoperiods of 18 h light (L):6 h dark (D) for 6 weeks, and then 8L:16D for 6 weeks. Experiencing this sequence of photoperiods causes photosensitivity in starlings, such that males subsequently exposed to day lengths longer than 11 h demonstrate increased gonad volume and plasma T concentrations indicative of spring breeding condition [48]. Males were placed in groups of 5 into outdoor aviaries (2.13 m × 2.4 m × 1.98) that contained nest boxes, perches, nesting material, a water bath, and food and water *ad libitum*. Birds were exposed to the 13L:11D natural light conditions of this time of year (April). Birds habituated to the aviaries for 12 days before behavioral observations began.

2.3. Behavioral observations

Each aviary was observed in a rotating order at one time each day over a period of 4 days. A single experimenter recorded observations of all birds in a given aviary for 20 min. Starlings tend to not perform multiple behaviors simultaneously or rapidly change locations while behaving, enabling observations of several birds to be performed by a single observer, as is common in studies of starling behavior such as [10,49,50]. To observe male courtship singing and behavior, a female starling was released into the aviary, and fresh nesting materials (grass clippings and green leaves) were placed in the aviary. One of 4 different females was released into the aviaries each day, i.e. the same female was used in each aviary for a single observation day, although a different female was used each day.

Singing behaviors observed were time singing (s) and number of complete songs (defined as songs lasting more than 10 s). Non-vocal courtship behaviors consisted of the sum of the number of times a male entered a nest box, looked in a nest box, gathered nest material, and wing waved; these behaviors are clear indicators of sexual motivation, as they are performed only by male starlings during the spring breeding season as part of mate attraction [50,51]. We also measured the number of times a bird displaced a conspecific, an agonistic behavior, specified as a male approaching within 5 cm of another male who then departed after the approach. Non-specific behaviors included bouts of feeding and preening, with a bout defined as occurring at least 2 s after a previous behavior, and calling, which is a non-song vocalization. Tissue was collected for NT analysis only from birds observed to sing at least once during the 4 observation days or on any of the 12 preceding days of habituation, resulting in $n = 12$.

2.4. Tissue Preparation for Immunohistochemistry

Immediately after the final observation period for an aviary, the female was removed and each male was rapidly decapitated. Brains were subsequently removed, submerged in 5% acrolein (Sigma