

## RESEARCH ARTICLE

# The neural distribution of the avian homologue of oxytocin, mesotocin, in two songbird species, the zebra finch and the canary: A potential role in song perception and production

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The avian homologue of oxytocin (OT), formerly called mesotocin, influences social behaviors in songbirds and potentially song production. We sought to characterize the distribution of OT peptide in the brain of two songbird species: canaries (*Serinus canaria*) and zebra finches (*Taeniopygia guttata*). To visualize OT, we performed immunocytochemistry using an antibody previously shown to identify OT in avian species. In both canaries and zebra finches, dense OT-ir perikarya were located in the paraventricular nucleus (PVN), preoptic area (POA), supraoptic nucleus (SON), and medial bed nucleus of the stria terminalis (BNSTm). We also observed morphologically distinct OT-ir cells scattered throughout the mesopallium. OT-ir fibers were observed in the PVN, ventral medial hypothalamus (VMH), periaqueductal gray (PAG), intercollicular nucleus (ICo), and ventral tegmental area (VTA). We also observed punctate OT-ir fibers in the song control nucleus HVC. In both male and female canaries, OT-ir fibers were present in the lateral septum (LS), but innervation was greater in males. We did not observe this sex difference in zebra finches. Much of the OT staining observed is consistent with general distributions within the vertebrate hypothalamus, indicating a possible conserved function. However, some extra-hypothalamic distributions, such as perikarya in the mesopallium, may be specific to songbirds and play a role in song perception and production. The presence of OT-ir fibers in HVC and song control nuclei projecting dopaminergic regions provides anatomical evidence in support of the idea that OT can influence singing behavior—either directly via HVC or indirectly via the PAG, VTA, or POA.

## KEYWORDS

neuroanatomy, nonapeptide, social behavior, songbird

**Abbreviations:** BNSTm, medial bed nucleus of the stria terminalis; ICo, intercollicular nucleus; IP, nucleus interpeduncularis; IT, isotocin; LS, lateral septum; MP, mesopallium; MT, mesotocin; OT, oxytocin; PAG, periaqueductal grey; POA, preoptic area; POM, medial preoptic nucleus; PVN, paraventricular nucleus; Rt, nucleus rotundus; SON, supraoptic nucleus; VT, vasotocin; VTA, ventral tegmental area; VMH, ventromedial nucleus of the hypothalamus; X, area X.

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## 1 | INTRODUCTION

The nonapeptide mesotocin (MT) is commonly expressed in amphibians, reptiles, and birds (Acher, 1993; Sawyer, 1977). Recent genomic analyses make it clear that MT is a different name for the same orthologous gene in mammals, oxytocin (OT), which resulted from a local duplication from vasotocin (Theofanopoulou et al., 2021). While the MT peptide differs from the mammalian OT peptide by a change in the 8th amino acid from arginine to isoleucine, we will refer to this peptide using the recently proposed universal gene nomenclature—oxytocin—to aid in translation of our findings across vertebrates and to mitigate ongoing confusion around the gene's evolutionary history and nomenclature (Saayman et al., 1986; Theofanopoulou et al., 2021).

OT is involved in the regulation of a variety of prosocial behaviors (Ross & Young, 2009). There is a broad body of work examining the role of OT, and its paralogue vasotocin, on differences in social behavior between two species of voles—the prairie vole and the montane vole (Froemke & Young, 2021; Insel & Shapiro, 1992; Young, 1999). These effects extend to other species as well, and intranasal administration of OT in pinyon jays increases the prosocial behavior of sharing food rewards (Duque et al., 2018). In marmosets, intranasal administration of OT increases the initiation of huddling with their social partner, while oral OT antagonist treatments have an obverse effect—reducing proximity and huddling (Smith et al., 2010). Furthermore, intranasal administration of OT increases affiliation and approach behaviors in dogs, both toward their owners and other dogs (Romero et al., 2014). However, not all studies of OT function support the prosocial role of OT, and there is some conflicting evidence indicating that OT may be involved in negative social behaviors, such as in aggression toward intruders (de Jong & Neumann, 2018; Goodson et al., 2015). Such differences may be due to social context; for example, in dominant male squirrel monkeys, OT administration increases sexual and aggressive behavior, while in subordinate monkeys it increases associative and marking behaviors (Winslow & Insel, 1991). This leads to the hypothesis that OT may play a role in determining social valence rather than simply promoting social behavior (Love, 2014; Shamay-Tsoory & Abu-Akel, 2016). The effects of OT on social behavior appear to be broad, affecting a variety of behaviors and multiple brain regions.

In songbirds, the role of OT in prosocial behavior has been repeatedly demonstrated in one of the most commonly studied species, the zebra finch. In this species, central infusions of OT increase the amount of time spent flocking with conspecifics, while OT antagonists have an opposite effect (Goodson et al., 2009). Antagonists of OT also inhibit pair bond formation (Pedersen & Tomaszycki, 2012). Likewise, infusing antisense OT mRNA specifically into the paraventricular nucleus decreases gregariousness and pair bonding in females (Kelly & Goodson, 2014). Therefore, it appears that OT is important for a variety of social behaviors in songbirds.

These mechanisms may be particularly relevant for song learning and production, as the process of learning and producing song is intertwined with the development and maintenance of social bonds (Chen et al., 2016; Sakata & Brainard, 2009; Sakata et al., 2008; Tomaszycki & Adkins-Regan, 2005). Song learning involves social interaction, as it is

affected by the responses of other birds—the song tutor, other singing birds, or other listening birds that respond via calls or nonvocal cues (Beecher & Burt, 2004; Carouso-Peck et al., 2020; King & West, 1988; Nelson & Marler, 1994; West & King, 1988). In addition, singing later in life is typically produced specifically in response to conspecifics—either to attract a mate or to defend a territory (Catchpole & Slater, 2008). Consequently, several research groups have hypothesized that song learning is influenced by nonapeptide receptors (Baran et al., 2017; Maney & Rodriguez-Saltos, 2016; Theofanopoulou et al., 2017). Indeed, treatment of hatchling zebra finches with a nonapeptide receptor antagonist results in adult song less similar to that of their tutor, indicating that OT influences song learning (Baran et al., 2017). The effects of OT also extend to crystallized song, as male zebra finches treated with OT antagonists exhibit a decrease in singing, specifically in a courtship context (Pedersen & Tomaszycki, 2012). Therefore, it appears that OT plays a significant role in song production throughout the lifespan.

How does OT modulate song learning and singing behavior? One potential mechanism is via modulation of activity in the ventral tegmental area (VTA) (Theofanopoulou et al., 2017). The VTA sends dopaminergic projections to several song control nuclei—HVC, the robust nucleus of the arcopallium (RA), and Area X—and is involved in song behavior (Appeltants et al., 2000, 2002; Castelino et al., 2007; Hara et al., 2007; Lewis et al., 1981; Lynch et al., 2008). There is evidence from mammalian studies that OT can modulate dopaminergic function in the context of male sexual behavior. In rats, OT fibers and receptors have been found in VTA, and the activity of OT in this region is important for male copulatory behavior (Melis et al., 2007; Succu et al., 2007, 2008; Vaccari et al., 1998). These OT fibers may originate in the hypothalamus, either within in the mPOA or PVN/SON (Theofanopoulou et al., 2017). Similar mechanisms may extend to the modulation of birdsongs in a courtship context by other dopaminergic or noradrenergic regions that project to the song control system and influence song, such as the periaqueductal gray (PAG) (Appeltants et al., 2000, 2002; Castelino et al., 2007; Haakenson et al., 2020; Tanaka et al., 2018). This role of OT, modulating dopamine function during song learning and song production, would be consistent with evidence in mammals that OT modulates midbrain dopamine to promote social behaviors (Charlet & Grinevich, 2017; Liu & Wang, 2003).

Despite the fact there is great interest in OT function in birds, there have been relatively few studies of the distribution of the OT peptide in avian species due, in part, to the historical lack of readily accessible reagents, such as specific antibodies. Past immunohistochemical investigations of OT peptide expression in birds were performed utilizing antigens produced in laboratory and that are no longer available (Goossens et al., 1977). More recently, several studies have sequenced the OT gene and characterized the expression of OT mRNA in a limited number of brain regions in songbirds via *in situ* hybridization (Barth et al., 1997; Tobari et al., 2022; Vicario et al., 2017). The gene for OT has been included in the zebra finch atlas (Tobari et al., 2022; Vicario et al., 2017; *Zebra Finch Expression Brain Atlas*, n.d.). However, these studies have not comprehensively mapped the distribution of the mRNA, and the levels of mRNA expression can differ from peptide abundance due

to regulatory posttranscriptional processes. Therefore, a thorough mapping of the peptide distribution will be useful for future studies, and determining a reliable method for examining the distribution of the peptide itself can also aid in future studies by broadening the potential methodologies available and allowing investigators to target examinations of OT at the desired molecular level (Kousounadis et al., 2015; Maier et al., 2009; Vogel & Marcotte, 2012).

In order to improve the understanding of OT's role in singing behavior and other social behaviors in songbirds, we sought to characterize the distribution of OT peptide in the brains of two commonly studied songbird species—the zebra finch (*Taeniopygia guttata*) and the canary (*Serinus canaria*). Our analysis focused on the song control and auditory systems known to be involved in song learning, perception, and production. We also specifically sought to determine if OT peptide is present in dopaminergic brain regions, to ascertain if there is support for the hypothesis that OT modulates dopaminergic regions.

## 2 | MATERIALS AND METHODS

### 2.1 | Experimental animals

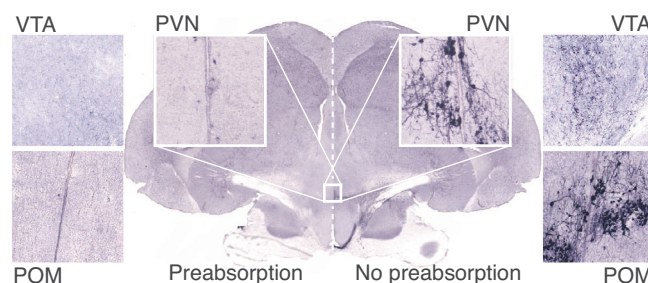
Adult zebra finches (three males, three females) and canaries of the American singer strain (three males, three females) were obtained from a local breeder (Maryland Exotic Birds, Pasadena, MD, USA). Upon arrival, birds were housed in group aviaries on a short-day photoperiod (8L:16D). Food and water were provided ad libitum. All procedures were approved by the University of Maryland, College Park Animal Care and Use Committee.

### 2.2 | Tissue preparation

Brains were collected following rapid decapitation without anesthesia and fixed for 2 h in 5% acrolein in phosphate-buffered saline (PBS). Brains were then washed in PBS for 15 min four times and cryoprotected in 30% sucrose. After 24 h at 4°C, brains were flash frozen on dry ice and stored at −80°C. Brain tissue was sectioned with a cryostat (Microm HM 500 OM) at 30 µm in the coronal plane. We collected four sets of sections, such that one set was collected every 120 µm. We performed immunocytochemistry to visualize OT on one set of sections while another set was Nissl-stained with thionin to visualize cell bodies and identify brain regions.

### 2.3 | Immunocytochemistry

Briefly, free-floating sections were washed in PBS (0.01 M, pH 7.5) and treated with 0.5% H<sub>2</sub>O<sub>2</sub> for 30 min to block endogenous peroxidases. After three rinses in PBS containing 0.3% Triton-X (PBST), sections were placed for 1 h in blocking solution (20% normal goat serum [NGS] in PBST). Sections were then incubated for 48 h at 4°C in 2% NGS and primary antibody (1:20,000, rabbit, Oxytocin, Immunostar,



**FIGURE 1** Photomicrographs demonstrating preabsorption with mesotocin (MT) peptide (left) compared to standard immunohistochemistry (right) in several representative regions—paraventricular nucleus (PVN), ventral tegmental area (VTA), and medial preoptic nucleus (POM).

cat#: 20068, Hudson, WI, USA) in PBST. Next, sections were incubated for 1 h in biotinylated goat anti-rabbit secondary antibody (1:250, Vector, cat#: BA-1000) in PBST. Antibody bound to OT peptide was visualized using Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA, USA) and 3,3'-diaminobenzidine tetrahydrochloride chromagen with nickel ammonium sulfate added, yielding a black reaction product (Sigma, cat#: D4293-50SET, St. Louis, MO, USA).

### 2.4 | Antibody validations

The primary antibody used to detect OT-ir, a rabbit polyclonal antibody directed against mammalian OT, has been previously utilized in studies of Thai hens (Chokchaloemwong et al., 2013; Sinpru et al., 2017). These authors performed preabsorption controls to validate the antibody for use in that species (Chokchaloemwong et al., 2013). They found that when they preabsorbed the primary antibody with OT peptide with the characteristic avian substitution of the 8th amino acid from arginine to isoleucine, specific immunoreactivity disappeared. When they preabsorbed with vasotocin, the staining was identical to what is observed with the standard antibody.

We tested the specificity of the antibody in our species by preabsorption with the MT antigen (avian OT) and closely related antigens (OT or vasotocin [VT]) in separate experiments. Prior to the immunohistochemistry protocol, antigen (10 µg/ml MT, OT, or VT) was added to a solution of primary antibody 1:20,000 in PBS with 0.3% Triton-X and 2% NGS. This solution, along with a separate control solution of antibody with no antigen, was left to incubate at 4°C for 24 h and then centrifuged at 12,000 rpm. This centrifugation step removed the pellet formed by antigen-antibody complexes from the solution but did not affect the unbound antibody, as evidenced by the fact that staining was ablated following preabsorption with MT and OT but not VT (see Figure 1). Tissue was then run through the protocol described previously, either with the preabsorbed antibody or standard primary antibody. Preabsorption and immunohistochemical-staining experiments were carried out in parallel using corresponding areas on consecutive sections to compare results from preabsorption to standard staining.

## 2.5 | Image analysis

We visualized microscopic images of the brain sections using a Nikon Eclipse microscope with an attached Nikon DS-Fi2 camera and Nikon Elements software (Nikon Instruments, Melville, NY, USA). The sections stained with thionin were used to prepare a series of coronal drawings through the canary and zebra finch brains. Photomicrographs of every third section were taken at 10× magnification, and the Nikon Elements software was used to automatically control the stage movement to take individual photomicrographs and then stitch them together into one large image of the entire section. The prominent structures in these sections, such as nuclei and fiber tracts, were drawn on a layer superimposed on these images with Adobe Illustrator. The same photomicrograph procedure was followed for the corresponding sections in the OT-ir series. The locations of OT-ir cell bodies and fibers were then drawn on another layer of the Adobe Illustrator file (v 26.0.3, Adobe, San Jose, CA, USA).

To quantify fiber density in the lateral septum, photomicrographs of this region were taken at 20× magnification. From these photomicrographs, the percentage of each photomicrograph covered by OT-immunoreactive fibers was calculated using Fiji (Schindelin et al., 2012). First, color deconvolution was performed on the image using user-inputted regions of interest (the boundary of the lateral septum) to separate out areas where there was a high density of staining. Next, the image was automatically set to a threshold (Yen), and the ImageJ “Measurement” function was performed to measure the percentage area with such staining.

The identification of anatomical structures and the associated nomenclature utilized in this study is based largely on the atlases of the canary (Stokes et al., 1974), pigeon (Karten & Hodós, 1967), and chick brain (Kuenzel & Masson, 1988; Puelles et al., 2018). Several names from these sources have been altered to reflect the revised avian nomenclature recommended in 2004 (Reiner, Perkel, Bruce, et al., 2004; Reiner, Perkel, Mello, et al., 2004). We employed the definitions proposed by Aste et al. (1998) for the bed nucleus of the stria terminalis and by Balthazart et al. (1996) for distinctions in the hypothalamus among the songbird preoptic area, the paraventricular nucleus, and the ventromedial nucleus.

## 3 | RESULTS

Dense immunostaining of fibers and perikarya was observed in vast areas of both the zebra finch and canary brains. All regions described as having OT-ir in the present results did not exhibit staining following preabsorption with MT (avian OT) or OT peptide, but still exhibited staining following preabsorption with VT as well as with the normal (not preabsorbed) antibody. Some background staining represented by slightly darkened tissue remained following preabsorption with MT in the arcopallium for unexplained reasons (such as the presence of a higher density of peptide or of another related antigen) and will therefore not be discussed here. Figure 1

illustrates representative images of brain regions from sections incubated with the antibody with or without preabsorption with MT peptide.

The overall distribution of OT-immunoreactive cells and fibers was qualitatively similar throughout the zebra finch and canary brains and is illustrated in Figures 2 and 3, respectively, see abbreviations. With the exception of the lateral septum, no gross difference in distribution was detected between males and females. One should, however, not exclude that a detailed quantitative study based on a larger number of subjects in each sex could identify more subtle differences.

OT-ir staining was detected in broad areas of the brain including several hypothalamic nuclei and the entire mesopallium. The nature of the immunoreactive structures present in these regions is variable, however. Hypothalamic nuclei essentially contained very densely stained perikarya where the signal was so dense that it did not, in general, allow for visibility of the unstained nucleus. Dense networks of fibers were also present at these levels. By contrast, in the mesopallium, the OT-ir material appeared to be concentrated at the periphery of cells containing a large unstained nucleus. At the more rostral brain levels, this type of staining appeared to extend more dorsally in both species into the mesopallium dorsale. This discrete distribution of OT-ir structures relates to functionally distinct brain areas as presented in the next sections.

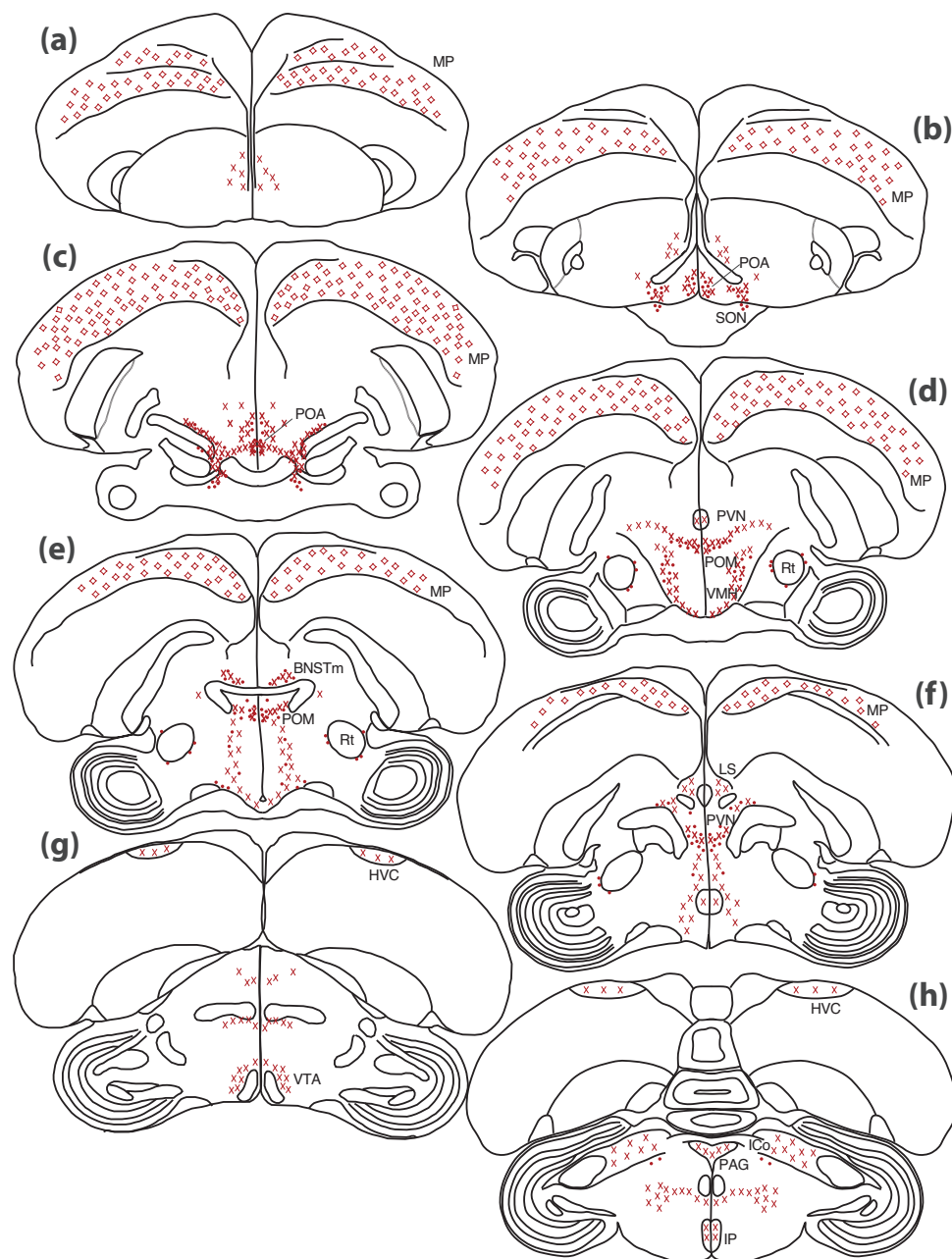
### 3.1 | Regions associated with song perception and production

By far, the largest number of cells immunoreactive for OT was observed scattered throughout the mesopallium (Figure 4a). Most of these cells however exhibited a distinct morphological phenotype where the positive staining was concentrated at the edge of the cell bodies. This region includes the caudomedial mesopallium (CMM), which is one of the secondary auditory areas essential for song perception. However, since these cells were present throughout the extent of the mesopallium, and not limited to CMM, it is likely they are not solely associated with song perception. We also observed punctate OT-ir fibers in HVC (Figure 4b), indicating that OT may act in this major song control region. In addition, OT-ir fibers were present in sections containing lateral Area X, but staining was positioned more ventral and medial relative to the visible Area X in Nissl sections (Figure 2a).

### 3.2 | Hypothalamic regions

We observed densely stained OT-ir fibers and perikarya in several hypothalamic regions—the periventricular nucleus (PVN), the preoptic area (POA), the supraoptic nucleus (SON), as well as in the adjacent telencephalic medial bed nucleus of the stria terminalis (BNSTm). A dense network of positive fibers was seen extending laterally from the PVN in both species. The target of these fibers could not be





**FIGURE 2** Schematic drawings of coronal sections through the zebra finch brain illustrating the distribution of OT-ir structures in a rostral to caudal order (a–h). OT-ir perikarya with staining throughout the cell body are represented by circles while perikarya with OT-ir material present only at the periphery are represented by open diamonds. OT-ir fibers are represented by x's. The density of symbols has been adjusted to give a qualitative estimate of the number of the immunoreactive structures.

identified. Representative photomicrographs from some of these regions can be found in Figure 5.

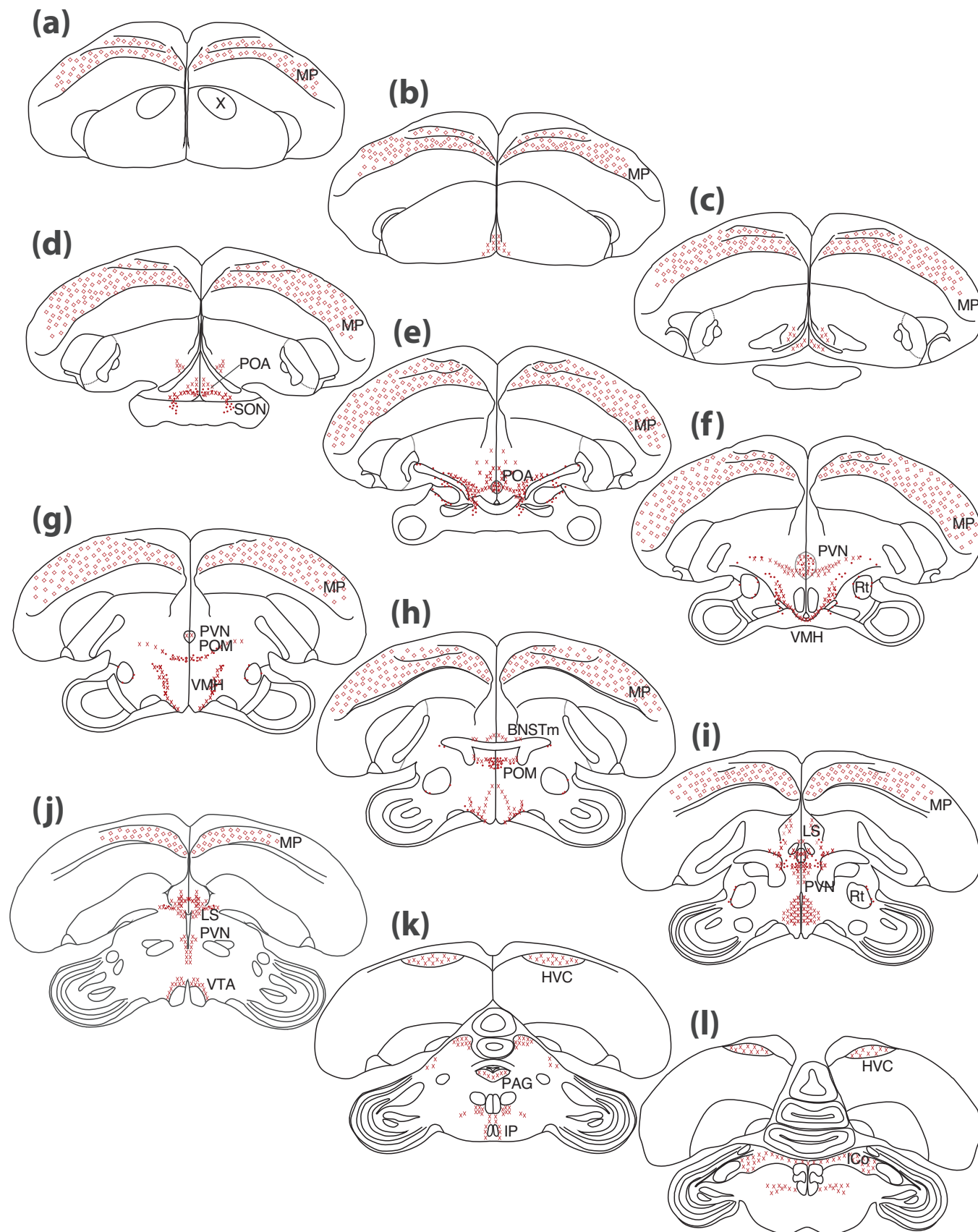
### 3.3 | Dopaminergic regions

OT-ir fibers but not cell bodies were observed in several dopaminergic regions, including the ventral tegmental area (VTA), the periaqueductal gray (PAG), and intercollicular nucleus (ICo). Representative photomicrographs from these regions can be found in Figure 6. The origin of

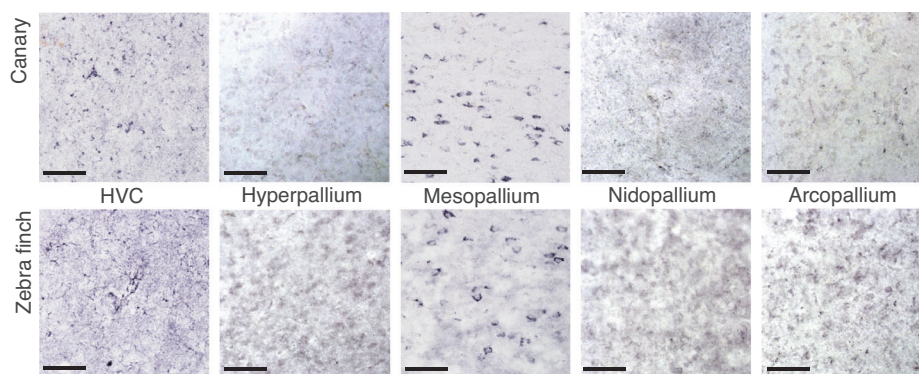
this innervation is not established at present but is likely located in one of the hypothalamic nuclei given the known anatomical relationships between these brain regions (Gordon et al., 2011; He et al., 2021; Jiang et al., 2019).

### 3.4 | Lateral septum

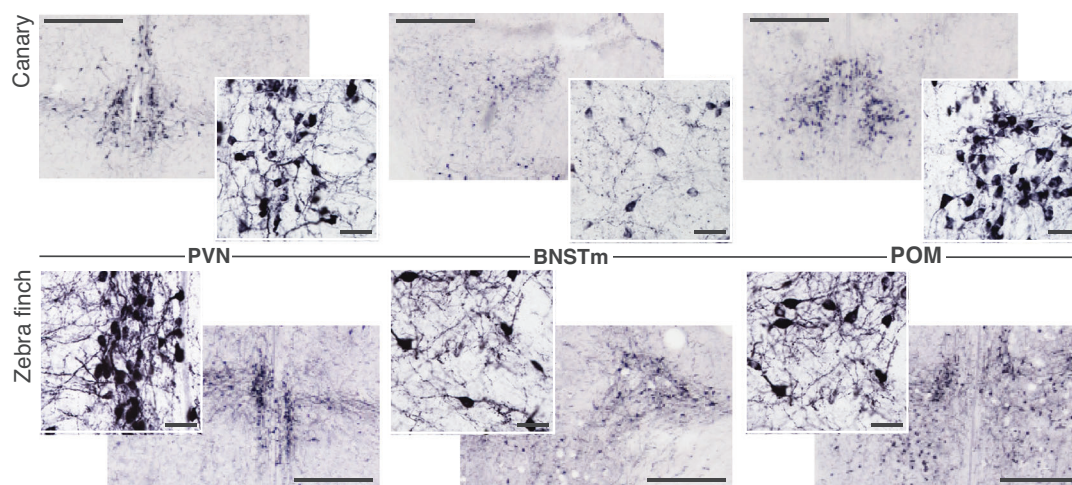
The lateral septum (LS) of both zebra finches and canaries was densely innervated by OT-ir fibers (Figure 7a). While both male and female



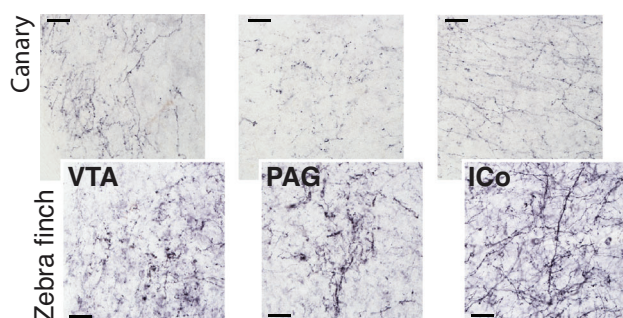
**FIGURE 3** Schematic drawings of coronal sections through the canary brain illustrating the distribution of OT-ir structures in a rostral to caudal order (a–l). OT-ir perikarya with staining throughout the cell body are represented by circles, while perikarya with OT-ir material present only at the periphery are represented by open diamonds. OT-ir fibers are represented by x's. The density of symbols has been adjusted to give a qualitative estimate of the number of the immunoreactive structures.



**FIGURE 4** Photomicrographs taken at 40× magnification demonstrating the presence of OT-ir fibers in HVC and OT-ir perikarya in the mesopallium of a canary (top) and zebra finch (bottom) along with comparison images taken from other pallial areas (hyperpallium, nidopallium, and arcopallium). Scale bars indicate 50  $\mu$ m.



**FIGURE 5** Photomicrographs demonstrating the presence of dense OT-ir perikarya and fibers in the paraventricular nucleus (PVN), medial bed nucleus of the stria terminalis (BNSTm), and medial preoptic nucleus (POM) of a canary (top) and zebra finch (bottom). Rectangular images were taken at 4× magnification, with scale bars indicating 500  $\mu$ m, and square images were taken at 40× magnification, with scale bars indicating 50  $\mu$ m.



**FIGURE 6** Photomicrographs taken at 10× magnification demonstrating the presence of OT-ir fibers in three dopaminergic regions—ventral tegmental area (VTA), periaqueductal gray (PAG), and intercollicular nucleus (ICo). Scale bars indicate 100  $\mu$ m.

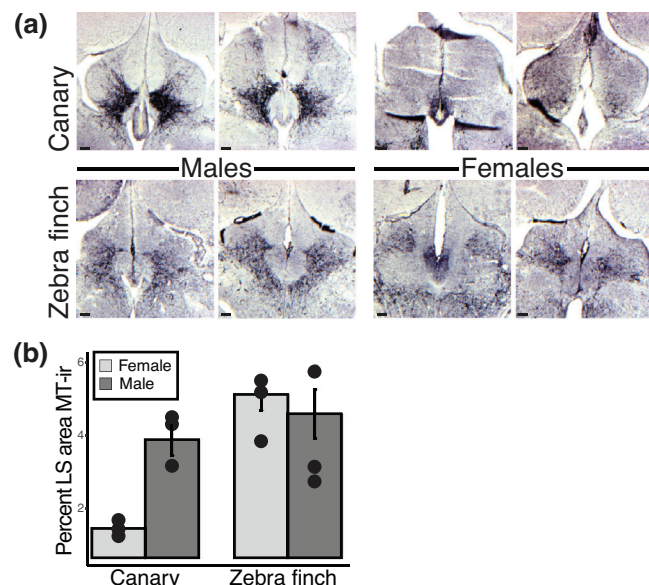
zebra finches appeared to have equal amounts of these fibers, the density of innervation appeared to be higher in male canaries compared to females (Figure 7b). Image analysis was therefore used to determine the fractional area covered by immunoreactive material in both

sexes in the two species. This clearly confirmed that in the three female canaries that were studied, the percentage of the area covered by OT-ir fibers was much lower than in all males of the same species and in zebra finches of both sexes. A two-way analysis of variance (ANOVA) with species and sex as factors detected a significant difference between species ( $F(1,8) = 8.16, p = .02, \eta^2 = 0.51$ ) and an interaction between species and sex ( $F(1,8) = 9.13, p = .017, \eta^2 = 0.53$ ) but no overall sex difference ( $F(1,8) = 1.87, p = .21, \eta^2 = 0.19$ ). This interaction appears to be due to the reduction in percent area covered by OT-ir fibers in female canaries but not in female zebra finches, as Tukey post hoc comparisons indicated that female canaries were 3% lower than male canaries ( $p = .05$ ) and 4% lower than female zebra finches ( $p = .01$ ).

### 3.5 | Additional regions

In addition to the above regions, scattered OT-ir cells were observed around nucleus rotundus (Rt), and OT-ir fibers were found in the nucleus interpeduncularis (IP).





**FIGURE 7** (a) Example photomicrographs of the septum of male and female canaries and zebra finches. Male canaries (top two left images) had a much greater density of OT-ir fibers than female canaries (top two right images). Zebra finches had similar densities in males and females. Scale bars signify 100  $\mu$ m. (b) Quantification of OT fiber density in the lateral septum. Individual points indicate the average percent area of the lateral septum covered by OT-ir fibers for each individual bird.

We did not observe OT-ir in regions involved in the oscine song system that were not previously mentioned—namely Area X, the robust nucleus of the arcopallium (RA), the dorsolateral nucleus of the anterior thalamus (DLM), or the lateral magnocellular nucleus of the anterior nidopallium (LMAN).

## 4 | DISCUSSION

In this study, an immunocytochemical procedure was utilized to characterize the distribution of fibers and cells immunoreactive for OT in the brain of male and female zebra finches and canaries. The presence of OT peptide in several brain regions, particularly hypothalamic regions, corresponds to the distribution of OT found in some nonoscine avian species, amphibians and reptiles, as well as the distribution of OT found in a variety of mammalian species (Buijs et al., 1978; Chokchaloemwong et al., 2013; Insel & Shapiro, 1992; Silveira et al., 2002; Sinpru et al., 2017; Smeets & González, 2001; Sofroniew et al., 1979; Thayanunphat et al., 2011; Thepen et al., 1987). These results are consistent with the hypothesis that many aspects of this nonapeptide system are conserved as one compares the OT system of species in which it was formerly called MT (birds, amphibians, and reptiles) to the system in fish (formerly IT) and mammals. In addition, we found support based on these anatomical findings for the idea that OT is involved in song learning and production and is linked to dopaminergic regions (Theofanopoulou et al., 2017).

We also identified a novel sex difference in the lateral septum of canaries.

### 4.1 | Conserved distribution in hypothalamic nuclei

The distribution of OT-ir neurons in hypothalamic nuclei of these two songbird species shares commonalities with that previously observed in nonoscine avian species. Peking ducks (*Anas platyrhynchos*) and Japanese quail (*Coturnix japonica*) have OT-ir perikarya in POA, SON, and PVN (Bons, 1980). In turkeys (*Meleagris gallopavo*), OT-ir cells have been found in the POA, BNST, and PVN (Thayanunphat et al., 2011). Likewise, in Thai hens (*Gallus domesticus*), OT-ir neurons and fibers are abundant in SON, medial preoptic nucleus (POM), and PVN (Chokchaloemwong et al., 2013; Sinpru et al., 2017). This peptide distribution also shares commonalities with the distribution of OT mRNA observed in songbirds. The zebra finch exhibits OT mRNA in BNSTm, PVN, and SON, while Bengalese finches (*Lonchura striata* var. *domestica*) and white-rumped munias (*Lonchura striata*) have OT mRNA in the SON, PVN, and lateral hypothalamus (Tobari et al., 2022; Vicario et al., 2017). Therefore, OT expression in these areas appears to be shared across avian taxa.

There are also similarities with the hypothalamic distribution of OT in amphibians and reptiles. In several frog species (including *Rana perezi*, *Xenopus laevis*, *Pleurodeles waltl*ans, and *Typhlonectes natans*), OT-ir cell bodies are present in two of the hypothalamic regions in which we observed OT in our songbirds—the POA and BNST (Smeets & González, 2001). Another frog species, the Cayenne caecilian (*Typhlonectes compressicauda*), has OT-ir cells in these regions as well, but also has a more extensive OT-ir cell distribution, with additional cells across the hypothalamus and midbrain tegmentum (Smeets & González, 2001). Studies of a variety of geckos and lizards reveal OT-ir cells in POA, SON, PVN, and VMN (Bons, 1983; Silveira et al., 2002; Thepen et al., 1987). Therefore, it seems that the presence of OT cells in the hypothalamus is conserved across the group Diapsida, with possibly some species-specific variations in the specific subdivisions of the hypothalamus that contain OT-ir. It remains unclear, however, whether these localized differences in distribution reflect a different sensitivity of the immunocytochemical procedures between studies, potentially associated with local differences in neuropeptide concentration, or simply divergences in the nomenclature of hypothalamic subregions. This brain area has a complex anatomy and authors often diverge in the nomenclature they use, even within the same species.

Recent evidence supports the idea that the OT nonapeptide in amphibians, reptiles and birds, formerly known as mesotocin, has a common origin with the OT nonapeptide in nondiapsids (formerly named isotocin in fish) that arose after the divergence of jawed vertebrates (Theofanopoulou et al., 2021). Therefore, comparison of the distribution of OT-ir across species can provide valuable insight into which regions ancestrally contained such nonapeptides.

In addition to the similarities in distribution of perikarya expressing OT across vertebrates, particularly in hypothalamic nuclei, the main tract of OT-ir fibers also appears to be conserved. Many species,



including Peking ducks, Japanese quail, Tokay geckos, jararaca snakes, garden dormice, guinea pigs, and Wister rats, exhibit OT-ir in the hypothalamo-hypophyseal tract, with bundled OT-ir fibers emerging from SON and/or PVN and converging in the median eminence and neurohypophyseal stalk (Bons, 1980; Buijs et al., 1978; Hermes et al., 1988; Silveira et al., 2002; Sofroniew et al., 1979; Thepen et al., 1987). This indicates that this tract may perform a functional role conserved across vertebrates.

## 4.2 | OT-ir localization in dopaminergic regions

The innervation by OT of dopaminergic areas that we observed has also been found in several other species. In the Tokay gecko, the jararaca snake, the midshipman fish, and the gulf toadfish, OT-ir fibers are present in the PAG (Goodson et al., 2003; Silveira et al., 2002; Thepen et al., 1987). In several frog species (*R. perezi*, *X. laevis*, *T. natans*, and *T. compressicauda*), OT fibers have been observed in many portions of the midbrain tegmentum containing dopaminergic cell bodies (Smeets & González, 2001). In addition, OT fibers have been observed in the PAG and VTA of several mammalian species, including the garden dormouse and crab-eating macaque (Caffé et al., 1989; Hermes et al., 1988).

## 4.3 | OT involvement in song processing and control nuclei

The results of this study support the hypothesis that OT may be involved in song learning and/or production, as we found evidence of OT peptide in some regions related to these functions (Theofanopoulou et al., 2017).

First, the localization of this nonapeptide in regions integral to processing and producing song supports the idea that OT is important in both aspects of vocal communication, the perception and production of sounds. The findings that the OT peptide is present in the auditory region CMM and in the song control nucleus HVC correspond with recent evidence showing that OT receptor mRNA is expressed in the auditory forebrain and song system of zebra finches (Davis et al., 2022). Likewise, in white-throated sparrows, OT receptor mRNA is highly expressed in HVC and CMM (Leung et al., 2011). The presence of OT-ir perikarya in the mesopallium, an area which includes the secondary auditory processing area CMM, would indicate that OT may be involved in the perception of song and could consequently help with the sensory portion of song learning. In fact, peripheral administration of an OT receptor antagonist in juvenile zebra finches decreases attention and preference for tutor song during learning (Pilgeram et al., 2021).

Besides these effects during song learning, OT action may influence song perception during adulthood. A similar function might occur in humans, as people with a GG OT receptor gene variant named OXTR rs53576 more easily hear and understand human speech in background noise (Tops et al., 2011). In addition, the OT-ir fibers found in the song control nucleus HVC, a major region involved in song production

and integration of sensory and motor information, may be influential in modulating the frequency or structure of song production.

Second, the presence of OT-ir somata and fibers in the preoptic area may be involved in regulating the frequency of production of a variety of vocalizations—both learned (song) and unlearned (calls). Past studies have implicated OT activity in the production of vocalizations in several non-vocal learning species which only produce innate/unlearned vocalizations. For example, OT knock-out mice pups produce fewer ultrasonic vocalizations than wild-type pups (Winslow et al., 2000). Similarly, rat pups in social isolation have differences in vocalization frequency with changes in OT activity; ultrasonic vocalizations are reduced following intracerebroventricular injection of OT, while after subcutaneous administration of OT vocalizations are increased at one dose (1  $\mu$ g) but decreased at another (10  $\mu$ g) (Insel & Winslow, 1991). Some of these effects are likely due to OT action in the POA, as this area has been specifically implicated as a modulator of the rate of vocal production (Alward et al., 2013; Nieder & Mooney, 2020). In the midshipman fish, OT in the POA differentially modulates vocalizations in different morphs. In females and sneak-spawning males, administration of OT in POA inhibited vocal bursts, while OT antagonist administration increased vocalizations. In contrast, males that acoustically court females did not exhibit differences in vocalizations following OT agonism/antagonism (Kelly & Goodson, 2014). In hamsters, OT in the POA has the opposite effect, as injection of OT into the MPOA of female hamsters increases ultrasonic vocalizations (Floody et al., 1998). While it is clear that OT action, specifically in the POA, can modulate the production of innate vocalizations, these effects are variable and may be dependent on a variety of external factors, such as social context. In songbirds, which produce the learned vocalizations of song in addition to unlearned calls, the POM also has an important function in the regulation of song production: lesions of this area decrease the rate of singing (Alger et al., 2009), while stereotaxic implants of testosterone increase it (Alward et al., 2013). Therefore, OT action in this region may modulate the frequency of both songs and calls.

Finally, the presence of OT-ir fibers in VTA and PAG provides evidence that OT may modulate dopaminergic input into the song system, consequently influencing singing (Theofanopoulou et al., 2017). VTA and PAG send dopaminergic projections to song control nuclei—HVC, the robust nucleus of the arcopallium (RA), and Area X—and dopamine metabolites in these two areas are correlated with song production (Appeltants et al., 2000, 2002; Castellino et al., 2007; Heimovics et al., 2011; Lewis et al., 1981). Damaging or inactivating these regions also influences song production, reducing directed song in the case of VTA and increasing latency to sing in PAG (Ben-Tov et al., 2021; Haaken-son et al., 2020; Hara et al., 2007). Furthermore, these two regions may also serve as a relay for the previously discussed effects of OT in POA, as the POM does not send direct projections to song control nuclei but does have reciprocal projections with VTA and PAG (Riters & Alger, 2004; Theofanopoulou et al., 2017). The presence of OT-ir in many of these regions—fibers in VTA, PAG, and HVC and perikarya in the POM—indicates that OT may modulate multiple processes along this neural pathway to influence song output. More research is needed to determine the specific effects of OT activity in the VTA and the PAG

on song and whether this activity is due to OT action in POM or directly in VTA/PAG.

#### 4.4 | Species-specific difference in lateral septum

The presence of immunoreactive fibers in the LS corroborates findings that OT action in the LS is involved in songbird social behaviors (Goodson et al., 2009). Interestingly, this region is the only site where qualitative differences between the two species were observed, although this does not, of course, preclude the existence of more subtle differences. Female canaries lacked the dense innervation of OT in the LS that was observed in male canaries and in both sexes in zebra finches.

One possible explanation for this difference is the short-day photoperiod that these birds were housed on. While female zebra finches are opportunistic breeders and do not rely on daylength as a reproductive cue, female canaries typically do not exhibit mating behavior under short daylengths (Follett et al., 1973; Steel & Hinde, 2009; Zann, 1996). Therefore, changes that occur in OT-ir neurons concurrently with changes in breeding condition may be responsible for this difference.

In rats, OT fibers innervating the LS originate from the PVN (De Vries & Buijs, 1983). Therefore, differences in OT-ir innervation of the LS could be due to seasonal changes in PVN OT neurons. OT action in PVN does appear to be involved in breeding behavior, since knock-down of OT production in the PVN of zebra finches results in female-specific decreases in pair bonding and nest cup ownership (Kelly & Goodson, 2014). In addition, in nonoscine species, the density of OT neurons in PVN does change in accordance with breeding condition: female turkeys and Thai hens exhibit an increase in the number of OT-ir neurons in the PVN, along with SON and POM, during the laying and incubating stages of breeding (Chokchaloemwong et al., 2013; Sinpru et al., 2017; Thayananuphat et al., 2011). The PVN neurons that are up- or downregulated in OT expression as a function of breeding condition may be the drivers of OT innervation in the LS and consequently increase or decrease OT-ir fibers in this region. This idea is supported by recent evidence that in a seasonally breeding oscine species, Gambel's white-crowned sparrow, there is an increase in nonapeptide receptor binding in the LS during egg laying (Meddle, 2021). Therefore, in seasonally breeding species, the projections of OT-ir fibers from PVN to the LS may be modulated by photoperiodic or other environmental conditions in order to limit these behaviors to the appropriate context for successful breeding. More studies are needed to determine if OT action in the LS of female canaries induces equivalent prosocial effects as those observed in female zebra finches and if there is seasonal variation in the extent of OT innervation of LS.

## 5 | SUMMARY AND CONCLUSIONS

This study characterized the distribution of the OT peptide in two species of songbird. We found that many regions expressing OT fibers and cells were the same as those observed in other vertebrate species, indicating that patterns of OT distribution are likely conserved across

a wide range of vertebrate species. Our results also provided support for the hypothesis that OT is involved in song learning and production, either by direct action in auditory or vocal motor regions or via indirect action through POA and dopaminergic regions (PAG and VTA) (Theofanopoulou et al., 2017). We also found a marked sex difference in the innervation of the canary LS by OT-ir fibers. Future research should investigate the functional role of OT in the regions we identified here and their influence on prosocial behavior.

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#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

#### DATA AVAILABILITY STATEMENTS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

#### PEER REVIEW

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