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Neural Distribution of Nonapeptide Receptors in Two Species of Songbird

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Neural Distribution of Nonapeptide Receptors in Two Species of Songbird

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B.A., Cornell University, 1999

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Abstract

Neural Distribution of Nonapeptide Receptors in Two Species of Songbird

By

Cary H. Leung

Vasotocin (VT) and its mammalian homologue, vasopressin, modulate many social behaviors across vertebrate groups. In songbirds, the effects of centrally administered VT vary according to species, which may reflect species-specific distributions of VT binding sites. Using two model songbird species, the white-throated sparrow (*Zonotrichia albicollis*) and zebra finch (*Taeniopygia guttata*), I labeled putative VT receptors using two radioligands, an iodinated vasotocin analog ([^{125}I]OVTA) and a V1a receptor antagonist ([^{125}I]LVA). [^{125}I]OVTA labeled receptors throughout the telencephalon, diencephalon, midbrain and brainstem, with a similar distribution in both species. In contrast, the binding of [^{125}I]LVA was restricted to the septal area, dorsal arcopallium, and the optic tectum in sparrow, and was essentially non-detectable in zebra finch. Competitive binding assays in the lateral septum showed that both ligands were effectively displaced by both VT and a related nonapeptide, mesotocin (MT), showing that these radioligands, which were developed to label mammalian nonapeptide receptors, label at least one population of related receptors in songbirds. Because multiple receptor subtypes are expressed in the avian brain, I developed and used species-specific riboprobes to show the neural distribution of three VT receptor subtypes, VT1, VT3 and VT4 in white-throated sparrow and zebra finch. I found that there were similarities in

distribution between species, and that the expression of each receptor subtype overlapped with one another in several brain regions. In sparrow, VT3 and VT4 were widely distributed in the brain, whereas VT1 distribution was more limited. In zebra finch, all three receptors were widely distributed in the brain. Based on the predicted amino acid sequences for these receptors, VT1 and VT4 likely bind with greater affinity to VT than MT, and VT3 likely binds with greater affinity to MT than VT. Because testosterone (T) is known to have a modulatory effect on the VT system, I tested for an effect of T on VT receptor expression in white-throated sparrow. There was no effect of T on VT receptor expression although plasma T was elevated in T treated birds and T treated birds sang more than control birds. No relationship between behavior and receptor expression, however, was detected. Furthermore, sex differences in VT receptor expression were not detected in either white-throated sparrow or zebra finch.

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General Introduction

Nonapeptides have been identified in organisms as varied as snails, worms, insects, fish, birds and mammals (Acher et al., 1995). These peptides are thought to have evolved over 700 million years ago (for a review, see Acher et al., 1980; Acher et al., 1995; Hoyle et al., 1999). Invertebrates, with a few exceptions, have one nonapeptide homolog. For example, annetocin is found in worms, conepressin is found in snails, sea hares and leeches, and inotocin is found in some insects (see Fig. 1). In vertebrates, there are two forms of nonapeptides, which are thought to have evolved from vasotocin (VT) as a result of gene duplication and subsequent mutation (Acher, 1995). Non-mammalian vertebrates have VT and either mesotocin (MT; birds, reptiles, amphibians and lungfishes), or isotocin (IT; bony fishes), whereas mammals typically have vasopressin (VP) and oxytocin (OT).

Over the last few decades, hundreds of studies have shown that VP and OT, and related nonapeptides, are key regulators of social behavior across diverse species. For example, VP and VT have been implicated in aggression, pair bonding, parental behavior, sexual behavior and social recognition in several species of fish, anurans, birds, and rodents (see Goodson and Bass, 2001; Caldwell et al., 2008). OT has been shown to influence maternal care and aggression, pair bonding, sexual behavior and social memory in rodents (see Neumann, 2008; Veenema and Neumann, 2008; Carter et al., 2008) and sexual behavior in birds (Kihlstrom and Danninge, 1972). MT has been implicated in familiarity and group-size preference in zebra finches (*Taeniopygia guttata*; Goodson et

al., 2009) and conepressin is involved in sexual behavior in a species of mollusk (*Lymnae stagnalis*; Van Kesteren et al., 1995).

In vertebrates, nonapeptides are found in brain regions known to be involved in social behavior. Because these regions are each connected to one another and each is involved in modulating several social behaviors, Newman (1999) proposed that together they form a social behavior network, in which the overall pattern of activity influences a behavioral response (Fig. 2). Social behavior is often intimately linked with reward and reinforcement behaviors, which involve many of the same brain regions of the social behavior network, as well as regions such as the ventral pallidum, nucleus accumbens and hippocampus. VP and OT, and their non-mammalian homologues, are found in the brain regions involved in social behavior and reward and reinforcement behavior across vertebrate groups, which suggests that the behavioral function of these nonapeptides may be conserved across diverse species.

Within these brain regions, neuropeptides can act as neurotransmitters, traveling quickly over short distances to excite a target neuron (Iversen, 1984; Schmitt, 1984). Unlike traditional neurotransmitters, whose effects on a target neuron last for fractions of a second, neuropeptides can change the excitability in a nearby neuron for several minutes or even hours (Mayeri et al., 1979). They can also act as neuromodulators, regulating the release of neurotransmitters (Iversen, 1984; Schmitt, 1984) and as neurohormones, which travel some distance before reaching and acting on a target neuron (Moore, 1987). When injected into discrete regions of the brain that are involved in social behavior or reward

and reinforcement behavior, these peptides can have pronounced effects on social behavior. For example, infusions of VP into the medial preoptic area (MPOA) in Syrian hamsters (*Mesocricetus auratus*) increase aggression and a territorial behavior called flank-marking (Ferris et al., 1984; Ferris et al., 1997). In the monogamous prairie vole (*Microtus ochrogaster*), a species that exhibits paternal care and forms strong pair-bonds, infusions of VP into the lateral septum increase paternal behavior (Wang et al., 1994).

Infusions of nonapeptides into the cerebrospinal fluid (CSF), which can transport peptides to many regions of the brain, can have species-specific effects on social behavior. The best-known example of species-specific effects is found in two closely related species of voles. Intracerebroventricular (i.c.v.) infusions of VP increase aggression in the monogamous prairie vole but not in the closely related non-monogamous montane vole (*Microtus montanus*; Young et al., 1997). The species-specific effects of VP are thought to be related to species differences in the neural distribution of a type of VP receptor, V1a, which is found in many of the brain regions involved in social behavior as well as reward and reinforcement. Prairie voles have higher levels of V1a receptor binding in the ventral pallidum whereas montane voles have more V1a receptors in the lateral septum (LS) than prairie voles (Young et al., 1997). Similarly, OT receptor distribution differs between prairie and montane voles, and is thought to underlie the species-specific effects of OT (Insel and Shapiro, 1992; Winslow, et al., 1993). The monogamous California mouse (*Peromyscus californicus*) has more V1a receptors in the LS compared to the non-monogamous white-footed mouse (*Peromyscus leucopus*; Bester-Meredith et al., 1999) and in tuco-tucos, a species of South

American rodent, the colonial *Ctenomys sociabilis* and the solitary *Ctenomys haigi* differ in levels of V1a and OT receptor binding in many regions including the LS and ventral pallidum (Beery et al., 2008). Furthermore, when nonapeptide receptor distribution in the rodent brain is genetically manipulated to resemble the pattern of a highly social monogamous species, affilitation behavior changes to resemble that in monogamous rodents (Young et al, 1999; Lim et al., 2004). Together, these studies demonstrate that nonapeptide receptor distribution may underlie species-specific behaviors.

Because of the diversity of songbird behaviors and the voluminous literature documenting them, songbirds present a unique opportunity to study the role of nonapeptides and their receptors in behavior across species. Like VP in rodents, VT can have species-specific effects on behavior in songbirds. For example, i.c.v. infusions of VT increase aggressive behaviors in zebra finches (*Taeniopygia guttata*; Goodson et al., 2004) and inhibit them in violet-eared waxbills (*Uraeginthus granatina*; Goodson, 1998a) and field sparrows (*Spizella pusilla*; Goodson, 1998b). Like the effects of VP in rodents, the species-specific effects of VT in songbirds may reflect species differences in nonapeptide receptor distribution.

The distribution of nonapeptide receptors in songbirds has been studied using several different radioligands that were developed to label mammalian nonapeptide receptors. For example, Voorhuis et al. (1988a) used tritiated VP ([^{3}H]VP) to label VT receptors in the canary brain. They found [^{3}H]VP binding in several regions implicated in social behavior, such as a song motor nucleus, the robust nucleus of the arcopallium (RA). They

also found [^{3}H]VP binding in brain regions involved in visual processing, such as the nucleus pretectalis (Pt) and the optic tectum (TeO). In another study in canary, Voorhuis et al. (1990) used an iodinated vasotocin analog, [^{125}I]OVTA, to label VT receptors. They reported the distribution of VT-like binding was limited to the dorsal arcopallium (Ad) around RA. To describe the distribution of VT receptors in several estrildid species that vary in territorial behavior, Goodson et al. (2006) used an iodinated V1a receptor antagonist, [^{125}I]LVA, that binds with high affinity to the mammalian V1a receptor. They found [^{125}I]LVA binding was limited to the LS in most of the species, and extended into some pallial layers and the lateral striatum in spice finch (*Lonchura punctulata*). Because of the disparate binding distributions between and within species, it is not clear whether there are species differences in the distribution of VT receptors or whether the radioligands are each binding to a different population of VT receptors.

In order to determine whether VT-like binding patterns differ between species, radioligand studies using more than one species would be required. This has been done with the use of a single radioligand in several species of songbird (Goodson et al., 2006). Determining whether VT-like binding patterns depend on the radioligand would require the use of multiple radioligands in a single species, which has been done only for the canary (Voorhuis et al., 1988a; 1990). In chapter one, therefore, I used two radioligands from previous studies (Goodson et al., 2006; Voorhuis et a., 1990), [^{125}I]OVTA and [^{125}I]LVA, to label VT receptors in two species of songbird, the white-throated sparrow (*Zonotrichia leucophyrs*) and zebra finch. This study helped determine whether there are species differences in receptor distribution, determine whether VT-like binding patterns

depend on the radioligand, and demonstrate where nonapeptides may be binding to receptors in the brain to modulate behavior.

The distribution of VT receptor binding may tell us where VT or MT may be binding to receptors, but such studies do not give us definitive information on the distribution of the specific receptor subtypes. There are four known VT receptor subtypes in birds. VT1, the first nonapeptide receptor discovered in birds, is found in the avian brain (Tan et al., 2000). VT1 is not closely homologous to any known mammalian nonapeptide receptors, although it is somewhat similar in structure to the V1a receptors in mammals (Baeyens and Cornett, 2006) and shares binding properties with the mammalian V1a receptor (Acharjee et al., 2004). VT2, which is homologous to the V1b receptor in mammals, is found in the pituitary but not in brain tissue (Cornett et al., 2003). VT3 bears the greatest similarity in structure to the mammalian OT receptor and shares some homology with non-mammalian MT receptors (Gubrij et al., 2005), and its mRNA has been detected in songbird brain (Leung, unpublished). Analyses of the chicken and zebra finch genome demonstrate evidence for a fourth nonapeptide receptor, VT4, which is thought to be the avian equivalent of the mammalian V1a receptor (Cornett, Jacobi and Mikhailova, unpublished), and I have detected the mRNA for this gene in songbird brain (Leung, unpublished). Detailed radioligand and cold ligand competition studies have not yet been conducted for most of these receptors and it is unclear which of them is mediating the behavioral effects of VT and where these receptors are distributed in the brain.

To address these questions, I created species-specific riboprobes for white-throated sparrow and zebra finch VT1, VT3 and VT4 and mapped the expression of these receptors using *in situ* hybridization. I chose these subtypes because they are expressed in the brain (Tan et al., 2000; Leung, unpublished). These studies helped clarify where the receptor subtypes are distributed, whether there are species differences in subtype distribution, and which receptor subtypes are labeled by VT-like radioligands. Furthermore, studying the distribution of these receptors helped determine which VT receptor subtypes may be involved in social behavior.

There is some evidence that VT receptors are involved in behavior in a sex-specific manner. For example, Goodson et al. (2004) found that i.c.v. infusions of a VT-like receptor antagonist decreased aggressive behavior during mate-competition in male zebra finches, but not in females. This sex-specific effect on behavior suggests that there may be a sex difference in the concentration of VT receptors in specific regions of the songbird brain. We therefore looked for a sex difference in VT receptor expression in regions with high levels of expression in white-throated sparrow and zebra finch.

Describing the distribution of nonapeptide receptors allows us the opportunity to study how they are regulated and how they are related to behavior. For example, VP injected into the MPOA, a region implicated in social behavior, is more effective at stimulating flank-marking behavior if animals are treated with testosterone (T; Albers et al., 1988). In females, flank-marking varies in intensity with the estrus cycle, and treatment with estradiol increases the VP-induced behavioral response (Huhman and Albers, 1993;

Albers et al., 1996). T may modulate the behavioral actions of VP by acting on nonapeptide receptors. For example, T increases the number of VP receptors in the hypothalamus in castrated Siberian and Syrian hamsters (*Phodopus campbelli*; Dubois-Dauphin et al., 1994; *Mesocricetus auratus*; Delville et al., 1996. Johnson et al., 1995; Young et al., 2000). In the MPOA, T increases both V1a receptor mRNA and receptor binding in castrated Syrian hamsters (Young et al., 2000).

Many behaviors that are affected by VT/VP are also regulated by gonadal steroids in birds. T increases aggressive behavior in several species of songbird including white-throated sparrows (Archawaranon and Wiley, 1988) and zebra finches (Arnold, 1975; Adkins-Regan, 1999). Specifically, T increases the number of attacks and chases among flocking white-throated sparrows (Archawaranon and Wiley, 1988), and increases number of chases as well as decreases latency to aggress in zebra finches (Arnold, 1975; Adkins-Regan, 1999). The effects of T on aggression may be explained by the modulation of the VT system by T. VT immunoreactive fibers (VT-IR) and VT-like binding are sensitive to T in brain regions implicated in social behavior. For example, T increases VT-IR in the LS in canaries and juncos (Voorhuis et al., 1988b; Plumari et al., 2004) and the BSTm in zebra finches (Kimura et al., 1999). In female canaries, T increases [^{3}H]VP binding in the song nucleus RA (Voorhuis et al., 1988a).

Because of the wealth of information showing a modulatory role of T on the VT/VP system, I looked for an effect of T on VT receptor expression in white-throated sparrow. I focused my analysis on regions previously reported to have VT receptors that are

sensitive to T (Voorhuis et al., 1988a) and regions that had high levels of expression. For example, because VP receptors are sensitive to T in the rodent hypothalamus, I also looked for an effect of T on regions of the avian hypothalamus that had VT receptor expression. Because T is also known to affect vocalizations in seasonal songbirds, I looked at the effects of T on spontaneous courtship vocalizations in sparrows and tested for a correlation between behavior and VT receptor expression. These studies allowed us to determine whether a particular VT receptor subtype is involved in courtship vocalizations and whether one subtype is involved in vocalizations more than the other subtypes.

The studies described here provide much needed information on the distribution of VT receptors in the avian brain at the transcription and translational levels. Because I used two species of songbird and two radioligands, I determined whether there are species differences in VT-like binding using the same ligand, and whether different radioligands result in different distribution pattern within species. By conducting *in situ* studies in the same two species, I was able to compare the distribution of receptor subtypes to the VT receptor binding patterns, which allowed me to determine which VT receptor subtypes are likely labeled using radioligands. These studies demonstrate where nonapeptide receptors are distributed in the avian brain, and where they may play a role in nonapeptide-modulated behaviors. The comparative nature of these studies provides information on whether nonapeptide receptor distributions are generally conserved across avian species and expands the number of species in which nonapeptide receptor distribution is known.

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Figure Legends

Figure 1. Nonapeptide homologs in vertebrates and invertebrates. (Redrawn from Donaldson and Young, 2008).

Figure 2. Social behavior network. CeA = central amygdala; BSTm = bed nucleus of the stria terminalis; LS = lateral septum, VMH = ventromedial hypothalamus; AH = anterior hypothalamus; MPOA = medial preoptic area. (Redrawn from Newman, 1999).

Figure 1.

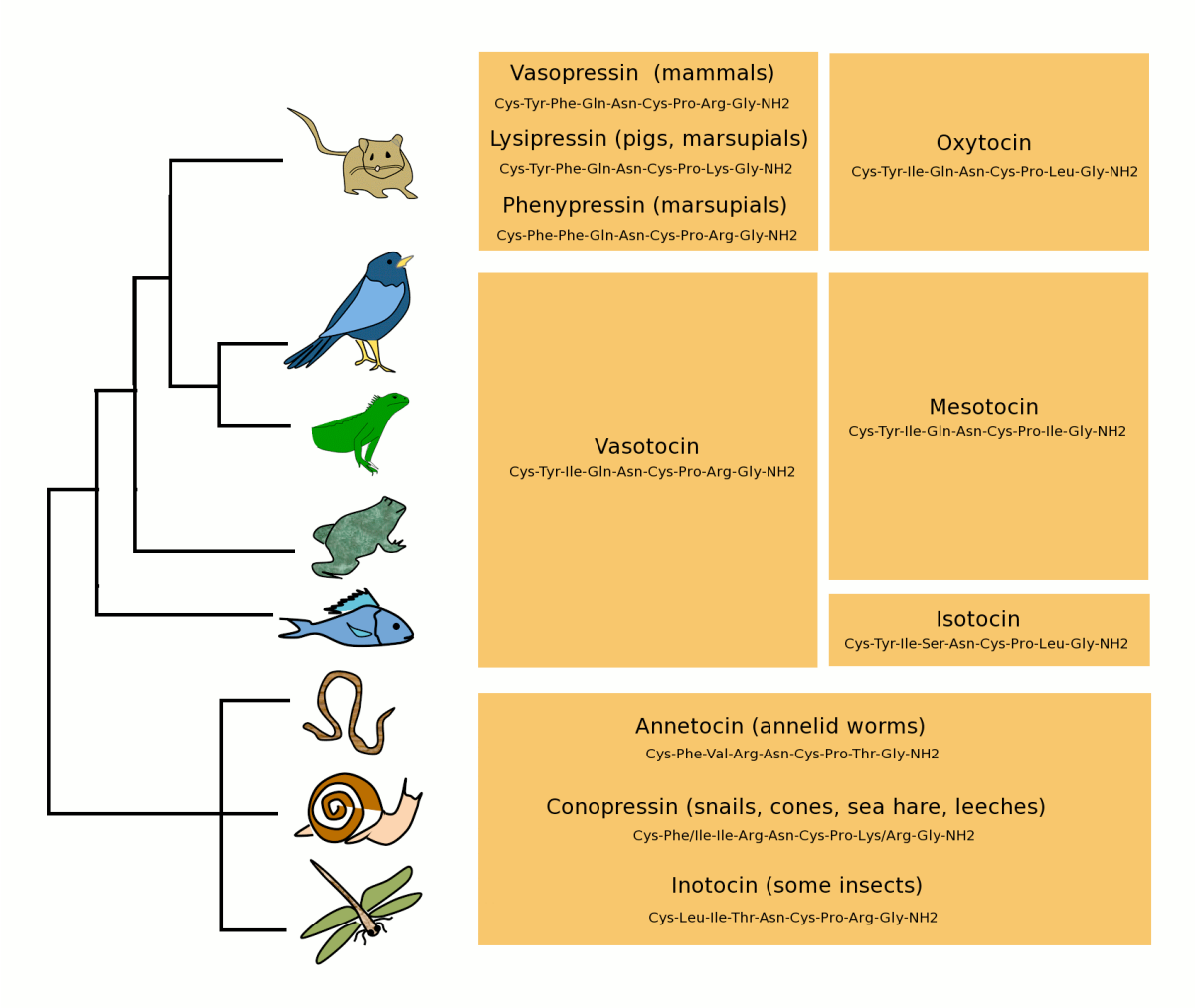
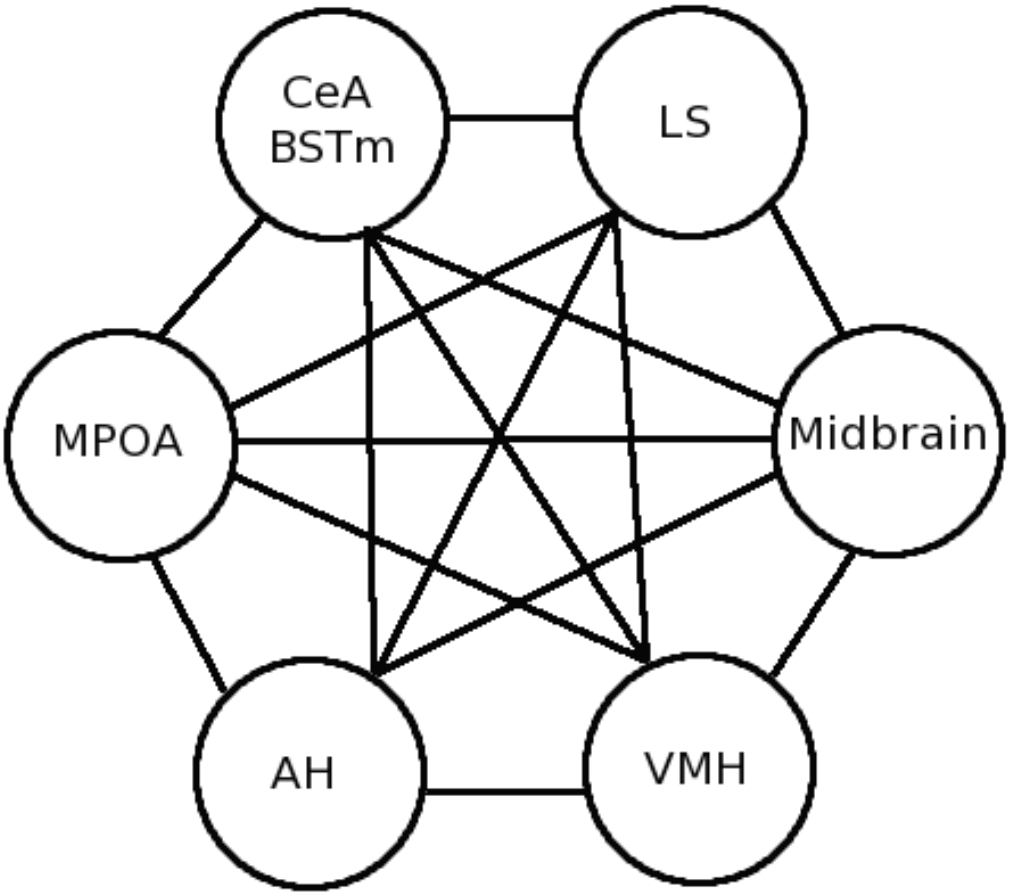

Vasopressin (mammals)
Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH2
Lysipressin (pigs, marsupials)
Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH2
Phenypressin (marsupials)
Cys-Phe-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH2
Oxytocin
Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH2
Vasotocin
Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Arg-Gly-NH2
Mesotocin
Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Ile-Gly-NH2
Isotocin
Cys-Tyr-Ile-Ser-Asn-Cys-Pro-Leu-Gly-NH2
Annetocin (annelid worms)
Cys-Phe-Val-Arg-Asn-Cys-Pro-Thr-Gly-NH2
Conopressin (snails, cones, sea hare, leeches)
Cys-Phe/Ile-Ile-Arg-Asn-Cys-Pro-Lys/Arg-Gly-NH2
Inotocin (some insects)
Cys-Leu-Ile-Thr-Asn-Cys-Pro-Arg-Gly-NH2

Figure 2.



Chapter One:

Neural Distribution of Nonapeptide Binding Sites in Two Species of Songbird

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Abstract

Vasotocin (VT) and its mammalian homologue, vasopressin (VP), are known to modulate many social behaviors in a variety of vertebrate species. Central infusions of VT have species-specific effects on vocalizations and aggression, which may relate to species-specific VT receptor distribution. Using two model songbird species, the white-throated sparrow (*Zonotrichia albicollis*) and zebra finch (*Taeniopygia guttata*), I labeled putative VT receptors using two radioligands, ^{125}I-ornithine vasotocin analog (^{125}I-OVTA) and ^{125}I-linear VP. ^{125}I-OVTA revealed VT-like binding in regions throughout the telencephalon, diencephalon, midbrain and brainstem in both sparrow and zebra finch, with few species differences in distribution. In contrast, the binding of ^{125}I-linear VP was restricted to the lateral septum (LS), medial septum, dorsal arcopallium and the optic tectum in sparrow brain and was close to non-detectable in the LS in zebra finch brain. No significant sex difference was found in ^{125}I-OVTA binding in the LS in zebra finch, although a strong trend was found with males having greater levels of binding than females. Competition experiments revealed that VT and the related neuropeptide mesotocin (MT), thought to be the avian homologue of oxytocin, were equally effective at displacing ^{125}I-OVTA in sparrow and zebra finch, but MT was significantly more effective at displacing ^{125}I-linear VP in sparrow. These results suggest that ^{125}I-OVTA and ^{125}I-linear VP may have different affinities and specificities for VT-like receptors. I am currently developing ribroprobes that will distinguish among the known avian neuropeptide receptors.

The neuropeptide vasotocin (VT) and its mammalian homologue vasopressin (VP) have been implicated in a variety of social behaviors across vertebrate groups (see Goodson and Bass, 2001). Among amphibians, fish and birds, VT has effects on vocalizations and sexual behavior in many species. For instance, VT increases calling behavior in four species of frog, *Hyla cinerea, Rata catesbeiana, Acris crepitans* and *Bufo cognatus* (Penna et al., 1992; Boyd, 1994; Marler et al., 1995; Propper and Dixon, 1997), increases courtship behavior in blue-headed wrasse, *Thalassoma bifasciatum* (Semsar et al., 2001) and modulates vocalizations in plainfin midshipman fish, *Porichthys notatus* (Goodson and Bass, 2000a; Goodson and Bass, 2000b).

In songbirds, VT has effects on aggressive behavior in several species. For example, septal VT infusion increases agonistic song in field sparrows (*Spizella pusilla*; Goodson, 1998). In zebra finches (*Taeniopygia guttata*), central infusions of VT increase aggressive behavior (displacements, threats, pecks and beak fences) whereas septal VT infusions increase beak fencing but not pecks or chases (Goodson et al., 2004a; Goodson and Adkins-Regan, 1999). These studies demonstrate that VT may have species-specific effects on aggressive behavior, and that the method of administration may affect where VT is binding in the brain and therefore what effect VT has on aggressive behavior.

VP has species-specific effects on aggression in two closely related species of rodent. ICV injections of VP increase levels of aggressive behavior in the monogamous prairie vole (*Microtus ochrogaster*) but not in the promiscuous montane vole (*Microtus montanus*; Young et al., 1997). These species-specific effects of VP may be explained by

species differences in VP receptor distribution (Young et al., 1997; Young et al., 2000) although region specific injections are needed to determine which brain regions are involved.

In mammals, the central actions of VP are mediated by a G-protein-coupled membrane receptor, V1a, which has been described throughout the brain in several rodent species (Phillips et al., 1988; Insel et al., 1994). These studies utilize an iodinated linear V1a receptor antagonist (^{125}I-linear VP; Phenylacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2), known for its specificity for mammalian V1a receptors (Schmidt et al., 1991). Using this same ligand, putative V1a-like receptor distribution has been described in several species of songbird (Goodson et al., 2006), although the specificity of this ligand for V1a-like receptors has not been demonstrated in birds. There are four known VT-like receptors, three of which may be in the avian brain (Tan et al., 2000; Cornett et al., 2003; Gubrij et al., 2005; Genbank accession number: EU124684). ^{125}I-linear VP therefore may bind to any combination of these receptors in the brain.

Interestingly, the binding patterns described by Goodson et al. (2006) in several species of *Estrildid* finches and waxbills do not overlap with the distribution of putative VT receptors reported in the canary brain using ^{3}H-VP (Voorhuis et al.,1988) or ^{125}I-ornithine VT analogue (^{125}I-OVTA; Voorhuis et al., 1990), an oxytocin (OT) receptor antagonist (Insel and Shapiro, 1992), suggesting that there could be species differences in VT receptor distribution. Alternatively, these species differences in VT receptor distribution may relate to differences radioligand specificity or affinity (or both) for different VT-like

receptors. Goodson et al. (2006) used a single concentration of VT and mesotocin (MT), the avian homologue of OT, to demonstrate that VT was more effective at competing off ^{125}I-linear VP in *Estrildid* finches and waxbills, whereas Voorhuis et al. (1988) used a single concentration of VT and VP to show that both neuropeptides were equally effective at competing off ^{3}H-VP in canary. In a much more detailed competition experiment, Voorhuis et al. (1990) showed that VT was more effective at competing off ^{125}I-OVTA than MT, VP and OT at several different concentrations. These studies collectively demonstrate that these radioligands may differ in their affinity for VT receptors and that more detailed competition experiments are needed in more than one species of songbird.

To address these issues, I examined the brain distribution of putative VT receptors in two different species of songbird using ^{125}I-OVTA and ^{125}I-linear VP and conducted detailed competition experiments with several concentrations of cold competitors. I chose the white-throated sparrow (*Zonotrichia albicolis*) because this species is a model organism used extensively in the field of behavioral endocrinology for understanding the hormonal basis of aggression, and the zebra finch because of its established role in the discovery of neural mechanisms underlying vocal learning and song production.

I then compared VT-like binding distribution to VT immunoreactive fiber (VT-ir) distribution in both white-throated sparrow and zebra finch (Maney et al., 2005; Maney, unpublished). In rodents, VP receptor distribution overlaps with VP-ir in some brain regions but not others (Dubois-Dauphin et al., 1990; Insel et al., 1994; Wang et al., 1996;

Young et al., 1997), yet the significance of the overlap or mismatch is rarely reported or discussed (but see Dubois-Dauphin et al., 1990). Because VT receptor distribution and VT-ir overlap has not been explored, I will describe the regions in which this does or does not occur and compare these regions to areas in which overlap and mismatch of VP receptor and VP-ir occur in rodents.

MATERIALS AND METHODS

Autoradiographic labeling of VT-like receptors

White-throated sparrows were captured in mist nets during fall migration in Atlanta, GA and sex was determined using PCR analysis (Griffiths et al., 1998). Birds were housed on short photoperiod (9L:15D) in indoor mixed-sex flight cages in the animal care facility at Emory University for 3 months and then transferred to long photoperiod (15L:9D) for 4 weeks. Zebra finches were obtained from a laboratory breeding colony at Georgia State University, where they had been housed in mixed-sex groups on long photoperiod (14L:10D). Sex was determined by visual inspection. All birds were anesthetized using isoflurane and rapidly decapitated. Brains were immediately removed, frozen on dry ice and stored at -80°C until sectioning. Seven sets of brain sections were cut in the coronal plane at 20μm on a cryostat and thaw-mounted on Superfrost plus slides (Fisher, Pittsburgh, PA). The sections were then stored at -80°C until use in autoradiography. All research was conducted in accordance with the NIH Principles of Animal Care and with federal and state laws and university guidelines.

Eight white-throated sparrows (males: N = 4; females: N = 4) and 12 zebra finches (males: N = 6; females: N = 6) were used to determine the distribution of VT-like binding. Two sets of adjacent brain sections from each bird were processed for receptor autoradiography following the protocol of Young et al. (1997). Slides were fixed in paraformaldehyde and then prewashed in 50mM Tris base (pH 7.4). One set of tissue from each bird was incubated in 50 pM ^{125}I-linear VP (^{125}I-Phenylacetyl-D-Try(Me)-Phe-Gln-Asn-Arg-Pro-Arg-Tyr-NH_2; cat. no. NEX310, PerkinElmer, Boston, MA) in 50mM Tris base with 10mM MgCl (pH 7.4) for 90 minutes. The second set of tissue was incubated in 50 pM ^{125}I-ornithine vasotocin analog (^{125}I-OVTA; ^{125}I-d$(CH_2)_5$[Tyr$(Me)_2$-Thr$_4$-Orn$_8$-Tyr$_9$-NH_2]; cat. no. NEX254; PerkinElmer, Boston, MA) in 50mM Tris base with 10mM MgCl (pH 7.4) for 90 minutes. The slides were then washed in 50 mM Tris with 10mM MgCl (pH 7.4) to remove non-specific binding, dipped in H_20 and dried with cold air using a conventional blow dryer. BioMax MR film (Kodak, Rochester, NY) was exposed to slides and ^{125}I autoradiographic standards (Amersham, Arlington Heights, IL) for 96 hours. Films were then developed in a Konica SRX101-A developer.

After exposure to film, slides were stained using toluidine blue (cat. no. 52040; Fisher Scientific) using a protocol adapted from Charlier et al. (2006) for brain sections processed for receptor autoradiography. Slides were soaked overnight in a 1.5% solution of toluidine blue at room temperature in a dark room. A fresh toluidine blue solution was used for each rack of slides. Slides were then dipped in the following: 1) deionized water, 2) 20% alcohol, 3) 70% alcohol, 4) 90% alcohol, 5) two washes of 100% alcohol, and 6)

two washes of xylene. Slides were coverslipped with DPX mountant (cat. no. 44581; Sigma-Aldrich, St. Louis, MO).

Sex comparison

I compared the binding intensity of ^{125}I-OVTA in the LS between male and female zebra finch brains processed for the distribution study described above (males: N = 6; females: N = 6). The LS, which has the darkest binding, was traced using AIS (version 6.0, Imaging Research Inc., Cambridge, UK) and its optical density was converted to a measure of binding intensity, nanocuries (nCi) per mg tissue equivalents, from the standard curve derived from coexposed standards. Total binding was calculated by averaging binding in the LS in sections throughout the brain. Nonspecific binding was measured in an area adjacent to the LS, within the same coronal section. Specific binding was calculated by subtracting nonspecific binding from total binding measured in the LS. An independent t test was used to compare binding intensity of ^{125}I-OVTA between the sexes.

Competitive binding assays

One male white-throated sparrow and eight male zebra finches were used for competitive binding assays with ^{125}I-OVTA and several concentrations of VT and MT (cat. nos. H-1785.0005 and H-2505.0005; Bachem, Torrance, CA) ranging from 10^{-10} to 10^{-5} M. Four female white-throated sparrows and six male zebra finches were used for competitive binding assays with ^{125}I-linear VP and several concentrations of VT and MT (Bachem, Torrance, CA) ranging from 10^{-10} to 10^{-5} M. Films exposed to slides were coded to

obscure the identity of the cold competitor used. Binding intensity was quantified using the methods described above for the sex comparison. Inhibition curves and inhibitory concentration 50% (IC_{50}) values for each cold competitor were calculated using nonlinear regression analysis in Prism version 5.0 (GraphPad Software, La Jolla, CA; see Motulsky and Christopoulos, 2003). To graph the curves, the intensity of labeling in nCi/mg tissue equivalents was normalized using the 100% value from the plateau for each curve as calculated in Prism. For each curve, one-site and two-site models were compared for goodness-of-fit using F-tests. The potency of VT and MT of radioligand binding was compared using F-tests.

Immunohistochemical labeling of VT fibers

Eight white-throated sparrows (males: N = 4; females: N = 4) were collected in walk-in traps in Williamsburg, VA and five male zebra finches were obtained from a breeding colony at Rockefeller University. All birds were housed on long photoperiod (15L:9D). Birds were decapitated under deep isoflurane anesthesia. Brains were then removed, fixed in 5% acrolein, and either cryoprotected and cut on a microtome or embedded in gelatin and cut on a vibrotome at 50 μm.

The distribution of VT-ir distribution was determined using immunohistochemistry as described by Maney et al. (2005). Alternate sections were incubated overnight in 20% normal goat serum at 4°C, then incubated for 48h in rabbit anti-VP diluted 1:2000 at 4°C (cat. no. 64717, lot no. 1143C, MP Biomedicals, Solon, OH). This antibody has been demonstrated to label VT via preadsorption studies (Boyd et al., 1992). Staining was

visualized using anti-rabbit IgG antibodies raised in goat (Vector, Burlingame, CA) followed by the ABC Elite kit (Vector) and diaminobenzidine (Sigma, St. Louis, MO). Sections were then mounted on gelatin-coated slides and coverslipped with DPX (Sigma).

RESULTS

Distribution of VT-like binding

A complete list of brain regions with VT-like binding in white-throated sparrow and zebra finch can be found in Tables 1 and 2. Individual variation in VT-like binding was observed in most brain regions. The following narrative describes the distribution of binding for each ligand in each species.

Binding of ^{125}I-OVTA binding in white-throated sparrow

*Telencephalon***.** As demonstrated in Fig. 1, VT-like binding as revealed by ^{125}I-OVTA was distributed throughout the pallial layers of the telencephalon. VT-like binding in the nidopallium (N) was homogenous in rostral sections, but became more heterogeneous in caudal sections. The dorsal region of the parahippocampal area (APH) had moderately high levels of binding whereas the neighboring hippocampus (Hp) had low levels of heterogeneous binding. Binding was darkest in the LS, medial septum (MS) and dorsal arcopallium (Ad). The nucleus taeniae of the amygdala (TnA) had moderate levels of binding, whereas the surrounding arcopallium (A) had very limited binding (see Fig. 1K). The HVC (proper name, located in the caudal N; not shown) had moderate levels of

binding whereas the robust nucleus of the arcopallium (RA) had relatively little binding (Fig. 1M). The RA was surrounded by a strong band of binding in the Ad that began just rostral to the RA, and continued caudal to the RA (Figs. 1M and 1O).

Diencephalon. Levels of ^{125}I-OVTA binding in the diencephalon were greatest along the walls of the third ventricle (3v; see Fig. 1E). Several thalamic nuclei had moderate levels of ^{125}I-OVTA binding as seen in Figs. 1E, 1G and 1I. These nuclei included the lateral anterior nucleus of the thalamus (LA), lateral geniculate nucleus (GLV), the lateral portion of the dorsolateral nucleus of the anterior thalamus (DLL), and the dorsomedial nucleus of the posterior thalamus (DMP).

Midbrain and brainstem. Within the midbrain (see Fig. 1I), moderate levels of ^{125}I-OVTA binding were found in the medial pretectal nucleus (PTM), an area immediately medial to the shell of the pretectal nucleus. Moderate levels of binding were found in the isthmo-opticus nucleus (IO; see Fig. 1M) and ventral tegmental area (VTA; not shown), whereas low levels of binding were found in the midbrain central gray (GCT), intercollicular nucleus (ICo), optic tectum (TeO), and several additional midbrain nuclei that are not shown in figures (see Table 1). Within the brainstem in Fig. 1M, low levels of ^{125}I-OVTA binding were detected in the locus coeruleus (LoC) and the motor nucleus of the trigeminal nerve (MV).

Binding of ^{125}I-linear VP in white-throated sparrow

The distribution of ^{125}I-linear VP binding was limited to the LS, MS, Ad and TeO, and was substantially lighter than the ^{125}I-OVTA binding in these regions. (see Figs. 1E-H and 1M-1N).

Binding of ^{125}I-OVTA in zebra finch

Telencephalon. The distribution of ^{125}I-OVTA binding in zebra finch brain was similar to ^{125}I-OVTA binding distribution described above in sparrow. Like in sparrow, binding in the LS was high relative to other regions in the brain. Binding in the MS, however, was essentially non-detectable. Moderate to low levels of binding were detected in the distinct pallial layers of the telencephalon (Fig. 2). As seen in Fig. 2B, the lateral magnocellular nucleus of the anterior nidopallium (LMAN) and Area X clearly lack binding whereas the surrounding N and medial striatum (StM) have moderate levels binding. In contrast to what was seen in sparrows, levels of VT-like binding in Ad were low in zebra finch.

Diencephalon. ^{125}I-OVTA binding was detected in the same thalamic nuclei as in the sparrow including the DLL, LA, GLV, DMP (see Table 2), although at lower levels.

Midbrain and brainstem. Within the midbrain (Fig. 2D-H), ^{125}I-OVTA binding was found in the LoC, nucleus of Edinger-Westphal (EW; not shown) and TeO. Within the brainstem, ^{125}I-OVTA binding in the MV was much more distinct in zebra finch (Fig. 2F) than it was in sparrow (Fig. 1M).

Binding of ^{125}I-linear VP in zebra finch

Although ^{125}I-linear VP has been used to label VT-like receptors in this species (Goodson et al., 2006), in our study this radioligand showed very little affinity for zebra finch brain tissue. Only one section from each of two birds exhibited weak binding in the LS. Sparrow tissue run in the same assay showed strong VT-like binding. I therefore excluded the zebra finch brains processed with this radioligand from our analysis.

Sex comparison

I did not find a statistically significant difference in ^{125}I-OVTA binding between males and females, but there was a compelling trend (with males higher than females, $t(10) = 1.89$, $p = .089$, Cohen's $d = 1.19$, see Fig. 3). The power to detect a difference between male and female zebra finches was 52%.

Competitive binding assays

Inhibition of radioligand binding in sparrow tissue

^{125}I-OVTA. In sparrow tissue, ^{125}I-OVTA was displaced by VT and by MT (Fig. 4). The data fit well to models in which each ligand interacted with more than one binding site, but both two-site models were rejected because the IC_{50} of the higher affinity sites fell below the range of cold competitor concentrations used in the assay (see Motulsky & Christopoulos, 2003). A one-site model was therefore accepted for both ligands (VT: $R^2 = 0.725$; MT: $R^2 = 0.789$). VT and MT inhibited ^{125}I-OVTA binding with essentially equal potency ($F_{(1, 122)} = 0.095$; $p = 0.756$ see Table 3).

^{125}I-linear VP. Binding of ^{125}I-linear VP was inhibited in sparrow tissue by both VT and MT (Fig. 4). The VT data could not be fit unambiguously to a two-site model. A one-site model was therefore accepted for VT ($R^2 = 0.7202$). The data from the MT competition assay did not fit well with a two-site model ($F_{(4, 71)} = 0.8214$; $p = 0.5158$) and a one-site model was accepted ($R^2 = 0.5835$). MT inhibited the binding of ^{125}I-linear VP with much greater potency than did VT ($F_{(1, 105)} = 15.613$; $p = 0.0001$; see Table 3). Although I do not know the exact affinities because I did not calculate dissociation constants in these experiments, the IC_{50} was an order of magnitude lower for MT than for VT is consistent with the hypothesis that MT had a higher affinity for ^{125}I-linear VP binding sites than did VT.

Inhibition of radioligand binding in zebra finch tissue

Because I did not obtain quantifiable binding of ^{125}I-linear VP in zebra finch, I report here the results for competition assays for ^{125}I-OVTA only. These data did not fit with two-site models (VT: $F_{(4, 79)} = 0.618$, $p = 0.651$; MT: $F_{(4, 79)} = 0.638$, $p = 0.637$), so a one-site model was accepted (VT: $R^2 = 0.678$; MT: $R^2 = 0.809$). VT and MT inhibited binding with essentially equal potency ($F_{(1, 167)} = 0.360$; $p = 0.549$; see Table 3).

Overlap of VT-like binding and VT-ir

In white-throated sparrow and zebra finch, VT-like binding overlapped with VT-ir in several regions in the telencephalon, diencephalon and midbrain (Tables 1 and 2). The regions with overlap and mismatch of ^{125}I-OVTA and VT-ir are remarkably similar in

white-throated sparrow and zebra finch. In both species, ^{125}I-OVTA binding overlapped with low to moderate levels of VT-ir in the LS, TnA, TeO, DLL, DMP, ICo, and LoC, and with high levels of VT-ir in the GCT, VTA and walls of the 3v. Binding of ^{125}I-linear VP overlapped with VT-ir in the LS, Ad, TnA and TeO in sparrow and in the LS in zebra finch.

DISCUSSION

Using two radioligands, ^{125}I-OVTA and ^{125}I-linear VP, I have described the distribution of putative VT receptors throughout the brain in two model songbird species, the white-throated sparrow and the zebra finch. I found that VT-like binding, as revealed by ^{125}I-OVTA, was widely distributed in the brain in both species whereas binding of ^{125}I-linear VP was found in a limited number of brain regions in white-throated sparrow and was essentially non-detectable in the zebra finch.

Species Comparisons

White-throated sparrow and zebra finch brain exhibited similar distributions of ^{125}I-OVTA binding, with only a few exceptions. ^{125}I-OVTA binding was found in the MS, PTM and IO in sparrow but not in zebra finch. Conversely, binding was found in the medial vestibular nucleus (VeM), the plexus of Horsley (PH) and the EW in zebra finch but not in sparrow. ^{125}I-linear VP binding was found in the LS, MS, Ad and TeO in sparrow but was observed only in the LS in zebra finch. In both white-throated sparrow and zebra finch, I found ^{125}I-OVTA binding and ^{125}I-linear VP binding in the LS and Ad.

Binding in these two regions is consistent with VT-like binding reported in other songbird species using these ligands (Voorhuis et al., 1990; Goodson et al., 2006). I did not detect ^{125}I-linear VP binding in the N, lateral striatum or ventral pallidum, as reported in spice finch (Goodson et al., 2006), although I found binding in other regions of the sparrow brain using this ligand (Table 1).

I compared the distributions of ^{125}I-OVTA and ^{125}I-linear VP binding I found in this study to the distribution of ^{3}H-VP binding reported in canary (Voorhuis et al., 1988) and found that binding overlapped in some, but not all, brain regions. For example, Voorhuis et al. (1988) reported ^{3}H-VP binding in the TeO, RA and VTA. I found ^{125}I-OVTA binding in the TeO in white-throated sparrow and zebra finch, ^{125}I-OVTA and ^{125}I-linear VP binding in the RA only in sparrow, and ^{125}I-OVTA binding in the VTA only in zebra finch. I did not observe any VT-like binding in the medial posterior hypothalamic nucleus, PT or the habenula, as reported in canary.

Species differences in VT-like binding could reflect species differences in VT receptor subtype distribution, as subtypes may differ in affinity and specificity for ^{125}I-OVTA, ^{125}I-linear VP and ^{3}H-VP radioligands. Currently, there are three VT-like receptor subtypes that may be expressed in the brain. These include a V1a-like receptor, homologous to the V1a receptor in mammals, an oxytocin-like (OT-like) receptor homologous to the OT receptor in mammals, and VT1, a VT receptor that bares little homology to the known mammalian neuropeptide receptors (Tan et al., 2000; Gubrij et al., 2005; Baeyens and

Cornett, 2006). ^{125}I-OVTA, ^{125}I-linear VP and ^{3}H-VP, therefore, may bind to any combination of these receptor subtypes.

I conducted competition experiments in white-throated sparrow and zebra finch to determine whether VT or MT was more effective at competing off ^{125}I-OVTA or ^{125}I-linear VP. Increasing concentrations of VT or MT were each effective at competing off both radioligands, but neither cold competitor was more effective at competing off ^{125}I-OVTA in sparrow or zebra finch, although MT was significantly more effective at competing off ^{125}I-linear VP than VT in sparrow. Competition experiments in canary arcopallial membrane revealed that VT is more effective at competing off ^{125}I-OVTA than MT (Voorhuis et al., 1990), and a single concentration of cold VT is more effective at eliminating ^{125}I-linear VP than MT in whole sections of brain in several species of finch and waxbill (Goodson et al., 2006). Together, these findings suggest that there may be species differences in affinity or specificity of VT-like receptors for VT and MT. Competition experiments for each radioligand using brain tissue from each species are necessary for interpreting binding studies.

Given that ^{125}I-linear VP specifically labels the mammalian V1a receptor (Schmidt et al., 1991) and ^{125}I-OVTA specifically labels the mammalian OT receptor (Elands et al., 1988), it is tempting to hypothesize that ^{125}I-linear VP binds to the V1a-like receptor and ^{125}I-OVTA binds to the OT-like receptor in birds. However, the molecular characteristics of the avian VT-like receptors are not well understood. The V1a-like receptor has not been cloned in any avian species to date, although the gene and protein sequences are

available in chicken and zebra finch, and the OT-like receptor has been described only in the myometrium of the chicken shell gland (Gubrij et al., 2005). The binding specificity of V1a-like and OT-like receptor subtypes for VT and MT or VP and OT is unknown. VT1 is known to bind with equal affinity to VT and VP, with less affinity for MT and OT (Tan et al., 2000) and VT2 has been shown to bind to VT with greatest affinity, followed by VP, MT and OT, although this receptor subtype has not been detected in the avian brain (Cornett et al., 2003). Future studies using *in situ* hybridization in several species of songbird will help determine the distribution of each VT receptor subtype.

VT-like binding in the zebra finch and sparrow was found in several of the brain regions in which V1a and OT receptor binding is found in rodents, such as the LS, medial amygdala (the mammalian equivalent of the TnA), and the Hp (Insel and Shapiro, 1992; Young et al., 1997; Bester-Meredith et al., 1999; Tribollet et al., 1999; Beery et al., 2008). VT-like binding was not detected in the bed nucleus of the stria terminalis, however, a region in which both V1a and OT receptor binding are found (Insel and Shapiro, 1992; Bester-Meredith et al., 1999; Young et al., 2000). The distribution of these neuropeptides receptors seems to differ across vertebrate groups, which suggests that these receptors may modulate behavior in a species-specific manner. For instance, differences in V1a and OT receptor binding in the LS is thought to relate to differences in parental and mating behaviors, aggression, and sociality in rodents (Insel and Shapiro, 1992; Young et al., 1997; Bester-Meredith et al., 1999; Beery et al., 2008). Goodson et al. (2006) found that ^{125}I-linear VP binding in the caudal division of the LS and the MS was greater in gregarious species of songbird compared to highly territorial species and

further hypothesized that receptor systems in the LS may have evolved in relation to sociality. Given that white-throated sparrows and zebra finches live in very different social structures, I did not see striking species differences in the LS with ^{125}I-OVTA, although ^{125}I-linear VP binding in the LS was much darker in white-throated sparrows compared to zebra finches.

I did not see striking species differences in binding in any thalamic regions, the TnA, or the Hp, although I cannot say for certain without a quantitative analysis. In rodents, species differences in V1a and OT receptor binding are found in these regions (Insel and Shapiro, 1992; Young et al., 1997; Beery et al., 2008). I did see, however, a great deal of individual variation in binding (see Tables 1 and 2), as has been reported for V1a receptor binding in prairie voles (Phelps and Young, 2003; Hammock and Young, 2005). Polymorphisms in the V1a receptor promoter region are thought to cause differences in V1a binding, ultimately causing individual variation in social behavior (Hammock and Young, 2002; Hammock and Young, 2005; Insel, 2006). Like rodents, birds exhibit a great deal of intraspecific variation in social behavior, which may relate to the intraspecific variation in levels of VT-like binding observed in our study. Future comparisons of behavioral data with receptor binding will be needed to test this hypothesis in birds.

Sex comparison

I conducted a sex comparison of ^{125}I-OVTA binding in the LS in zebra finches and found a strong trend such that males had higher levels of binding than females. This finding is

similar to that reported in Goodson et al. (2006), who found sex differences in zebra finch in the septohippocampal septum, a subregion of the LS, using ^{125}I-linear VP. VT-like binding in the RA increases in response to testosterone (T) treatment (Voorhuis et al., 1988), which suggests that a sex difference in binding may be driven by sex differences in gonadal hormones. It is unclear, however, whether T increases VT-like binding in the LS. In zebra finches, plasma T is lower in males than females (Adkins-Regan et al., 1990), and therefore unlikely the direct causal mechanism of this sex difference. Circulating levels of estradiol (E2) in male zebra finches can be higher than females (Adkins-Regan et al., 1990), but it is unknown whether E2 increases VT-like binding in birds. In rodents, there is little evidence for an effect of E2 on V1a receptor binding (Kalamatianos et al., 2004; Moore et al., 2004), although E2 does increase OT receptor binding in rats (Coirini et al., 1991; Bealer et al., 2006). Experiments testing the effect of E2 on VT-like binding will be necessary to determine what is causing the sex difference in VT-like binding in zebra finch.

Overlap and Mismatch of VT-like binding and VT-ir

The role of neuropeptide receptors in modulating behavior is likely determined by the delivery and binding of corresponding neuropeptides. Overlap and mismatch of receptors and fiber distribution may provide insight into the function of receptors in a given brain region. VT-like binding and VT-ir overlapped in many brain regions in white-throated sparrow and zebra finch (see Tables 1 and 2). The overlap of receptors and fibers suggests that VT may function as a traditional fast acting neurotransmitter or neuromodulator in these regions of the brain (Iversen, 1984). Neurotransmitters may also

travel great distances through extracellular space (Schmitt, 1984), which would explain the VT-like binding and VT-ir mismatch in some brain regions; VT may be travel a greater distance to reach its target receptor. In rodents, VP-ir overlaps with V1a receptor binding in the LS and MeA (Wang et al., 1996; Young et al., 1997), the mammalian homologue of the TnA. Similarly in white-throated sparrow and zebra finch, VT-ir overlaps with ^{125}I-OVTA binding in the LS and TnA, and with ^{125}I-linear VP binding in the LS in sparrow and zebra finch. Such consistency across species suggests that the role of these neuropeptides as neurotransmitters or neuromodulators in these brain regions may be conserved across vertebrate groups.

Summary

Comparative studies of putative VT receptor distribution were undertaken to describe the brain distribution of VT-like binding in two species of model songbirds. Distribution of putative VT receptors was relatively similar in white-throated sparrow and zebra finch, although some species differences were observed. VT-like binding was found in some, but not all brain regions with V1a and OT receptor binding described in rodents and VT-like binding overlapped with VT-ir in some but not all regions in which V1a and OT receptor binding overlapped with VP-ir and OT-ir in rodents (Insel and Shapiro, 1992; Wang et al., 1996; Young et al., 1997). Future experiments are needed to determine the receptor subtypes labeled using ^{125}I-OVTA and ^{125}I-linear VP and to determine the relationship between VT-like binding and individual variation in behavior.

ACKNOWLEDGMENTS

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Abbreviations

A	arcopallium
Ad	dorsal arcopallium
APH	parahippocampal area
BSTm	bed nucleus of the stria terminalis
CcS	caudocentral septum
CS	superior central nucleus
DLL	lateral portion of the dorsolateral nucleus of the anterior thalamus
DMP	dorsomedial nucleus of the posterior thalamus
E	entopallium
EW	nucleus of Edinger-Westphal
GCt	midbrain central gray
GLV	lateral geniculate nucleus
HA	apical hyperpallium
HD	densocellular hyperpallium
Hp	hippocampus
HVC	proper name
ICo	intercollicular nucleus
IO	isthmo-optic nucleus
IPN	interpeduncular nucleus
LA	lateral anterior nucleus of the thalamus
lMAN	lateral magnocellular nucleus of the anterior nidopallium
LoC	locus coeruleus
LS	lateral septum
LSc.d	caudal division of the LS, dorsal zone
LSc.l	caudal division of the LS, lateral zone
LSc.v	caudal division of the LS, ventral zone
LSc.vl	caudal division of the LS, ventrolateral zone
LSt	lateral striatum
M	mesopallium
MLd	lateral mesencephalic nucleus
MS	medial septum
MSt	medial striatum
MV	motor nucleus of the trigeminal nerve
N	nidopallium
Nc	caudal nidopallium
OMd	dorsal nucleus of the oculomotor nerve
OMv	ventral nucleus of the oculomotor nerve
PH	plexus of Horsley
PTM	medial pretectal nucleus
PVN	paraventricular nucleus
RA	robust nucleus of arcopallium
Rt	rotund nucleus
SGP	periventricular grey and fibrous tectal layers
SH	septohippocampal septum
SN	substantia nigra

TeO	optic tectum
TnA	nucleus taeniae of the amygdala
v	ventricle
VeM	medial vestibular nucleus
VMH	ventromedial hypothalamus
VTA	ventral tegmental area

Table 1. The distribution of receptors that bind [^{125}I]-ornithine vasotocin analogue ([^{125}I]-OVTA) or [^{125}I]-linear vasopressin antagonist ([^{125}I]-LVA) is compared to the distribution of vasotocin immunoreactivity (VT-IR) in white-throated sparrow brain. Plus signs indicate relative levels of VT-like binding or VT-IR. Minus signs indicate the absence of VT-like binding or VT-IR. Slashes indicate individual variation in levels of VT-like binding or VT-IR.

Brain Region	[^{125}I]-OVTA	[^{125}I]-LVA	VT-IR
A	-/+/++	-	+
Ad	++/+++	+	-
APH	+/++	-	-
Area X	-	-	-
BSTm	-	-	+/++
CS	-/+	-	++
DLL	+/++	-	++
DMP	-/+	-	++
E	-	-	-
EW	-/+	-	-
GCt	-/+	-	+/++
GLV	+/++	-	+
HA	+/++	-	-
HD	++	-	-
Hp	-/+	-	-
HVC	+/++	-	-
ICo	-/+	-	++
IO	-/+/++	-	+
IPN	-/+	-	-
LA	+/++	-	+
lMAN	-	-	-
LoC	-/+/++	-	++
LS	++/+++	++	++/+++
LSt	-	-	-
M	+/++	-	-
MLd	-	-	-
MS	-/++/+++	+	-/+
MSt	-/+	-	+
MV	+/++	-	-
N	+/++	-	-
Nc	-/+/++	-	-
OMd	-/+	-	-
OMv	-/+	-	-
PH	-	-	-
PT	-	-	++
PTM	-/+/++	-	+
PVN	-	-	++/+++
RA	-/+/++/+++	-/+/++	+
Rt	-	-	+
SGP	-/+	-	++
SN	-	-	+/++
TeO	+	++	+/++
TnA	+/++/+++	-	+
VeM	-	-	-
VMH	-	-	+
VTA	-	-	+/++

*Plus signs indicate relative densities of VT-like binding or fibers. Minus signs indicate the absence of VT-like binding or fibers. Slashes indicate individual variation.

Table 2. The distribution of receptors that bind [^{125}I]-ornithine vasotocin analogue ([^{125}I]-OVTA) is compared to the distribution of vasotocin immunoreactivity (VT-IR) in zebra finch brain. Plus signs indicate relative levels of VT-like binding or VT-IR. Minus signs indicate the absence of VT-like binding or VT-IR. Slashes indicate individual variation in levels of VT-like binding or VT-IR.

Brain Region	[^{125}I]-OVTA	VT-IR
A	-/+/++	+
Ad	+/++/+++	-
APH	-/+/++	-
Area X	-	-
BST	-	++
CS	++	++
DLL	-/+/++	+
DMP	+/++/+++	++
E	-/+	-
EW	-	-
GCt	-/+	+
GLV	-/+	+
HA	+/++	-
HD	-/+	-
Hp	-	-
HVC	-/++	-
ICo	-/+/++	+
IO	-	++
LA	-/+	+
lMAN	-	-
LoC	+/++	+
LS	++/+++	++
LSt	-	-
M	-/+	-
MLd	-	-
MS	-/+	-
MSt	-/+	+
MV	+	-
N	+/++	-
Nc	+/++/+++	+
OMd	+	-
OMv	-/+	-
PH	+/++	+
PTM	-	+
PT	-	+
PVN	-	++
RA	-	+
Rt	-	++
SGP	-/+	+
SN	-	+
TeO	+/++	++
TnA	-/+/++	+
VeM	+	+
VMH	-	+
VTA	-/+/++	++

*Plus signs indicate relative densities of VT-like binding or fibers. Minus signs indicate the absence of VT-like binding or fibers. Slashes indicate individual variation.

Table 3. $LogIC_{50}$ values and standard errors for vasotocin (VT) and mesotocin (MT) used as cold competitors in competition binding assays. VT and MT inhibited binding of [^{125}I]-ornithine vasotocin analogue ([^{125}I]-OVTA) with essentially equal potency; MT was a significantly more potent inhibitor of [^{125}I]-linear vasopressin antagonist ([^{125}I]-LVA) than VT. The binding profile for each ligand is described by the following model: Y = 1/((1+10^(X-logIC_{50})).

Species and ligand	VT logIC_{50} (nM)	MT logIC_{50} (nM)	p-value
Sparrow, [^{125}I]-OVTA	-8.75 ± 0.10	-8.70 ± 0.09	n.s.
Sparrow, [^{125}I]-LVA	-8.24 ± 0.18	-9.40 ± 0.14	0.0045
Zebra finch, [^{125}I]-OVTA	-7.91 ± 0.12	-7.93 ± 0.10	n.s.

Figure Legends

Fig. 1. Binding of [^{125}I]-ornithine vasotocin analogue ([^{125}I]-OVTA; A, C, E, G, I, K, M, O) or [^{125}I]-linearized vasopressin antagonist ([^{125}I]-LVA; B, D, F, H, J, L, N, P) in comparable sections in the brain of a white-throated sparrow. Anterior-Posterior (AP) coordinates follow Stokes et al. (1974).

Fig. 2. Binding of [^{125}I]-ornithine vasotocin analogue ([^{125}I]-OVTA) in the brain of a zebra finch. Anterior-Posterior (AP) coordinates follow Nixdorf-Bergweiler and Bischof (2007).

Fig. 3. Binding of [^{125}I]-ornithine vasotocin analogue ([^{125}I]-OVTA) in the lateral septum (LS) of male and female zebra finches. Males tended to have higher levels of binding than females. Although this difference was not statistically significant ($p = .089$), the effect size was large ($d = 1.19$).

Fig. 4. Inhibition curves showing dose-dependent displacement of (A) [^{125}I]-ornithine vasotocin analogue ([^{125}I]-OVTA) in white-throated sparrow, (B) [^{125}I]-linearized vasopressin antagonist ([^{125}I]-LVA) in white-throated sparrow and (C) [^{125}I]-OVTA in zebra finch by unlabeled neuropeptides vasotocin (VT) and mesotocin (MT). In each graph, the data points at the lowest plotted concentration represent binding in the absence of cold competitor. Log IC_{50} values are given in Table 3.

Fig. 5. The distribution of cells and fibers immunoreactive for vasotocin (A, C, E) compared to the distribution of binding of [^{125}I]-ornithine vasotocin analogue (B, D, F) in the lateral septum. The midline is visible on the left side of each photo. Nomenclature for subdivisions of the LS follow Goodson et al. (2004b).

Figure 1.

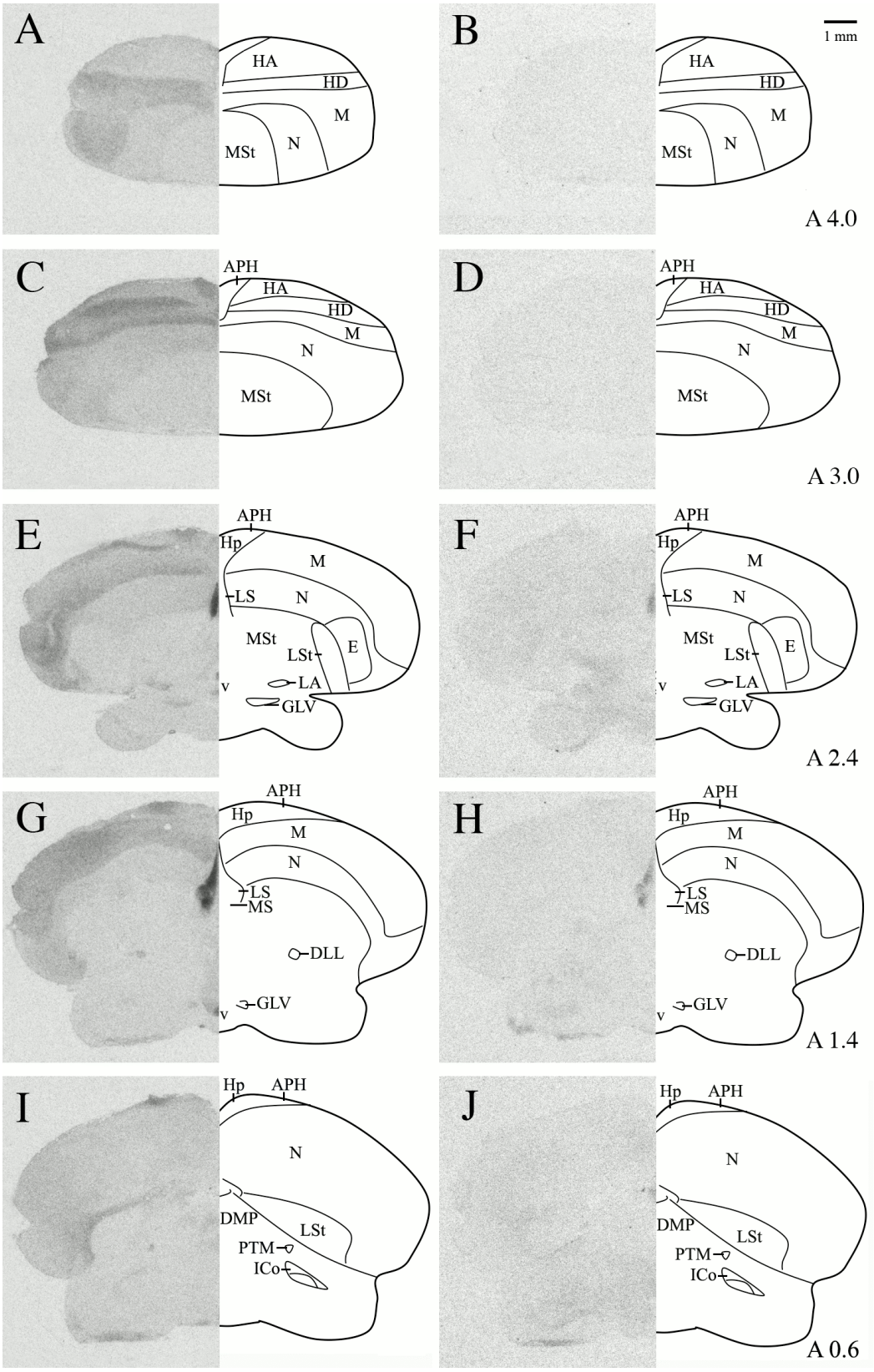
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Figure 1, cont.

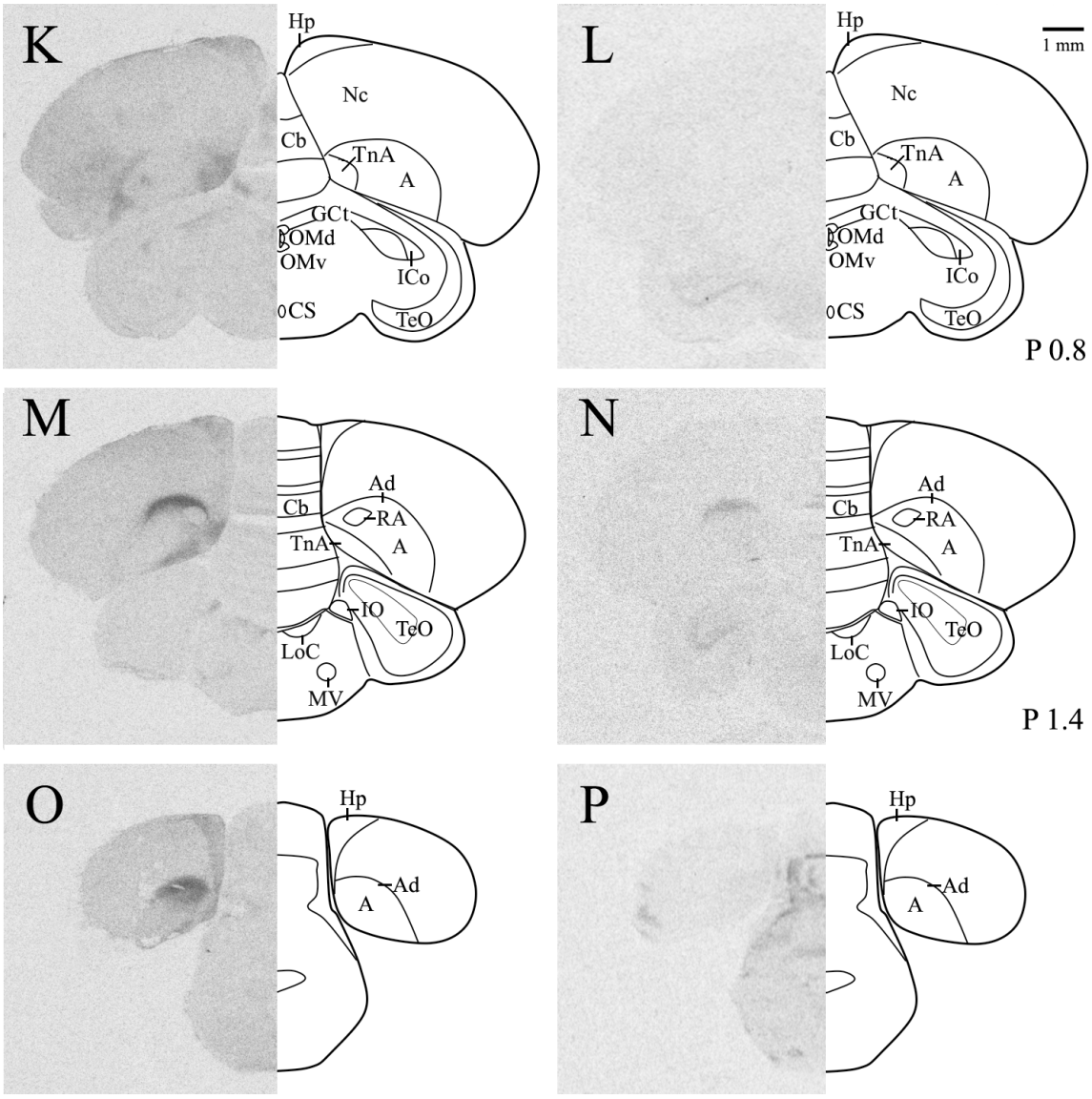
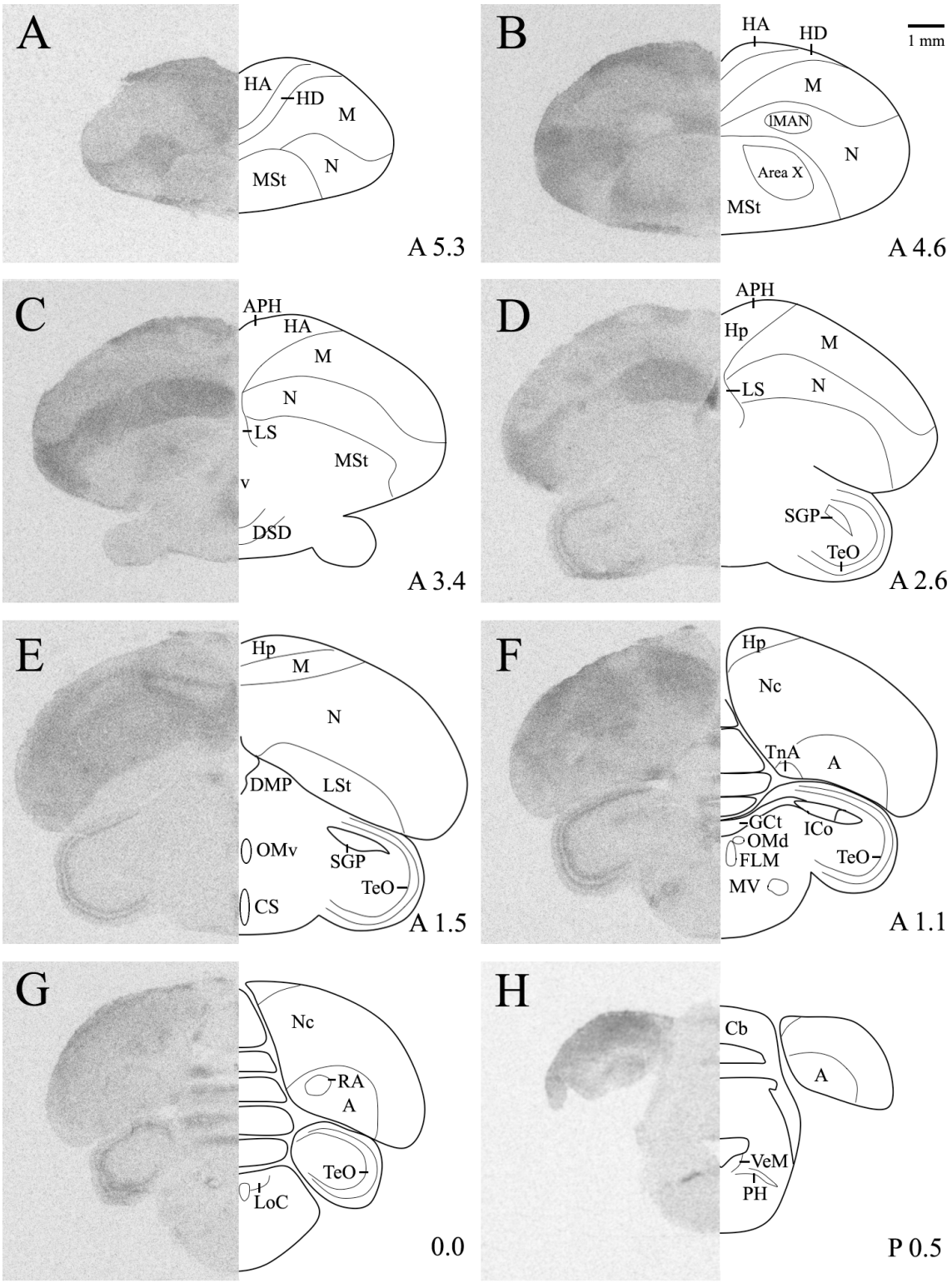


Figure 2.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TeO
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Figure 3.

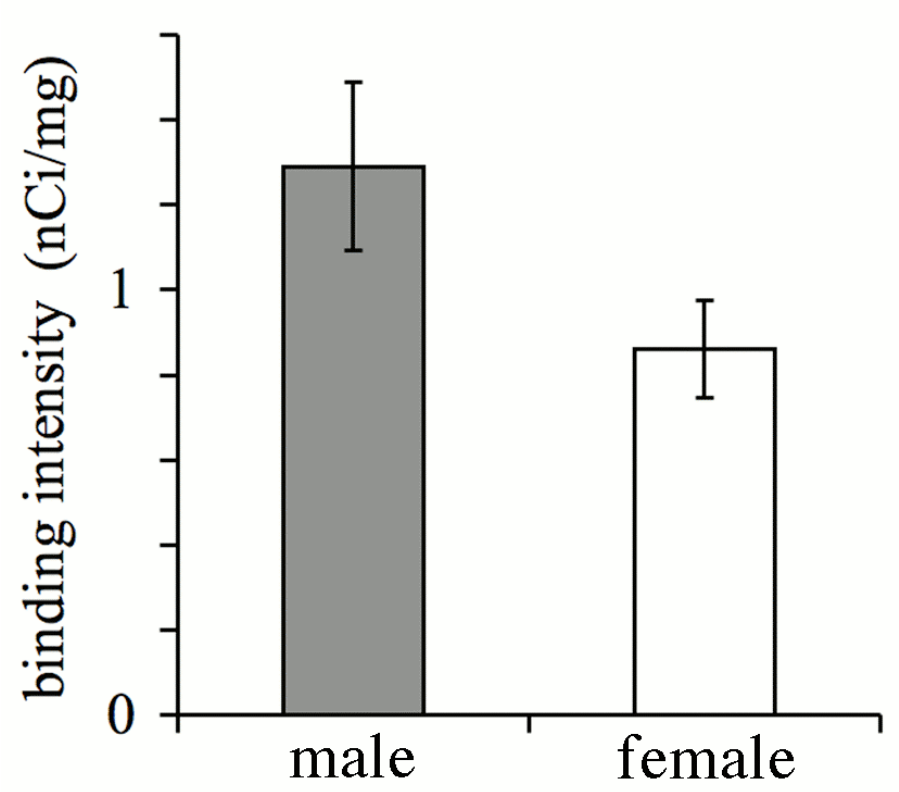


Figure 4.

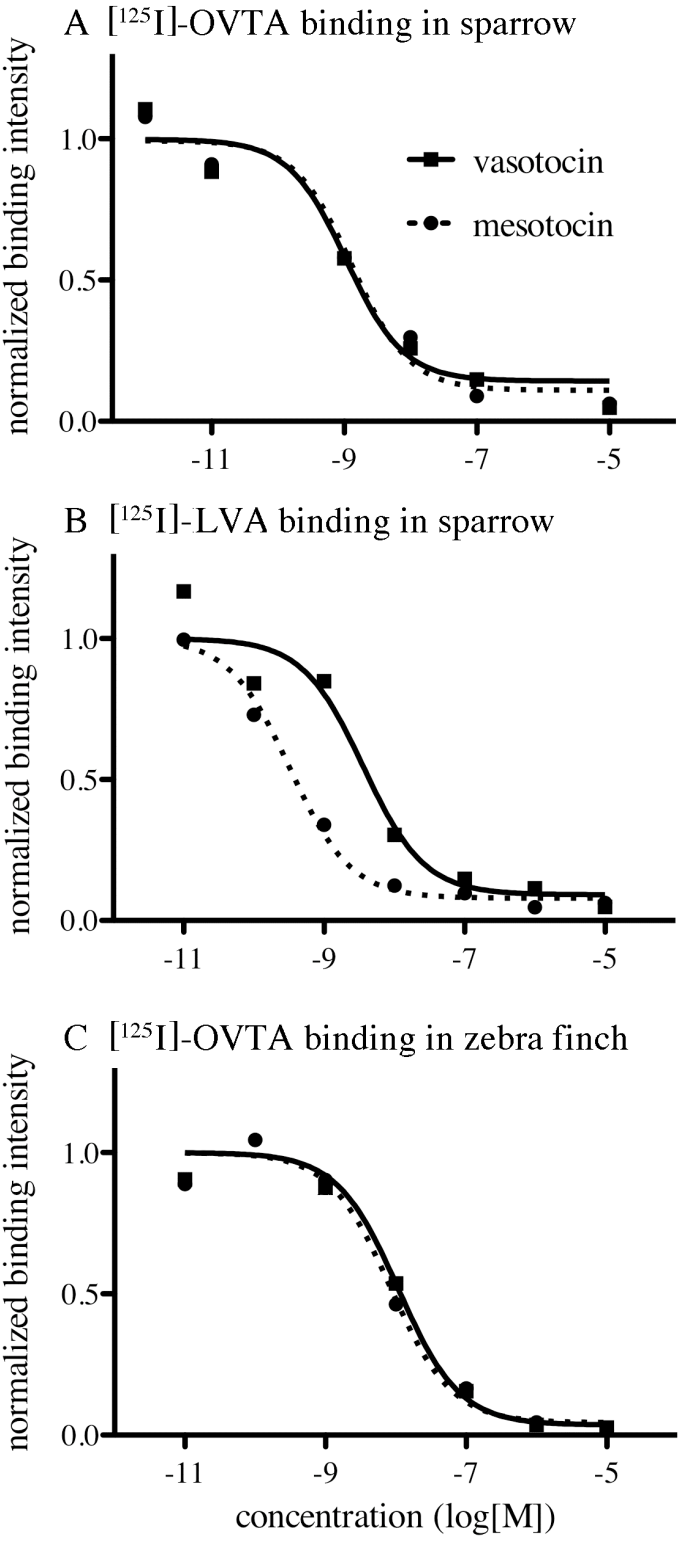
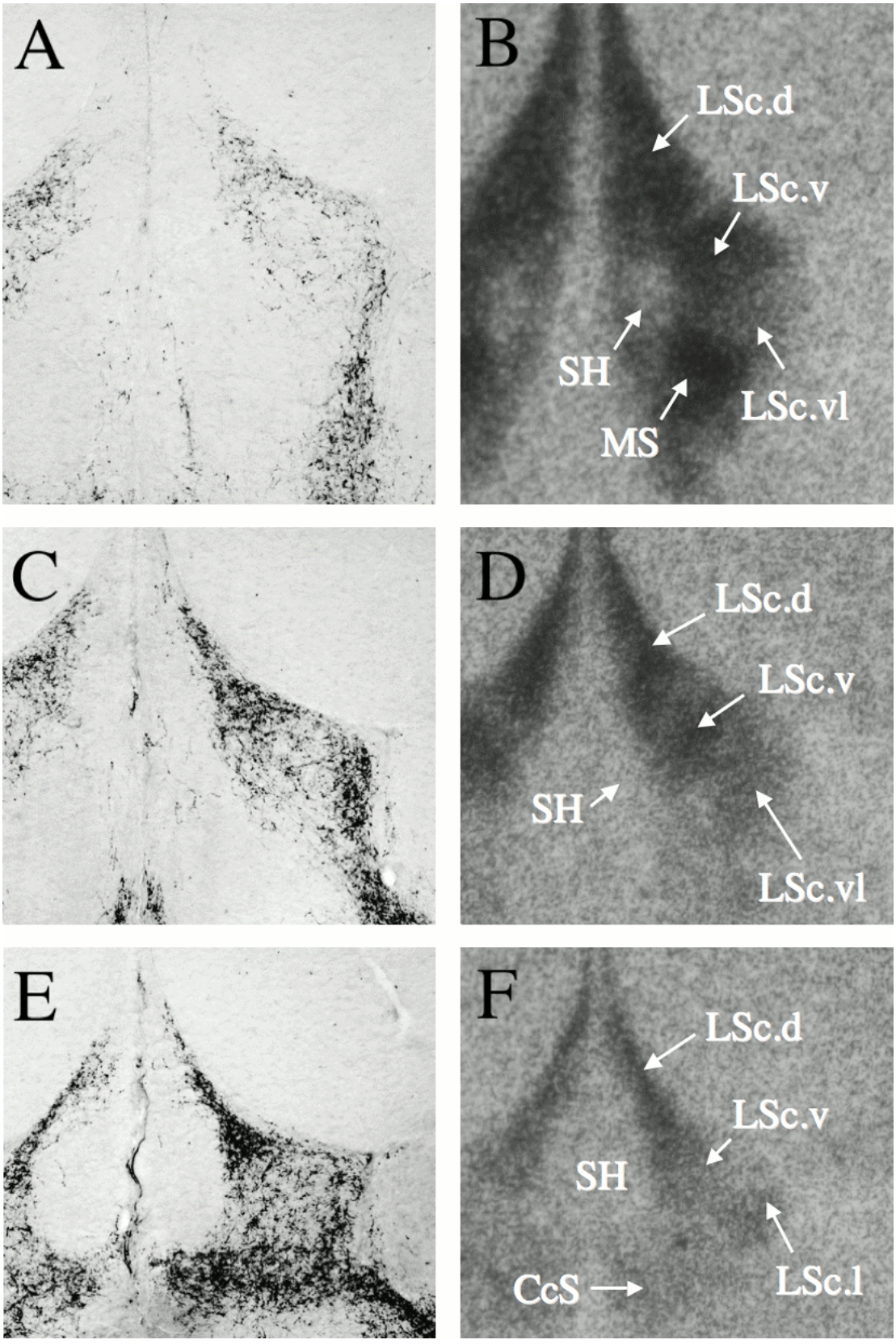

A [125I]-OVTA binding in sparrow
normalized binding intensity
1.0
0.5
0.0
-11
-9
-7
-5
vasotocin
mesotocin
B [125I]-LVA binding in sparrow
normalized binding intensity
1.0
0.5
0.0
-11
-9
-7
-5
C [125I]-OVTA binding in zebra finch
normalized binding intensity
1.0
0.5
0.0
-11
-9
-7
-5
concentration (log[M])

Figure 5.


A
B
LSc.d
LSc.v
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MS
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C
D
LSc.d
LSc.v
SH
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LSc.d
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Chapter Two:

Neural Distribution of Nonapeptide Receptor mRNA in Two Species of Songbird

Abstract

Vasotocin (VT) and its mammalian homologue (VP) are involved in social behaviors in across vertebrate groups. In birds, the behavioral effects of VT are likely mediated by more than one VT receptor subtype. I previously mapped the neural distribution of VT receptors in two species of songbird, the white-throated sparrow (*Zonotrichia albicollis*) and zebra finch (*Taeniopygia guttata*) with the use of two radioligands. Because multiple receptor subtypes are likely expressed in the avian brain, I hypothesize that VT-like binding represents several VT receptor subtypes. Here in this study, I used species-specific riboprobes to show the neural distribution of three VT receptor subtypes, VT1, VT3 and VT4 in white-throated sparrow and zebra finch. I found that there were similarities in distribution between species, and that the expression of each receptor subtype overlapped with one another in several brain regions. In sparrow, VT3 and VT4 were widely distributed in the brain, whereas VT1 distribution was more limited. In zebra finch, all three receptors were widely distributed in the brain. Based on the predicted amino acid sequences for these receptors, I believe that VT1 and VT4 may bind with greater affinity to VT than MT, and VT3 may bind with greater affinity to MT than VT. Because testosterone (T) is known to modulate VT-dependent behaviors, we looked for an effect of T on VT receptor expression. Although I found an effect of T on song, I did not see any effects of T on VT receptor expression and I did not see a relationship between behavior and receptor expression in sparrow. Furthermore, I did not find any sex differences in VT receptor expression in the brain regions of interest in either species.

Vasotocin (VT) and its mammalian homologue, vasopressin (VP), have been implicated in social behaviors across vertebrate groups. For example, these neuropeptides have been shown to affect vocalizations, aggression, sexual behavior, parental behavior and pair-bonding behavior in fish, anurans, birds and rodents (see Goodson and Bass, 2001; Caldwell et al., 2008). VT and VP are produced mainly in the bed nucleus of the stria terminalis (BSTm), the paraventricular nucleus (PVN) and the medial preoptic area (POA), and their fibers project to many other regions of the brain (De Vries et al., 1985; Kiss et al., 1987; Voorhuis and de Kloet, 1992). In rodents, the central actions of VP are mediated by a G protein coupled receptor, V1a, which shows remarkable variability in distribution between closely related species of rodent (Insel et al., 1994; Young et al., 1997; Bester-Meredith et al., 1999; Beery et al., 2008). For example, the neural distribution of V1a receptor binding and V1a receptor mRNA differs between monogamous prairie voles (*Microtus ochrogaster*) and non-monogamous montane voles (*Microtus montanus*; Young et al., 1997). In songbirds, patterns of VT receptor binding in the lateral septum (LS), a region implicated in territorial behavior (Goodson et al., 1999), differ between colonial and territorial species of *Estrildid* finches (Goodson et al., 2006; 2009). Comparative studies examining species-specific VP and VT receptor distributions and how they relate to social behavior can help us better understand the evolution of social behavior across vertebrate species.

There are four known VT receptor subtypes in birds. VT1, the first nonapeptide receptor discovered in birds, is found in the avian brain (Tan et al., 2000). VT1 is not closely homologous to any known mammalian nonapeptide receptors, although it is somewhat

similar in structure to the V1a and V1b receptors in mammals (Baeyens and Cornett, 2006) and shares binding properties with the mammalian V1a receptor (Acharjee et al., 2004a). VT2, which is homologous to the V1b receptor in mammals, is found in the pituitary but not in brain tissue (Cornett et al., 2003). VT3 bears the greatest similarity in structure to the mammalian OT receptor and shares some homology with non-mammalian MT receptors (Gubrij et al., 2005), and its mRNA has been detected in songbird brain (Leung, unpublished). Analyses of the chicken and zebra finch genome demonstrate evidence for a fourth nonapeptide receptor, VT4, which is thought to be the avian equivalent of the mammalian V1a receptor (Cornett, Jacobi and Mikhailova, unpublished), and I have detected the mRNA for this gene in songbird brain (Leung, unpublished). Detailed radioligand and cold ligand competition studies have not yet been conducted for most of these receptors and it is unclear which of them is mediating the behavioral effects of VT.

I recently mapped the distribution of VT receptors in white-throated sparrows (*Zonotrichia albicollis*) and zebra finches (*Taeniopygia guttata*) with the use of two radioligands, an iodinated VT analog [^{125}I]OVTA and iodinated V1a antagonist [^{125}I]LVA (see chapter 1). [^{125}I]OVTA labeled receptors throughout the telencephalon, diencephalon, midbrain and brainstem, with a similar distribution in both species. [^{125}I]LVA binding, in contrast, was limited to the LS, dorsal arcopallium (Ad) and the optic tectum (TeO) in white-throated sparrow, and was essentially non-detectable in zebra finch. Because multiple receptor subtypes are likely expressed in the avian brain, I hypothesize that the distribution patterns of receptor binding reported in chapter one

represent a heterogenous group of VT receptors. To address this issue, I used *in situ* hybridization to describe the neural distribution of VT1, VT3 and VT4 in zebra finch and white-throated sparrow. I selected these particular VT receptors because they are expressed in the avian brain (Tan et al., 2000; Leung, unpublished). I chose the zebra finch because it has been a model system in neuroscience for decades and has become important for studies in behavioral genetics and genomics with the completion of the zebra finch genome. *Zonotrichia* sparrows such as the white-throated sparrow have long been ideal systems in which to study photoperiodicity and endocrinology, and have recently become a model for studying the genetics of social behavior (Maney, 2008; Thomas et al., 2008). Because testosterone (T) is known to upregulate VT receptors (Voorhuis et al., 1990) and T is known to affect VT-dependent behaviors such as song in seasonally-breeding songbirds, I looked for an effect of T on the expression of VT1, VT3 and VT4 and tested for a correlation between VT receptor expression and behavior in male white-throated sparrows.

MATERIALS AND METHODS

Animals

All research was conducted in accordance with NIH principles of animal care, federal and state laws and university guidelines. Twenty-three white-throated sparrows (males: N = 15; females: N = 8) were captured in mist nets during fall migration in Atlanta, Georgia and sex was determined by PCR analysis (Griffiths et al., 1998). Birds were housed in individual cages (38 x 38 x 42 cm) and placed inside large walk-in sound-attenuated booths (Industrial Acoustics, Bronx, NY). Twelve zebra finches (males: N = 6; females:

N = 6) were obtained from a laboratory breeding colony at Georgia State University, where they were housed in mixed-sex groups on long photoperiod (14L:10D) for at least 6 months. Sex was determined by plumage characteristics.

Hormone Manipulation in Sparrows

Each white-throated sparrow received one subcutaneous silastic implant (length 12mm, inner diameter 1.47 mm, outside diameter 1.96 mm, Dow Corning, Midland, MI) sealed at both ends with silicone adhesive (brand name). Eight males and eight females received an empty implant (C) and eight males received an implant filled with testosterone (T; Steraloids, Newport, RI). The dose of T was based on a previous study conducted in this genus (Tramontin et al., 2003). Birds were then visually isolated from each other for 4 weeks on short photoperiod (9L:15D).

Behavioral Testing in Sparrows

On the morning prior to the day of sacrifice, each bird was relocated into a sound attenuation booth equipped with a microphone and video camera. Each T treated male was placed in a booth with a C treated male, and each C treated female was placed in a booth with another C treated female. All birds remained individually housed and were visually isolated from their neighbor at all times. On the following morning at lights on, video and audio recordings were made of each bird for 120 min. Behaviors scored included number of songs, tseets and bouts of preening. Behavior were scored as follows: Song consists of pure whistled notes in a specific pattern without detectable harmonics (Falls and Kopachena, 1994); Tseets begin with a downward slur and have a narrow

frequency range (Falls and Kopachena, 1994); A bout of preening was scored as a period of preening in which the bird did not lift its head or stop preening.

Tissue

All birds were anesthetized with isoflurane in pairs to counterbalance for treatment and sex within 20 min after the behavioral recording (sparrows) or removal from colony (zebra finches). I collected a blood sample from the jugular vein for radioimmunoassay (RIA) and then rapidly decapitated each bird. Brains were immediately removed, frozen on dry ice, and stored at -80°C until sectioning. Seven sets of brain sections were cut in the coronal plane at 20 μm on a cryostat and thaw-mounted on Superfrost plus slides (Fisher, Pittsburgh, PA). The sections were then stored at -80°C until use for *in situ* hybridization.

Testosterone Assay

Plasma T was assayed at the Endocrine Core Lab in Yerkes Primate Research Center using a commercially available RIA kit from Diagnostic Systems Laboratories (DSL-4000, Webster, TX). Normal assay range for this kit is 0.05-28ng/ml per 50ul. The inter-assay coefficients of variation were 5.95% at 0.68ng/ml and 4.14% at 5.67ng/ml and the intra-assay coefficient of variation was 6.3% (n = 6 repetitions).

Cloning and In Situ Hybridization

Total RNA was extracted from sparrow and zebra finch brain using a RNEasy minikit (Qiagen, Valencia, CA) and reverse-transcribed into single strand cDNA using

SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). Approximately 2ug of cDNA was used for each polymerase chain reaction (PCR). The primers used for VT1 amplication were 5' primer ATGTGTTCATGCTTCACCTCAG and 3' primer ATCCTCAGGGTCTCTGTCTACG. The primers used for VT3 amplication were 5' primer ACATCACCTTCAGGTTCTACGG and 3' primer ATGTAGATCCACGGGTTACAGC, and the primers used for VT4 amplication were 5' primer GCTCGTACGGGATGATCG and 3' primer GGGAAGCTCTGTAGGCAGTC. Amplication was performed in an Eppendorf Mastercycle Gradient PCR machine (Westbury, NY), with the following parameters: 94°C melting for 30 sec, 58°C annealing for 30 sec, and 72°C extension for 40 cycles. The resulting fragments (863bp for VT1, 680bp for VT3 and 534bp for VT4) were cloned into pCRII vectors using the TOPO TA Cloning kit (Invitrogen) and sequenced by Retrogen (San Diego, CA). Plasmids containing the inserts were grown in One Shot Mach 1-T1 Chemically competent E. coli bacteria (Invitrogen), isolated with the QIAprep Spin Miniprep kit (Qiagen) and linearized using either BamHIII or NotI for generating antisense or sense probes.

Antisense cRNA probes were synthesized using a T7/SP6 Riboprobe In Vitro Transcription Kit (Promega, Madison, WI) incorporating ^{35}S-UTP (PerkinElmer, Waltham, Massachusetts). The transcription reaction contained 120ng of linearized cDNA template, 2.5ul of 5X transcription buffer, 1.25ul of 100 mM dithiothreitol (DTT, Fisher), 1.25ul each of 2.5mM ATP, CTP and GTP, 3.5ul of ^{35}S-UTP (PerkinElmer), 0.5ul of RNAsin, and 1ul of T7 or SP6 RNA polymerase. The reaction was allowed to proceed for 45 minutes at 37°C. 0.5ul of DNase RQ1 was then added to the mixture and

the reaction proceeded for 15 minutes at 37°C. 3ul of transfer RNA (10mg/ml tRNA, Roche Applied Science, Indianapolis, IN) was added to the mixture and the final reaction volume was brought up to 50ul with RNase-free water. The probes were then purified using Illustra ProbeQuant G-50 Micro Columns (GE Healthcare, Piscataway, NJ), and stored at -80°C until the day of use.

At the time of the *in situ* assay, slides were defrosted and dried with cold air from a conventional blow dryer. Slides were fixed in 4% paraformaldehyde (pH 7.4) at room temperature for 5 min and rinsed twice for 5 min in 1X PBS. After a dip in ddH20, slides were washed in 0.1M triethanolamine/.25% acetic anhydride for 10 min, rinsed in 2x SSC for 3 min, then dehydrated for 3 min in 70% ethanol (ETOH), 3 min in 95% ETOH, and 3 min in 100% ETOH, delipidated for 3 min in chloroform, then rehydrated for 3 min in 100% ETOH and 3 min in 95% ETOH.

Riboprobes were heat denatured at 80°C for 3 min, placed on ice for 5 min, and diluted to a final concentration of 1 x 10^5 cpm/ml in hybridization buffer (50% deionized formamide, 20% dextran sulfate, 20% TE buffer (10mM Tris, pH 8, 1 mM EDTA, pH 8), 10 mM Tris, pH 8.0, 10 mM dithiothreitol (DTT), 0.5M NaCl, 1 mM EDTA, pH 8.0, 3mg tRNA, and 1X Denhardt's solution). Hybridization buffer was then applied to the sections and hybridized overnight at 51-55°C depending on the GC content of each probe.

The following day, sections were washed twice for 15 min in 4X SSC with 2mM DTT, then incubated in RNase A (.03mg/ml, Roche Applied Science) diluted in 0.5M NaCl, 10 mM Tris-HCl, pH 8.0, and 1mM EDTA (RNase buffer) for 30 min at 37°C, and washed with RNase buffer with 1mM DTT for 30 min at 37°C. Slides were then washed for 30 min at room temperature in 2X SSC with 1mM DTT, followed by a 30 min wash in .1XSSC with 1mM DTT at 55°C in a shaking hot water bath, and a 3 min wash in .1XSSC (without DTT). Sections were then dehydrated in 50% ETOH with 300mM ammonium acetate (NH_4Ac), 85% ETOH with NH_4Ac, 100% ETOH (no NH_4Ac), and air-dried passively in the hood. Kodak BioMax maximum-resolution film (Rochester, NY) was exposed to slides for 9 days. Film was then developed in a Konica SRX101-A developer.

After exposure to film, slides were stained with toluidine blue (Fisher) according to the protocol adapted from Charlier et al. (2006).

Effects of T treatment

I looked for an effect of T on VT receptor expression in regions that had the highest levels of expression and areas in which VT receptors are known to be upregulated by T (Voorhuis et al., 1990). In sparrow brain, I quantified VT1 expression in the nucleus pretectalis (Pt), VT3 expression in the LS, medial portion of the ventromedial hypothalamus (VMH-med) and the arcopallium (A), which contains the robust nucleus of the arcopallium (RA), and VT4 expression in the nucleus taeniae of the amygdala (TnA), ventral tegmental area (VTA) and the lateral portion of the VMH (VMH-lat).

Sex comparison in sparrow and zebra finch

I previously reported a trend for a sex difference in the intensity of [^{125}I]OVTA binding in the septum of zebra finch (see chapter 1). Because levels of VT3 expression were very high in the septum for both sparrow and zebra finch, I looked for a sex difference in VT3 mRNA in this region for both species.

Quantification

Films were scanned at 1200 dpi using a Canon scanner (Canoscan 8400F) and Image J 1.41o (National Institutes of Health) was used to trace each region of interest in order to measure levels of expression (Burbridge et al., 2008). Specific optical density (OD) was quantified bilaterally in three coronal sections containing the region of interest. Background OD was quantified in an area adjacent to the region of interest that contained no obvious receptor expression. After background OD was subtracted from specific OD for each section, I averaged the values across the three sections (Jones et al., 2007). Higher levels of expression result in a lower optical density (black = 0, white = 256), so the absolute value was used in the statistical analysis.

Statistical analysis

All statistical analyses were conducted using SPSS 17.0 for Mac OS (SPSS Inc., Chicago). Because *in situ* hybridization was conducted in separate runs for each receptor subtype, I could not compare expression among the subtypes. I therefore analyzed the data for each subtype separately. To assess the effects of hormone treatment and sex on

mRNA expression, I conducted mixed-factorial ANOVAs with region of interest as a within-subjects factor and either hormone or sex as a between-subjects factor. Where significant main effects or interactions were found, pairwise t-tests were conducted to test for effects in each region of interest. In the behavioral study conducted on sparrows, Mann-Whitney U tests were conducted to look an effect of T on songs, tseets or preening. These tests were followed by the sequential Bonferroni correction for multiple tests (Rice, 1989). To assess the relationship between VT receptor expression and song, tseets or preening, I tested for a significant correlation between each behavior and each region of interest for each receptor subtype using Spearman's rho. I then conducted the sequential Bonferroni correction for multiple tests (Rice, 1989). The effects of T-treatment on plasma T in male white-throated sparrows were assessed using a t-test.

Photomicrograph production

I created TIFF photomicrographs for figures using a Spot camera and Spot Basic Imaging Software (version 4.6; Diagnostic Instruments Inc., Sterling Heights, MI). Figures were assembled in Gimp ver 2.6.6.

Amino acid sequence alignment

I aligned the partial amino acid sequences of white-throated sparrow and zebra finch VT1, VT3 and VT4 with the published sequences for bullfrog (*Rana catesbeiana*) VT1 and MT receptors (Accession numbers: AY277925 and AY277924; Acharjee et al., 2004b), chicken (*Gallus gallus*) VT1, VT3 and VT4 receptors (Accession numbers: AF147743, Tan et al., 2000, NM_001031569, Gubrij et al., 2005, and EU124684,

Cornett, Jacobi and Mikhailova, unpublished), Norway rat (*Rattus norvegicus*) V1a and OT receptors (Accession numbers: NM_053019, Morel et al., 1992; NM_012871, Rozen et al., 1995) and house mouse (*Mus musculus*) V1a and OT receptors (Accession numbers: NM_016847, Kikuchi et al., 1999, NM_001081147, Kubota et al., 1996). Amino acid residues were numbered according to their position on the receptor and the amino acid numbers for the individual amino acids were assigned based on the standard numbering system proposed by Ballesteros and Weinstein (1995). The transmembrane domains (TMDs) and extracellular loop (ECL) were determined based on the putative TMDs and ECL in Acharjee et al. (2004b).

RESULTS

Distribution of VT receptor mRNA

A complete list of brain regions in which I detected VT receptor mRNA can be found in Tables 1 and 2. Individual variation was observed in most regions. The following sections describe the distribution of mRNA for each VT receptor subtype in each species. In order to show examples of each receptor subtype within the same individual, I used sequential sections from one male sparrow in Fig. 1 and one male zebra finch in Fig. 2. Because there was a great deal of individual variation, I included the range of variation in Tables 1 and 2 from all birds in the study.

Distribution of VT1 receptor mRNA in white-throated sparrow

Within the telencephalon, VT1 was detected in the LS and septohippocampal septum (SH; see Table 1) and low to moderate levels of VT1 were seen in the medial magnocellular nucleus of anterior nidopallium (MMAN; see Fig. 1A arrow) and nidopallium (N; see Fig. 1P upper arrow). VT1 expression was highest in the nucleus pretectalis (Pt; See Fig. 1J arrow), and as seen in Table 1, low or moderate in the mediobasal hypothalamus (MBH), lateral anterior nucleus of the thalamus (LA), and ventral pallidum (VP). Low to moderate levels of VT1 expression were found in the midbrain central gray (GCt; Fig. 1S arrow) and low levels of VT1 expression were found laterally in the substantia nigra pars compacta (SNc) (Fig. 1P arrow).

Distribution of VT3 receptor mRNA in white-throated sparrow

VT3 receptor mRNA was the most widely distributed of the three receptor subtypes examined. The most robust signal detected in the telencephalon was in the LS (Fig. 1E,H), MS (Fig. 1H), and Ad (Fig. 1W). Low to moderate levels were found in the N and M (Fig. 1E,H), and levels found in the MSt (Fig. 1B), Hp (Fig. 1K), and TnA (Fig. 1T) were low or non-detectable. In the diencephalon, the GLV and DMP had VT3 expression ranging from low to high levels (Fig. 1E,Q). Moderate to high levels of expression were found in the MBH (Fig. 1K) and Hb (Fig. 1N), whereas low to moderate levels were found in the DLL (Fig. 1E), and low levels were detected in the Pt (Fig. 1K), VMH-lat, VMH-med, PVN and POM (Fig. 1H). High levels of VT3 expression were detected in the OMd and OMv (Fig. 1T; Table 1), whereas low levels were seen in the TeO and low

levels were found in the GCt, ICo and MLd (Fig. 1T). High levels of expression were seen in the trochlear nucleus (nIV) and the motor nucleus of the trigeminal nerve (MV). Low to moderate levels of expression were seen in the locus coeruleus (LoC, Fig. 1W) and superior central nucleus (CS, Fig. 1T). Low levels were detected in the IPN (Fig. 1O).

Distribution of VT4 receptor mRNA in white-throated sparrow

VT4 expression was limited to the LMAN, MMAN, BSTm and Ad in the telencephalon (see Table 1). Levels of expression in the LMAN and MMAN were very high (Fig. 1C) whereas levels in the Ad were low or non-detectable (see Table 1). Expression levels in the BSTm ranged from nondetectable to moderate (see Table 1). The highest levels of VT4 were in the FPL (Fig. 1F), MBH (Fig. 1L), DMP (Fig. 1R), and TeO (Fig. 1I,L). Moderate to high levels of expression were found in the GLV (Fig. 1F). VT4 expression was detected in a region that may overlap the nucleus of the dorsal lateral lemniscus (LLd) and semilunar nucleus (SLu; Fig. 1O, R). Moderate levels were seen in the MV and low to moderate levels were seen in the SNc (Fig 1O,R) and EW (Fig. 1U). Expression was also detected in the VTA (Fig. 1O top arrow) and IPN (Fig. 1O bottom arrow).

Distribution of VT1 receptor mRNA in zebra finch

Like in sparrow, VT1 expression was greatest in the Pt (Fig. 2J). Within the telencephalon, moderate levels were detected in the INP (Fig 2G) and low to high levels in the MBH (Fig. 2J). VT1 was detected at low to moderate levels in the M (Fig 2A,D), at

low levels in the HA (Fig. 2A) and POM (Fig 2D), and at low or nondetectable levels in the N (Fig 2A,D), MS and SH (Fig. 2J). A region in the dorsal thalamus, which may overlap the dorsal intermediate ventral anterior nucleus (DIVA), had moderate levels of VT1 expression (Fig. 1M).

Distribution of VT3 receptor mRNA in zebra finch

Within the telencephalon, VT3 expression was highest in the LS and N (Fig. 2H,K). Within the diencephalon, high levels of expression were detected in the OMv and OMd (not shown in figures). VT3 expression was also detected in the POM and several brainstem nuclei (see Table 2).

Distribution of VT4 receptor mRNA in zebra finch

VT4 expression was expressed at low or moderate levels in several regions, including the VMH-lat, VMH-med (Fig. 2I), MBH (Fig. 2L), DMP, SNc (Fig. 2O), TeO (Fig. 2I,L) and Nc. Low levels were detected in the LS and POM.

Testosterone assay in male sparrows

Plasma T levels were significantly higher in T-treated males than in blank-implanted males ($t(13) = 2.73$, $p = .017$; Cohen's $d = 1.52$; T males: 18.13 ± 5.94 ng/mL, C males: 0.68 ± 0.34 ng/mL). Testes were fully regressed in all males.

Behavior in sparrows

Behaviors exhibited during the two hour recording are plotted in Fig. 3A-C. Mann-Whitney U tests followed by the sequential Bonferroni correction for multiple tests revealed that T treated males produced more songs than C treated males (Mann-Whitney U Test: U = 7.0, N_1=8 T treated males, N_2 = 7 C treated males, P = .006, α = .01, Cliff's d = .75), but tseets and bouts of preening did not differ between the two groups (tseets Mann-Whitney U Test: U = 11.0, N_1=8 T treated males, N_2 = 7 C treated males, P = .045, α = .025, Cliff's d = .61; bouts of preening Mann-Whitney U Test: U = 10.5, N_1=8 T treated males, N_2 = 7 C treated males, P = .042, α = .025, Cliff's d = .63). Note that the effect sizes for tseets and bouts of preening were both medium with T males producing more tseets than C males, and C males preening more frequently than T males (see Fig. 3B-C).

T effects on VT receptor mRNA

I looked for an effect of T in brain regions that exhibited high levels of VT receptor mRNA, as well as in the VTA and RA, which are regions known to have VT receptors with sensitivity to T. I therefore looked for an effect of T on VT1 receptor expression in the Pt, which had the highest intensity of signal in the brain for this VT receptor subtype. An independent t test revealed no effect of treatment on VT1 expression in the Pt (T males: M $\pm$ SE = 84.20 $\pm$ 9.05 optical density (OD); C males: M $\pm$ SE = 89.52 $\pm$ 4.25 OD, t(13) = -.507, P = .621, Cohen's d = .28). I also looked for an effect of T on VT3 expression in the septum, the VMH-med and the arcopallium, which includes RA. These regions had the highest levels of expression for VT3. A mixed factorial ANOVA with

brain region as the within-subjects factor (LS, VMH-med, and A) and hormone treatment as the between-subjects factor revealed a main effect of brain region $F(2,26) = 22.24$, $P < .001$, but the main effect of treatment was not significant $F(1,13) = .108$, $P = .747$. The interaction between brain region and treatment was not significant, $F(2, 26) = .157$, $P = .855$, so no further statistical tests were conducted. Finally, I looked for an effect of T on VT4 expression in the VTA, TnA and the VMH-lat, which had the highest levels of expression for this subtype. A mixed factorial ANOVA with brain region as the within-subjects factor (VTA, TnA and VMH-lat) and hormone treatment as the between-subjects factor revealed a main effect of brain region $F(2,26) = 76.28$, $P < .001$ but no main effect of treatment [$F(1,13) = .099$, $P = .758$)] or interaction between brain region and treatment $F(2, 26) = .733$, $P = .653$. No further statistics, therefore, were conducted. Although I was predisposed to Type I errors by conducting separate analyses for each subtype, note that I did not find any effects of treatment.

Behavior and receptor expression in sparrows

Spearman correlations followed by the sequential Bonferroni correction for multiple tests were conducted to test for a relationship between receptor expression and songs, tseets or bouts of preening in each brain region of interest. No significant correlations, however, were found between expression and behavior (see Table 3).

Sex differences in sparrows

I looked for an effect of sex in the same brain regions as those examined in the analysis for the effects of T. An independent t test revealed no effect of sex on VT1 expression in

the Pt (males: M ± SE = 89.52 ± 4.25 OD; females: M ± SE = 85.10 ± 4.25 OD, t(13) = .569, *P* = .579, Cohen's d = .34). For VT3, a mixed factorial ANOVA with brain region as the within-subjects factor (LS, VMH-med, and A) and sex as the between-subjects factor revealed a main effect of brain region $F(2,26) = 22.24$, $p < .001$ but no main effect of sex $F(1,13) = .558$, $P = .468$. The interaction between brain region and sex was not significant, $F(2, 26) = .157$, $p = .855$, so no further statistical tests were conducted. For VT4, a mixed factorial ANOVA with brain region as the within-subjects factor (VTA, TnA and VMH-lat) and sex as the between-subjects factor revealed a main effect of brain region $F(2,26) = 44.934$, $p < .001$ but no main effect of sex $F(1,13) = .344$, $P = .567$. The interaction between brain region and sex was not significant, $F(2, 26) = .602$, $p = .555$, so no further statistical tests were conducted. Although I was predisposed to Type I errors by conducting separate analyses for each subtype, note that I did not find any effects of sex.

Sex differences in zebra finch septum

Because I previously reported a trend for a sex difference in [^{125}I]OVTA binding in zebra finches in the septum (see chapter 1), I looked for a sex difference in VT3, which had high levels of expression in this region. Because expression for VT1 and VT4 was low or non-detectable in the septum, I did not look for a sex difference for these subtypes in this region (see Table 2). An independent t test revealed no effect of sex on VT3 expression in the septum (males: M ± SE = 12.24 ± 2.20 OD; females: M ± SE = 12.28 ± 2.37 OD, t(10) = -.015, *P* = .99, Cohen's d = .10).

Amino acid sequence alignment

Because I generated partial nucleotide sequences for the sparrow and zebra finch VT1, VT3 and VT4 receptors, I was able to deduce the corresponding amino acid sequences and compare these to homologous sequences in other vertebrate species (see Fig. 4). I found that VT4 in both sparrow and finch contains a polar residue $Asp^{7.29}$ in the critical position in extracellular loop 3 (ECL3) that determines ligand selectivity for VT in the bullfrog (Cho et al., 2008). I found that VT3 in both species of songbird has the same nonpolar residues as the MTR in bullfrog (Cho et al., 2008) at the two critical positions that determine ligand selectivity for MT ($Val^{6.54}$ and $Pro^{7.29}$). In both sparrow and finch, VT1 has the same nonpolar residues as the chicken VT1 receptor at the critical positions for determining ligand selectivity for MT ($Ala^{6.54}$ and $Leu^{7.29}$) and for VT ($Leu^{7.29}$).

DISCUSSION

Using species-specific riboprobes, I mapped the neural distribution of VT1, VT3 and VT4 receptor mRNA in two model species of songbird, the white-throated sparrow and the zebra finch. In sparrow, the distribution of VT3 and VT4 expression was widespread, whereas the distribution VT1 mRNA was more limited (see Fig. 1). In zebra finch, the distributions of the three receptors were widespread (see Fig. 2). The distribution patterns for each subtype were similar between species. Within species, the patterns of VT1, VT3 and VT4 expression were each unique, although they overlapped with one another in several brain regions, many of which are known to be involved in social or motivated

behavior (see Tables 1 and 2). For example, in sparrow, VT1, VT3 and VT4 are expressed in the LS and GCt, VT3 and VT4 are both found in the BSTm and VMH, and VT1 and VT4 are found in the SNc and VP (see Table 1). In zebra finches, VT1, VT3 and VT4 are found in the LS, POM and VTA (see Table 2). Such findings suggest that these receptor subtypes could play a role in modulating social and motivational behaviors across avian species.

The expression of all three receptors in the LS is particularly interesting because this region is often the target of nonapeptide infusions in behavioral studies (Goodson, 1998a,b; Goodson and Adkins-Regan, 1999; Goodson et al., 2009). Ligands infused into the LS may be binding to one or more of the VT receptors expressed in this region. The LS is featured prominently in the literature on nonapeptide receptors for its role in social behavior (Insel et al., 1994; Young et al., 1997; Insel and Young, 2000) and both V1a receptor mRNA and binding are found in the LS in mammals (Young et al., 1997; Young et al., 1999). VT-like binding is found in the LS in several species of songbird, including the white-throated sparrow and zebra finch (Goodson et al., 2006; Goodson et al., 2009; see chapter 1). Because all three receptors are expressed in the LS, VT-like binding in the LS may represent more than one subtype.

I previously reported that MT and VT are equally effective at displacing the iodinated vasotocin analog [^{125}I]OVTA in white-throated sparrow and zebra finch LS (see chapter 1). This finding suggests that MT and VT could be acting as endogenous ligands in the LS. MT is detectable in the LS via radioimmunoassy (Robinzon et al., 1988) and VT

fibers are found throughout the LS (Kiss et al., 1987; Voorhuis and de Kloet, 1992; Panzica et al., 1999; Maney et al., 2005). Therefore, both MT and VT could be binding to receptors that I have identified in this brain region. The binding properties of the VT receptor subtypes are known only for VT1. With the use of [^{3}H]VP as a competitor, Tan et al. (2000) showed that chicken VT1 has higher affinity for VT than MT. Because of the similarities in sequence in sparrow and finch VT1 compared to chicken VT1, I predict that these receptors bind with greater affinity to VT than MT (see Table 4).

Because VT3 and VT4 are closely homologous to nonapeptide receptors in other vertebrate species, and the binding affinities for many of these receptors are well-characterized, I can speculate about the affinity of VT3 and VT4 for endogenous ligands. In bullfrogs, there are two known nonapeptide receptors, VTR and MTR (Acharjee et al., 2004b). VTR is homologous to the mammalian V1a receptor and MTR is homologous to the mammalian OT receptor (Acharjee et al., 2004b). By substituting different residues into these receptors, Cho et al. (2008) found that the residue important for determining binding affinity for VT is located in the extracellular loop (ECL3) at position 7.29 between transmembrane domains (TMD) VI and VII, and the two residues important for determining binding affinity for MT are located in TMD VI and ECL3 at positions 6.54 and 7.29 (see Fig. 4). Because I generated partial nucleotide sequences from this region for VT1, VT3 and VT4 in white-throated sparrow and zebra finch, I deduced the amino acid sequences and aligned them to homologous sequences in other species of vertebrates, including the bullfrog (Fig. 4). After comparing the residues at positions 6.54 and 7.29 across species, I predicted the potential binding properties of the sparrow and

zebra finch VT receptor subtypes based on the binding properties of the bullfrog VT and MT receptor. For example, the binding affinity of the bullfrog VT receptor for VT depends on the polar residue $Glu^{7.29}$ in extracellular loop 3 (ECL 3). Substituting $Glu^{7.29}$ for a nonpolar residue causes a shift in affinity from VT to MT (Cho et al., 2008). The avian VT4 receptor has a polar residue in the critical position in ECL 3 ($Asp^{7.29}$), suggesting that like the anuran VT receptor, VT may be the endogenous ligand for this receptor (see Table 4). Interestingly, the residue in position 7.29 in sparrow, finch and chicken VT4 is identical to that in the Norway rat and house mouse (Fig. 4), which suggests that the binding properties of the avian VT4 receptor may be similar to those of the mammalian V1a receptor.

The amino acid sequences of VT1 in both sparrow and zebra finch contain nonpolar residues at the two critical positions for determining affinity for MT ($Ala^{6.54}$ and $Leu^{7.29}$), although they are not the same exact residues as those in bullfrog MTR. This suggests that VT1 may share some binding properties with the anuran MTR. In both sparrow and zebra finch VT1, the residue in position 6.54, alanine, is identical to that in the chicken VT1, but the residue in position 7.29, is not, although the residues in both species are nonpolar and are isomers of one another (leucine in sparrow and finch VT1, and isoleucine in chicken VT1; see Fig. 4). This suggests that the sparrow and zebra finch VT1 receptor may share some binding properties with the chicken VT1 receptor. As mentioned earlier, chicken VT1 binds with greater affinity to VT than MT (Tan et al., 2000). I speculate, therefore, that sparrow and zebra finch VT1 may have affinities for MT and VT (see Table 4).

The results of our sequence analysis for VT1 and VT4 may aid in the interpretation of behavioral studies. Several studies show that septal infusions of VT and VT antagonists have behavioral effects in many species of songbird, including field sparrows (*Spizella pusilla*) and zebra finches (Goodson et al. 1998a,b; Goodson and Adkins-Regan, 1999). Because I have shown that VT1 and VT4 are expressed in the white-throated sparrow and zebra finch LS, and predict that these subtypes bind with affinity to VT (see Table 4), it is possible that these receptors are involved in behavior in these species. Maney et al. (1997) found that VT injected into the third ventricle induces vocalizations, including song, in female white-crowned sparrows (*Zonotrichia leucophrys*). In the congeneric white-throated sparrow, I found expression of VT1 and VT4 in the mediobasal hypothalamus, a region that surrounds the site of injection, which suggests that these receptors may be involved in the production of vocalizations in this genus.

In the bullfrog MT receptor, nonpolar residues $Val^{6.54}$ and $Pro^{7.29}$ are located within transmembrane domain VI (TMD VI) and ECL 3, respectively, and are responsible for dictating the affinity of this receptor for MT (Cho et al., 2008). The amino acid sequences for the VT3 receptor in sparrow and zebra finch contain the same key residues, $Val^{6.54}$ and $Pro^{7.29}$, as the MT receptor in anurans (Cho et al., 2008), which suggests that the avian VT3 receptor may preferentially bind to MT over VT (see Table 4). Interestingly, the mammalian OT receptors likewise have the same residues at positions 6.54 and 7.29, which suggests that VT3 may also have binding properties similar to those of OT receptors. Goodson et al. (2009) found that infusions of OT antagonist and MT into the

lateral ventricle affect social preference in zebra finches. Because I found VT3 and VT1 expression in the LS, a region surrounded by the lateral ventricles and thus would easily receive OT antagonist or MT infused into the ventricular space, these subtypes may be involved in the activation of social preference in this species.

I looked for a sex difference in VT receptor mRNA in brain regions with high levels of receptor expression in sparrow, and in the zebra finch LS, where I previously detected a trend for a sex difference in [^{125}I]OVTA binding (see chapter 1). I did not, however, find any effect of sex in the regions analyzed for either sparrow or zebra finch (see Results). These findings are consistent with the mammalian literature, which demonstrates that V1a receptor and OT receptor binding and V1a expression do not differ between males and females (Tribollet et al., 1990; Insel and Shapiro, 1992; Insel et al., 1994; Szot et al., 1994; Phelps and Young, 2003; Beery et al., 2008; but see Delville and Ferris, 1995). There is evidence, however, for a sex difference in OT receptor mRNA in the VMH of several species of rodent (Bale and Dorsa, 1995; Wang et al., 2000), although the direction of the sex difference may differ between species. In rats, males have greater levels of OT receptor expression than do females in the VMH (Bale and Dorsa, 1995). In prairie and montane voles, females have greater levels of OT receptor mRNA than males in the VMH, but only after parturition (Wang et al., 2000). Although hormone profiles for voles are not known, the latter finding may be a result of changes in gonadal hormones following parturition, as OT receptor mRNA is sensitive to estradiol (Bale and Dorsa, 1995). Furthermore, there are no sex differences in OT receptor mRNA in voles that are sexually-naïve (Wang et al., 2000). These findings suggest that sex differences in VP and

OT receptor binding and mRNA may not be detected unless gonadal steroids are involved in a sex-specific manner.

White-throated sparrows are seasonal breeders and typically have high levels of circulating gonadal steroids during the breeding season. Because I obtained our sparrows during fall migration when circulating sex steroids are low, I looked to see if treatment with T would increase levels of VT receptor expression. Although T treatment significantly increased plasma T, I did not find an effect of T on receptor expression in any brain region of interest for any VT receptor subtype. I was somewhat surprised with this finding because I sampled regions in which VT-like binding is sensitive to T in canary (Voorhuis et al., 1988). T increases the expression of V1a receptors and the intensity of V1a receptor binding in the medial preoptic nucleus of the hypothalamus (Young et al., 2000) and VP binding in the mPOA in castrated Syrian hamsters (Delville et al., 1996), and T has also been shown to increase the levels of OT binding in the VMH in castrated rats (Tribollet et al., 1990). I did not, however, find an effect of T on VT receptor expression in either of two hypothalamic regions (VMH-med and the VMH-lat). I did find an effect of T on behavior; I found an effect of T on song and a moderate trend for an effect of T on tseets. These findings are consistent with those reported by Maney et al. (2009). There was no relationship, however, between behavior and receptor expression. Although there are no previous studies demonstrating behavior and receptor expression relationships in birds, V1a receptor expression increases in the PVN in male and female prairie voles postpartum when compared to sexually-naïve controls (Wang et

al., 2000) and rats that exhibit high anxiety have higher V1a receptor expression in the PVN than low anxiety rats (Wigger et al., 2004).

In summary, I have mapped the mRNA distribution of three nonapeptide receptors in two species of songbird. Because I found expression for all three receptors in the LS, [^{125}I]OVTA and [^{125}I]LVA binding in the white-throated sparrow and zebra finch LS (as described in Goodson et al., 2006; see chapter 1) may represent all three receptor subtypes in both species of songbird. Note that in chapter one, I found that MT inhibited the binding of an iodinated V1a receptor antagonist, [^{125}I]LVA, in the sparrow LS more than did VT, and that Goodson et al. (2006) found VT inhibited [^{125}I]LVA in the zebra finch LS more than MT, which suggests that receptor subtypes may be expressed at different levels in the LS depending on the species. The analysis of the amino acid residues of VT receptor subtypes in white-throated sparrow and zebra finch suggests that VT4 may have greater affinity for VT than MT, VT3 may have greater affinity for MT than VT, and VT1 may have affinity for VT and MT (see Fig. 4, Table 4). Although I found an effect of T on song, I did not find any relationship between T and VT receptor expression, or a relationship between behavior and VT receptor expression. Future experiments can now target agonists and antagonists at specific receptor subtypes in a particular brain region in order to better characterize their role in behavior.

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Abbreviations

A	arcopallium
Ad	dorsal arcopallium
APH	parahippocampal area
BSTm	bed nucleus of the stria terminalis
Cb	cerebellum
CS	superior central nucleus
DIVA	dorsal intermediate ventral anterior nucleus
DLL	lateral portion of the dorsolateral nucleus of the anterior thalamus
DMP	dorsomedial nucleus of the posterior thalamus
EW	nucleus of Edinger-Westphal
FPL	lateral forebrain bundle
GCt	midbrain central gray
GLV	lateral geniculate nucleus
HA	apical hyperpallium
Hb	habenula
HD	densocellular hyperpallium
Hp	hippocampus
HVC	proper name
ICo	intercollicular nucleus
IO	isthmo-optic nucleus
INP	intrapeduncular nucleus
IPN	interpeduncular nucleus
LA	lateral anterior nucleus of the thalamus
LLd	nucleus of the dorsal lateral lemniscus
LMAN	lateral magnocellular nucleus of the anterior nidopallium
LoC	locus coeruleus
LS	lateral septum
LSt	lateral striatum
M	mesopallium
MLd	lateral mesencephalic nucleus
MS	medial septum
MSt	medial striatum
MV	motor nucleus of the trigeminal nerve
N	nidopallium
Nc	caudal nidopallium
NCM	caudomedial nidopallium
nIV	trochlear nucleus
OMd	dorsal nucleus of the oculomotor nerve
OMv	ventral nucleus of the oculomotor nerve
PMI	paramedial internal nucleus of the thalamus
POM	medial preoptic nucleus
PPT	pedunculopontine tegmental nucleus
Pt	nucleus pretectalis
PTM	medial pretectal nucleus
PVN	paraventricular nucleus

RA	robust nucleus of arcopallium
SH	septohippocampal septum
SLu	semilunar nucleus
SNc	compact substantia nigra
SNr	reticulated substantia nigra
TeO	optic tectum
TnA	nucleus taeniae of the amygdala
VMH-lat	lateral ventromedial hypothalamus
VMH-med	medial ventromedial hypothalamus
VP	ventral pallidum
VTA	ventral tegmental area

Table 1. The Distribution of VT1, VT3 and VT4 Receptor mRNA in White-Throated Sparrow Brain

Brain Region	VT1	VT3	VT4
Area X	-	-	+
Ad	-	++/+++	-/+
APH	-	+	-
BSTm	-	+	-/+/++
Cb	-	++	-
CS	-	+/++	+/++
DLL	-	+/++	-
DMP	-	++/+++	++/+++
EW	-	-	+/++
GCt	+/++	+	+
GLV	-	+/++	+
Hb		++	-
Hp	-	+	-
ICO	-	+	+
IO	-	+/++	+
IPN	-	+	-/+
LA	+/++	+	+
LLd	-	-/+	+/++
LMAN	-	-	+++
LoC	-	+/++	-/+
LS	-/+	+/++/+++	-/+
M	-	+/++	-
MBH	+/++	++/+++	++/+++
MLD	-	-/+	-
MMAN	+/++	-	++/+++
MS	-	+/++/+++	-/+
MSt	-	-/+	+
MV	-	++/+++	++
N	+/++	+/++	-
NCM	-	+/++	-
nIV	-	+++	-
OMd	-	+++	-
OMv	-	+++	-
PMI	-	-	++
POM	-	+	-
PPT	-	-	+
Pt	+++	+	-
PTM	-	-	+
PVN	-	++	-/+
RA	-	-/+	-
SH	-/+	+	-
SLu	-	-/+	+/++/+++
SNc	+	-	+/++
TeO	-/+	+/++	++/+++
TnA	-	-/+	-
VMH-lat	-	+	++
VMH-med	-	+	++

VP	+	-	+
VTA	-	-	+/++

*Plus signs indicate relative expression of VT receptor mRNA. Minus signs indicate the absence of VT receptor mRNA. Slashes indicate individual variation. Because each subtype was run in a separate assay, symbols pertain to levels of expression observed within each particular subtype, and not in relation to other subtypes.

Table 2. The Distribution of VT1, VT3 and VT4 Receptor mRNA in Zebra Finch Brain

Brain Region	VT1	VT3	VT4
Area X	-	-	-/+
Ad	-/+	-/+	-
APH	-	+	-
BSTm	-	-	-/+
CS	-	+/++	-
DIVA	+/++	-	-
DMP	-	+	+
EW	-	-	-
FPL	-	-	-/+
GCt	-	-	+
GLV	-	-	-
HA	+	-	-
Hb	+/++	+	-
HD	+/++	-	-/+
Hp	-	-	-
HVC	-	+	-
IO	-	-	-
INP	++	-	-
IPN	-/+	-	-
LA	-/+	-	-
LoC	-	-	+
LS	-/+	+/++	-/+
LSt	+	-	-
M	+/++	-	-
MBH	+/++/+++	-	+
MLD	-	-	-
MS	-/+	-	-
MSt	-	-	-/+
MV	-	++	-
N	-/+	+	-/+
Nc	+	+	+
nIV	-	+/++	-
OMd	-	++/+++	-
OMv	-	++/+++	-
POM	+	-/+	+
PMI	+	-	+
Pt	++/+++	-	-
PVN	-	-	-
SH	-/+	+	-/+
SNc	-	-	+
SNr	-/+	-	+
TeO	+	+	+/++
TnA	-/+	-	-
VMH-lat	-	-	-/+/++
VMH-med	-/+	-	+
VP	-	-	-
VTA	-/+	+	+/++

*Plus signs indicate relative expression of VT receptor mRNA. Minus signs indicate the absence of VT receptor mRNA. Slashes indicate individual variation. Because each subtype was run in a separate assay, symbols pertain to levels of expression observed within each particular subtype, and not in relation to other subtypes.

Table 3. Spearman's rho and *P* values from Spearman correlations between VT receptor expression and behaviors followed by the sequential Bonferroni correction for multiple tests ($\alpha = .003$). No significant correlations were detected.

Receptor	Brain Region	Rho or *P* value	Song	Tseets	Bouts of preening
VT1	Pt	rho	-0.185	-0.187	-0.304
		P	0.398	0.392	0.158
VT3	Arcopallium	rho	-0.338	-0.038	0.042
		P	0.115	0.864	0.85
VT3	LS	rho	-0.25	-0.362	-0.114
		P	0.251	0.09	0.603
VT3	VMH-med	rho	-0.207	-0.279	-0.018
		P	0.344	0.198	0.935
VT4	TnA	rho	-0.339	-0.124	0.042
		P	0.113	0.574	0.848
VT4	VMH-lat	rho	-0.171	-0.494	-0.319
		P	0.436	0.017	0.138
VT4	VTA	rho	-0.335	-0.297	0.048
		P	0.119	0.168	0.827

Table 4. Avian nonapeptide receptors, homologous mammalian receptors and predicted binding affinity for each nonapeptide receptor subtype in white-throated sparrow and zebra finch.

Avian nonapeptide receptor	Homologous receptor in mammals	Predicted binding affinity for ligands
VT1	none	VT > MT
VT3	OT receptor	VT < MT
VT4	V1a receptor	VT > MT

Figure Captions

Figure. 1.

VT1 (A,D,G,J,M,P,S,V), VT3 (B,E,H,K,N,Q,T,W), and VT4 (C,F,I,L,O,R,U,X) expression in comparable sections in the brain of a white-throated sparrow. Anterior-posterior (AP) coordinates follow Stokes et al. (1974). Arrows point to regions of interest that are labeled in the figure and mentioned in the text.

Figure 2.

VT1 (A,D,G,J,M), VT3 (B,E,H,K,N), and VT4 (C,F,I,L,O) expression in comparable sections in the brain of a zebra finch. Anterior-posterior (AP) coordinates follow Nixdorf-Bergweiler and Bischof (2007).

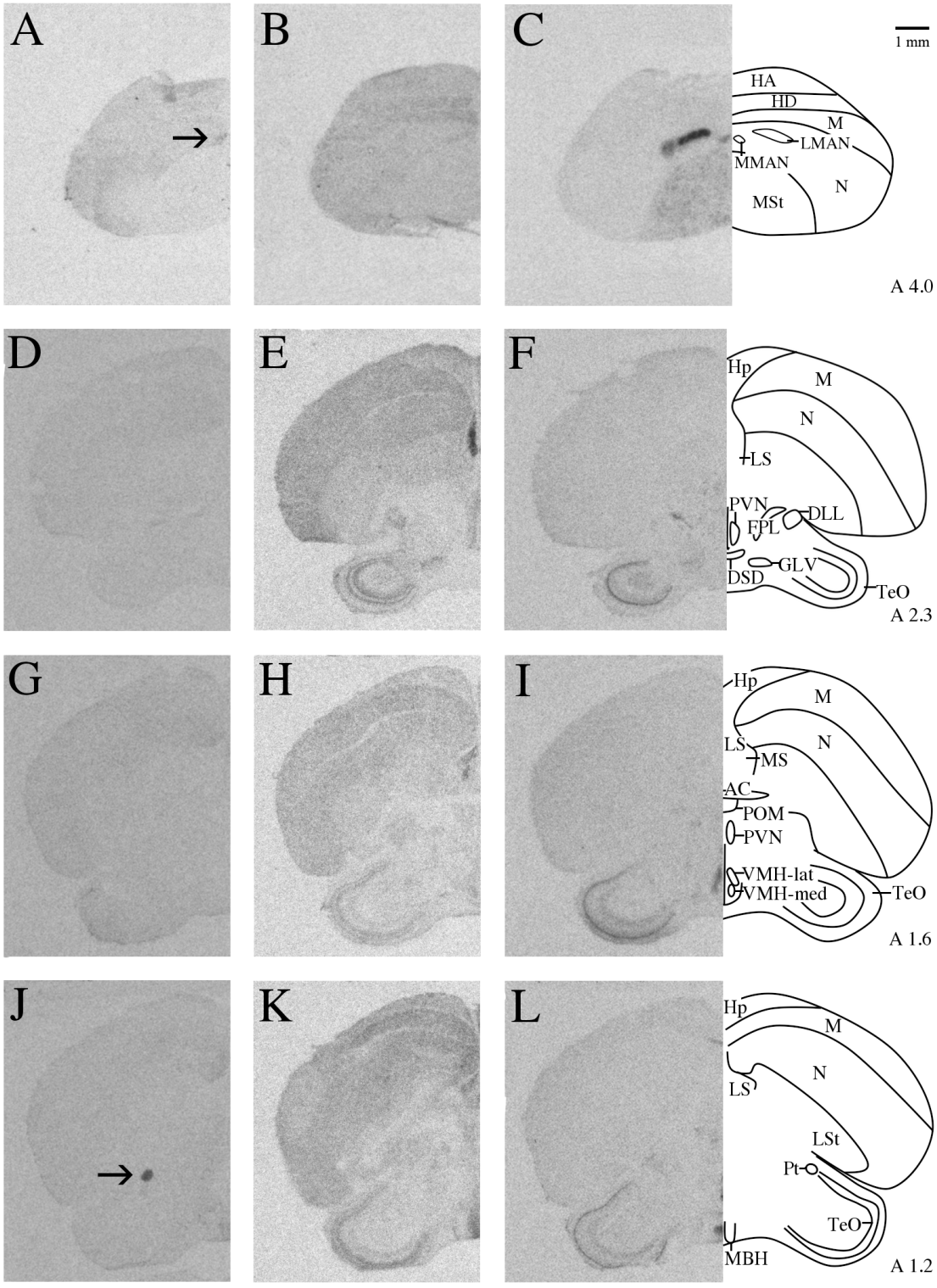
Figure 3.

Number of (A) songs, Cliff's d = .75, (B) tseets, Cliff's d = .61, and (C) bouts of preening, Cliff'd d = .63, in testosterone treated males (T males) and control males (C males). *P = .006

Figure 4.

Comparison of amino acid sequences from transmembrane domain (TMD) VI to TMD VII for chicken VT1, VT3 and VT4, white-throated sparrow VT1, VT3 and VT4, zebra finch VT1, VT3 and VT4, bullfrog VT1 and MTR, Norway rat V1aR and OTR, and house mouse V1aR and OTR. Arrows point to the residues important for determining ligand sensitivity. A = alanine (Ala); D = aspartic acid (Asp); E = glutamic acid (Glu); I = isoleucine (Ile); L = leucine (Leu); P = proline (Pro); V = valine (Val).

Figure 1.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A 4.0
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GLV
DSD
TeO
A 2.3
G
H
I
Hp
M
LS
N
MS
AC
POM
PVN
VMH-lat
VMH-med
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Pt
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MBH
A 1.2

Figure 1, cont.

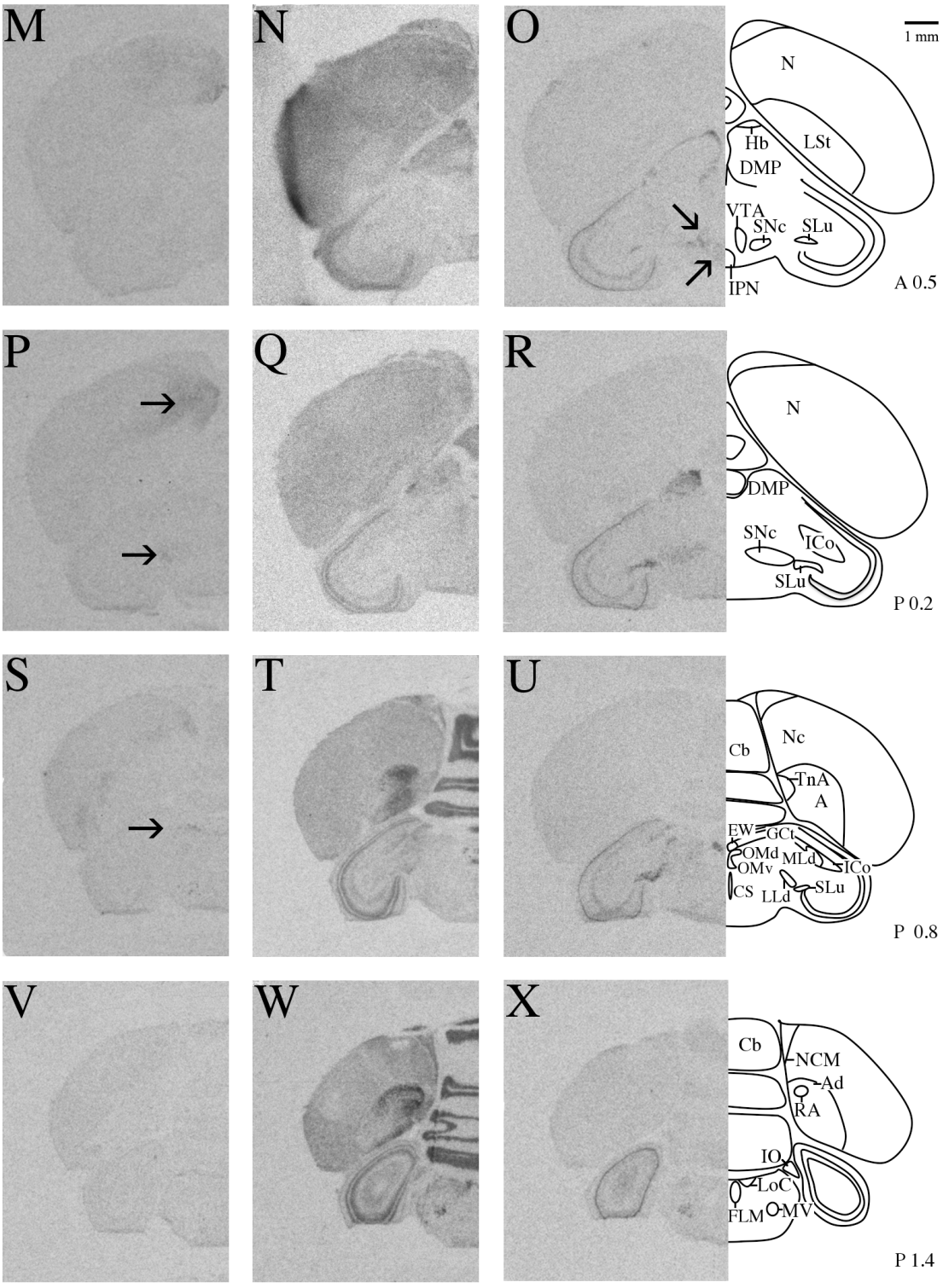
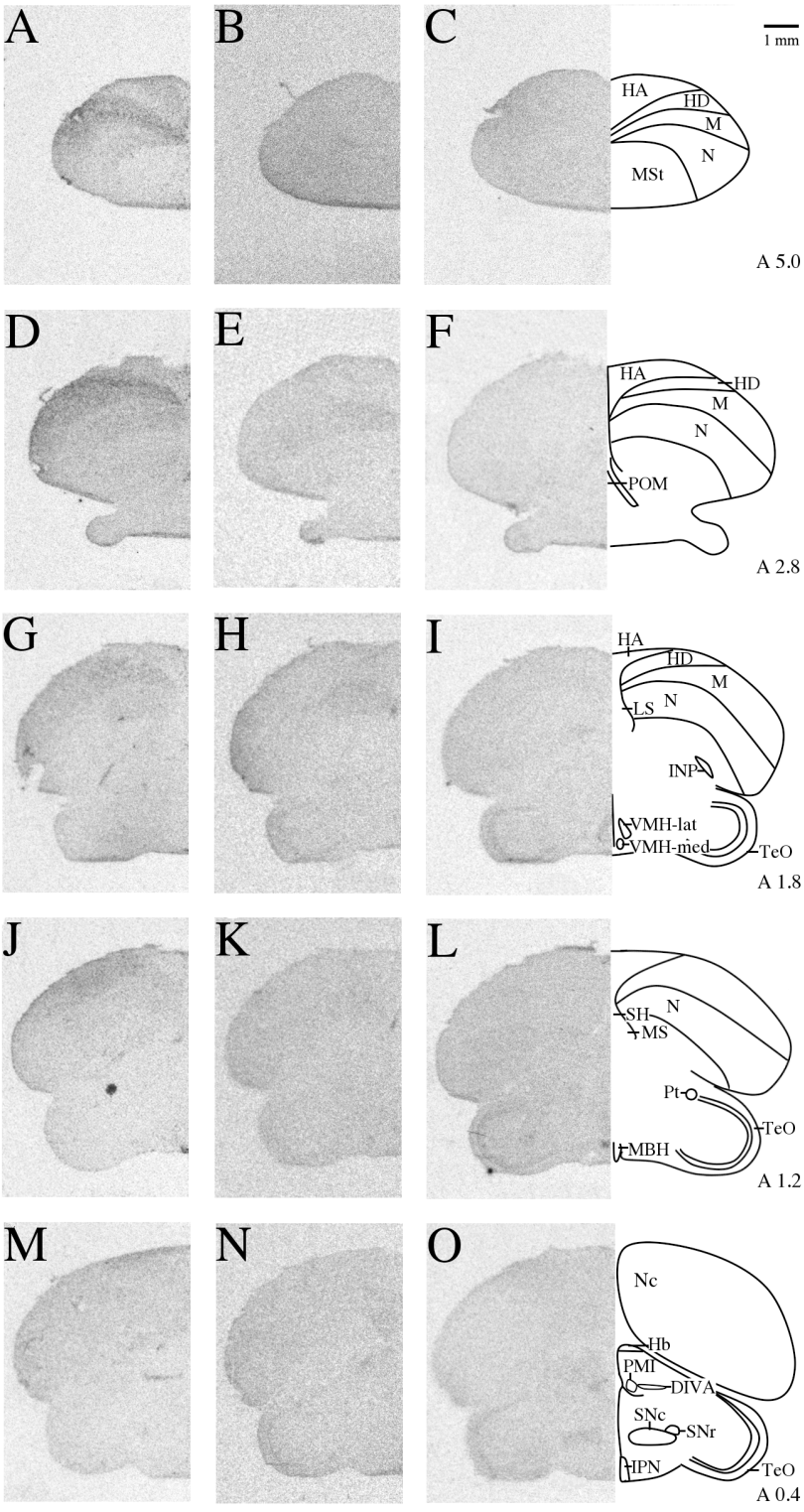


Figure 2.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G
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I
HA
HD
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LS
N
INP
VMH-lat
VMH-med
TeO
A 1.8
J
K
L
SH
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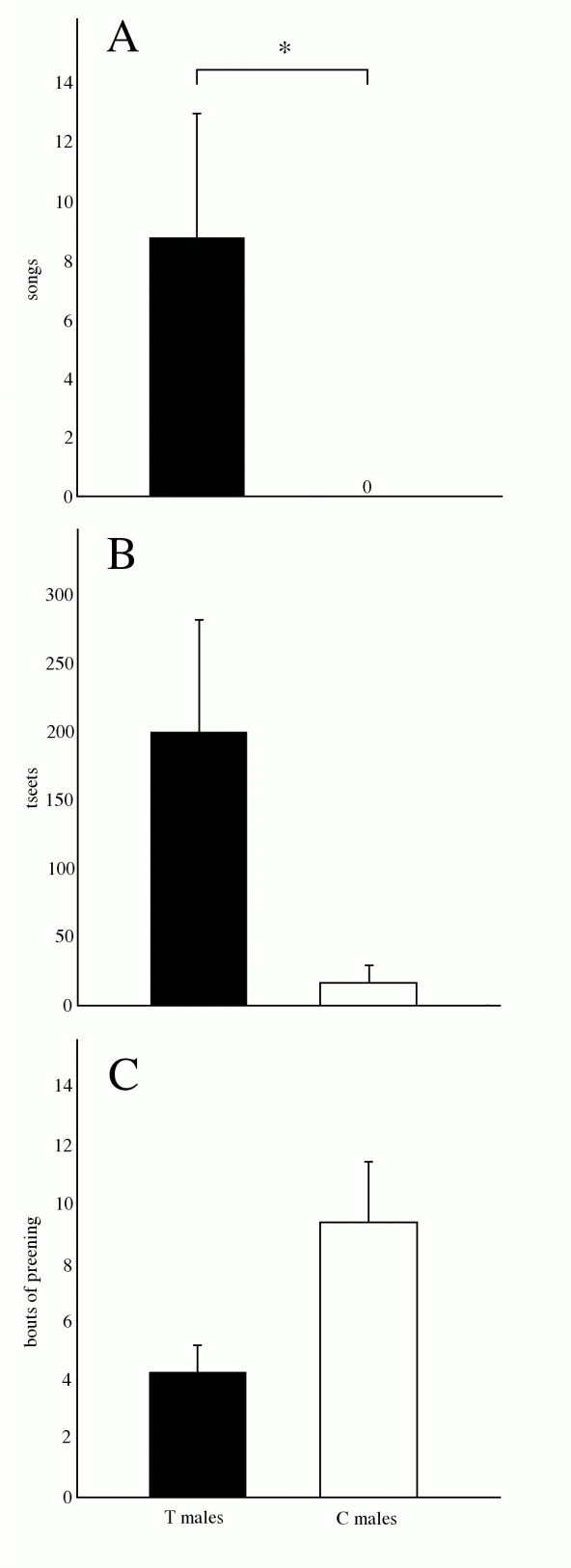
Figure 3.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tseets
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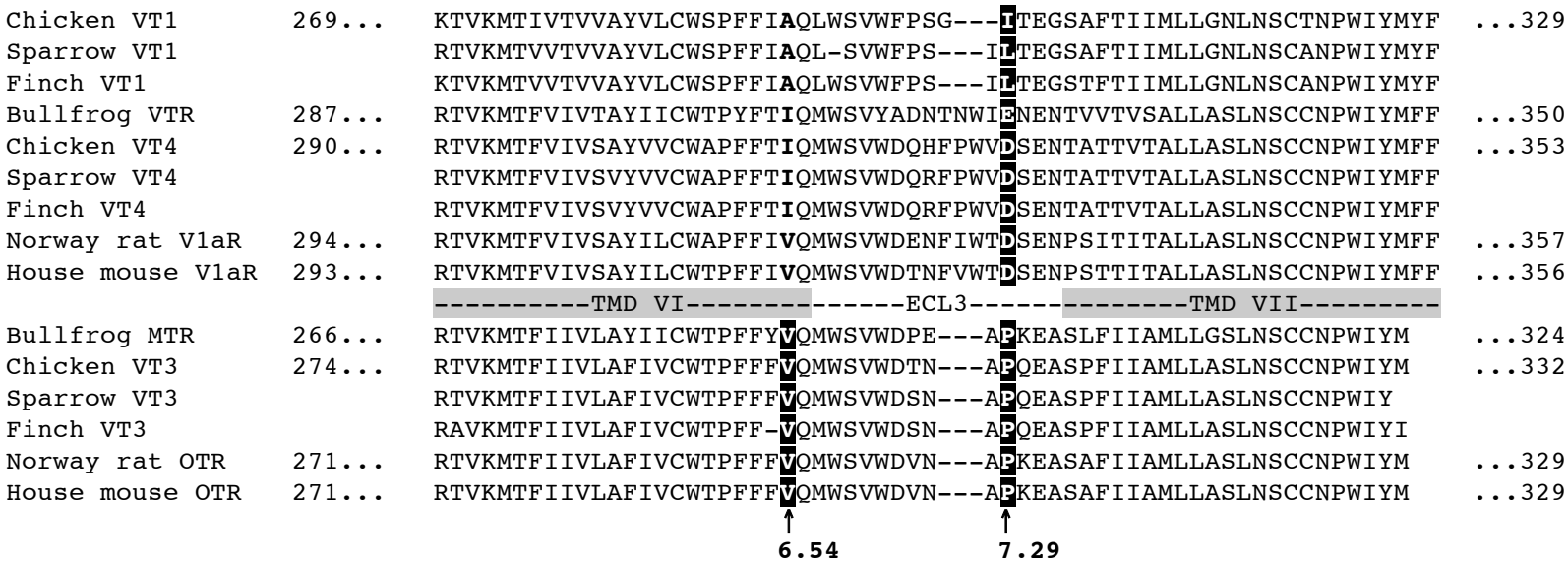
bouts of preening
T males
C males

Figure 4.


Chicken VT1 269... KTVKMTIVTVVAYVLCWSPFFIAQLWSVWFPSG---ITEGSAFTIIMLLGNLNSCTNPWIYMYF ...329
Sparrow VT1 RTVKMTVVTVVAYVLCWSPFFIAQL-SVWFPS---ILTEGSAFTIIMLLGNLNSCANPWIYMYF
Finch VT1 KTVKMTVVTVVAYVLCWSPFFIAQLWSVWFPS---ILTEGSTFTIIMLLGNLNSCANPWIYMYF
Bullfrog VTR 287... RTVKMTFVIVTAYIICWTPYFTIQMWSVYADNTNWIENENTVVTVSALLASLNSCCNPWIYMFF ...350
Chicken VT4 290... RTVKMTFVIVSAYVVCWAPFFTIQMWSVWDQHFPWVDSENTATTVTALLASLNSCCNPWIYMFF ...353
Sparrow VT4 RTVKMTFVIVSVYVVCWAPFFTIQMWSVWDQRFPWVDSENTATTVTALLASLNSCCNPWIYMFF
Finch VT4 RTVKMTFVIVSVYVVCWAPFFTIQMWSVWDQRFPWVDSENTATTVTALLASLNSCCNPWIYMFF
Norway rat V1aR 294... RTVKMTFVIVSAYILCWAPFFIVQMWSVWDENFIWTDSENPSITITALLASLNSCCNPWIYMFF ...357
House mouse V1aR 293... RTVKMTFVIVSAYILCWTPFFIVQMWSVWDTNFVWTDSENPSTTITALLASLNSCCNPWIYMFF ...356
----------TMD VI---------------ECL3------------TMD VII---------
Bullfrog MTR 266... RTVKMTFIIVLAYIICWTPFFYVQMWSVWDPE---APKEASLFIIAMLLGSLNSCCNPWIYM ...324
Chicken VT3 274... RTVKMTFIIVLAFIVCWTPFFFVQMWSVWDTN---APQEASPFIIAMLLASLNSCCNPWIYM ...332
Sparrow VT3 RTVKMTFIIVLAFIVCWTPFFFVQMWSVWDSN---APQEASPFIIAMLLASLNSCCNPWIY
Finch VT3 RAVKMTFIIVLAFIVCWTPFF-VQMWSVWDSN---APQEASPFIIAMLLASLNSCCNPWIYI
Norway rat OTR 271... RTVKMTFIIVLAFIVCWTPFFFVQMWSVWDVN---APKEASAFIIAMLLASLNSCCNPWIYM ...329
House mouse OTR 271... RTVKMTFIIVLAFIVCWTPFFFVQMWSVWDVN---APKEASAFIIAMLLASLNSCCNPWIYM ...329
6.54
7.29

General Discussion

The behavioral effects of the nonapeptide vasotocin (VT) have been well-studied in many non-mammalian vertebrates. In amphibians, VT has been implicated in various forms of social behavior related to reproduction in both males and females. For example, VT regulates sexual receptivity in female leopard frogs (*Rana pipiens*; Diakow, 1978) and bullfrogs (*Rana catesbeiana*; Boyd, 1994), male courtship behaviors in rough-skinned newts (*Taricha granulosa*; Moore and Miller, 1983) and advertisement calling in male bullfrogs (Boyd, 1994). In fish, VT is involved in sexual behavior. For example, VT modulates spawning reflex activity in killifish (*Fundulus heteroclitus*; Pickford and Strecker, 1977) and courtship vocalizations in plainfin midshipman fish (*Porichthys notatus*; Goodson and Bass, 2000).

In birds, VT is involved in social communication related to mate defense and mate attraction. In particular, intracerebroventricular (i.c.v.) infusions of VT increase aggressive behaviors in male zebra finches (*Taeniopygia guttata*; Goodson et al., 2004) and territorial song in female white-crowned sparrows (*Zonotrichia leucophrys*; Maney et al., 1997), and subcutaneous injections of VT increase female-directed song in male canaries (*Serinus serinus*; Voorhuis et al., 1991). In contrast, i.c.v. infusions of VT inhibit territorial behavior in field sparrows (*Spizella pusilla*; Goodson, 1998a) and the violet-eared waxbill (*Uraeginthus granatina*, Goodson, 1998b). These studies demonstrate that the role of VT in modulating behavior may be species-specific in birds.

Birds present a unique opportunity to understand the role of the VT system in modulating species-specific behavior because of the extensive variation in social behavior across species. Furthermore, much is known about the distribution of VT in several species of songbird, including the canary, junco (*Junco hyemalis*), white-throated sparrow (*Zonotrihia albicollis*) and zebra finch (Kiss et al., 1987; Voorhuis and de Kloet, 1992; Panzica et al., 1999; Maney et al., 2005). To better understand the effects of VT on behavior, we need to identify where VT acts in the brain. Identifying where VT receptors are distributed, and whether there are species differences in the location of these receptors, will help us better understand how nonapeptides modulate behavior in a species-specific manner.

Nonapeptide receptor binding

In chapter one, I mapped the neural distribution of binding sites of two radioligands in two model species of songbird, the white-throated sparrow and the zebra finch. I used two synthetic radioligands that were developed to label mammalian nonapeptide receptors and that have been used previously to label nonapeptide receptors in songbirds (Voorhuis et al., 1990; Goodson et al., 2006). I found that the binding distribution of the iodinated vasotocin analog, [^{125}I]OVTA, was similar between white-throated sparrow and zebra finch, and in both species, a great deal of individual variation was observed. I detected binding of the iodinated V1a antagonist, [^{125}I]LVA, only in white-throated sparrow brain. The distribution of this radioligand was more limited compared to that of [^{125}I]OVTA binding. In another study in songbirds, [^{125}I]LVA binding was detected in the zebra finch brain (Goodson et al., 2006) and its distribution was similar to that in sparrow

(see chapter 1 Fig. 1). These studies demonstrate that there are similarities in binding distribution between species and that radioligands differ from one another in binding distribution, which suggests that they may be labeling different populations of receptors.

Nonapeptide receptor mRNA

Because radioligand binding studies do not allow us to discriminate among the three VT receptor subtypes expressed in the avian brain (Tan et al., 2000; Leung, unpublished), I mapped the distribution of VT1, VT3 and VT4 using *in situ* hybridization (see chapter 2). Like the distribution of [^{125}I]OVTA binding in chapter one, the distribution of each receptor subtype was similar in the white-throated sparrow and zebra finch. One of the most striking similarities between sparrow and finch VT receptor expression was VT1 expression in the nucleus pretectalis (Pt; see chapter 2 Figs.1J,2J), a brain region involved in visual processing. I also detected VT3 expression in both sparrow and finch LS (see chapter 2 Figs. 1E,2H), a region involved in social behavior.

There were some species differences, however, in VT receptor distribution that were quite salient. In white-throated sparrow, I detected VT3 receptor expression in and around a song nucleus called the robust nucleus of the arcopallium (RA), whereas in zebra finch, VT3 expression was essentially nondetectable in these regions (see chapter 2 Fig 1W for sparrow; zebra finch not shown). This finding suggests that VT3 receptor mRNA may modulate song production in sparrow but not in finch. Another striking species difference in VT receptor distribution was found in VT4 receptor expression in an auditory song nucleus, the lateral nucleus of the anterior magnocellular nidopallium

(LMAN). In sparrow, levels of VT4 expression were high (see chapter 2 Fig.1C), whereas in zebra finch, expression of VT4 was not detected in LMAN (not shown). Both LMAN and RA are part of the established song circuit in birds; LMAN is involved in song learning and projects to RA, which is involved in song production. Because VT plays a species-specific role, species differences in VT receptor expression may provide a mechanism for the species-specificity.

VT receptor mRNA overlap with VT-like binding

Comparing the distribution of each VT receptor subtype with the distribution of VT-like binding can help us better interpret which receptors are represented by radioligand binding (see Figs. 1 and 2; Tables 1 and 2). I found that VT receptor mRNA is expressed in many of the same brain regions as those exhibiting VT-like binding. For example, the distribution of VT3 mRNA overlapped greatly with the distribution of [^{125}I]OVTA binding sites in regions that had high levels of binding in both white-throated sparrow and zebra finch. In sparrow, the intensity of [^{125}I]OVTA binding and VT3 expression was greatest in the LS and dorsal arcopallium (Ad), and both binding and expression were homogenously distributed throughout the pallial layers of the telencephalon. In zebra finch, [^{125}I]OVTA binding and VT3 expression were both highest in the LS and homogenously distributed throughout the nidopallium. This overlap suggests that [^{125}I]OVTA binding patterns may represent, at least in part, the VT3 receptor subtype in both species.

The overlap between [^{125}I]OVTA binding and receptor expression was not limited to VT3. In fact, [^{125}I]OVTA binding overlapped with the mRNA of all three nonpeptide receptors in several regions implicated in social behavior, such as the LS and midbrain central gray in sparrow, and the LS in zebra finch. Similarly, [^{125}I]LVA binding in sparrow (see chapter 1) and zebra finch (Goodson et al., 2006) overlapped with all three receptor subtypes in the LS.

In some cases, the distribution of VT receptor expression did not overlap with the distribution of receptor binding (see Fig. 1). As mentioned earlier, I detected high levels of VT1 expression in the Pt (see chapter 2 Fig. 1J), a brain region involved in visual processing. Low levels of VT3 expression were also detected in Pt, but its signal was not quite as salient as that of VT1 (see chapter 2 Fig. 1K). I did not detect [^{125}I]OVTA or [^{125}I]LVA binding in this brain region in sparrow or finch (see chapter 1). Voorhuis et al. (1988), however, reported [^{3}H]VP binding in the Pt in canary. These findings suggest that [^{3}H]VP may bind to VT1 and VT3 receptors in the Pt. Because I observed high levels of VT1 in this region, [^{3}H]VP binding may mainly represent VT1 receptors.

In studies comparing the neural distribution of nonapeptide receptor binding to that of nonapeptide receptor mRNA in mammals is somewhat more straightforward because the radioligands used in these studies are designed to bind to specific mammalian nonapeptide receptors such as V1a and OT receptors. For example, Young et al. (1997) found V1a receptor mRNA and binding overlapped in all the brain regions they examined, including the LS, ventral pallidum and cortex in prairie voles (*Microtus*

ochrogaster) and montane voles (*Microtus montanus*). In rhesus macaques (*Macaca mulatta*), V1a receptor binding overlaps with V1a receptor mRNA in the LS and cortex, but not in the ventral pallidum (Young et al., 1999), and in rats, OT receptor binding and OT receptor mRNA are found in all of these brain regions (Elands et al., 1988; Yoshimura et al., 1993). I showed that in songbirds, VT-like binding and VT receptor mRNA overlap in these brain regions (see Tables 1 and 2). Together, these studies demonstrate that there is some conservation in the distribution of nonapeptide receptor proteins and mRNA across vertebrates.

Predicting binding affinities of receptors

Little is known about the binding affinities of VT receptors in birds. To date, the binding affinity for endogenous ligands has been characterized only for chicken VT1. With the use of [^{3}H]VP as a competitor, Tan et al. (2000) showed that chicken VT1 has higher affinity for VT than MT. Because of the similarities in the sequence of VT1 receptors among sparrow, finch and chicken, I speculate that sparrow and finch VT1 bind with greater affinity to VT than MT. I was also able to make predictions about the endogenous ligands for VT3 and VT4 by comparing the partial sequence to the bullfrog MTR and VTR, whose binding properties for MT and VT have been well-characterized (Cho et al., 2008). The analysis of the sequences for sparrow and finch VT3 and VT4 revealed that VT3 likely binds with greater affinity to MT than VT, and VT4 likely binds to VT with higher affinity than it does to MT (see chapter 2 Table 4).

In chapter one, I showed in competition experiments that [^{125}I]OVTA and [^{125}I]LVA binding are inhibited by VT and MT in white-throated sparrow and zebra finch (see chapter 1). For example, [^{125}I]OVTA binding in sparrow and zebra finch LS was inhibited equally by VT and MT. In contrast, [^{125}I]LVA binding was inhibited more by MT than VT in sparrow LS. I previously concluded that the data from these competition experiments fit best to models in which VT and MT interact with one binding site (see chapter 1). Because I found that three receptor subtypes are expressed in the LS (see chapter 2), I now believe that [^{125}I]OVTA and [^{125}I]LVA are likely labeling multiple receptors and the receptor subtypes likely differ from each other in their affinities for these two radioligands.

Because I mapped the neural distribution of VT1, VT3 and VT4 in white-throated sparrow and zebra finch (see chapter 2), I may be able to predict which subtypes are represented by VT-like binding in other studies on songbirds. For example, Voorhuis et al. (1990) reported [^{125}I]OVTA binding in the arcopallium around RA, which is the binding pattern that I found in sparrow using the same radioligand (see chapter 1). Because [^{125}I]OVTA binding overlaps greatly with VT3 expression in sparrow and finch, I predict that [^{125}I]OVTA binding largely represents VT3 receptors across songbird species. As mentioned earlier, I found [^{125}I]OVTA was equally inhibited by VT and MT in sparrow and finch LS. Voorhuis et al. (1990), however, found that [^{125}I]OVTA was inhibited more by VT than MT in the canary arcopallium. Because VT receptor subtypes likely have different affinities for VT, MT and radioligands, the ratio of the subtypes relative to one another in a given brain region may determine how well VT or MT inhibit

radioligand binding. It is possible that the concentrations of these receptor subtypes in the LS and arcopallium differ and thus, may explain the differences observed between competition studies.

Behavioral studies with nonapeptide receptor antagonists

The traditional method for testing whether a substance is involved in regulating a particular behavior is the subcutaneous injection of the substance and then subsequent observation of behavior, with the assumption that the injected chemical has entered the brain. Nonapeptide agonists and antagonists may not be able to reach the brain through this method because of the blood-brain barrier; the effects of these substances on behavior, therefore, are studied after they are infused directly into the ventricular system or into a specific brain region. Because there are no synthetic antagonists designed to bind to non-mammalian nonapeptide receptors, the behavioral function of VT receptors in songbirds has largely been explored with the use of synthetic ligands designed to bind with affinity to specific mammalian receptors such as the V1a and OT receptor. For example, Goodson and Adkins-Regan (1999) showed that infusions of V1a receptor antagonist into the zebra finch LS reduced aggressive behavior, such as the number of chases and pecks, during mate competition, whereas VT infused into the LS increased aggressive behavior. This study provides evidence that VT receptors in the lateral septum are likely involved in modulating aggressive behavior in this species.

In another study in zebra finches, Kabelik et al. (2009a) found that i.c.v. infusions of VP receptor antagonist modulate aggressive behavior in a context-specific manner. They

found that the antagonist treatment reduced aggressive behavior such as chases and pecks in zebra finches during periods of mate competition, but increased these aggressive behaviors during periods when birds were involved with nest defense. This dual effect of the antagonist may relate to a context-dependent role of VT receptors in modulating behavior. During mate competition, inhibition of VT receptors may decrease levels of aggression whereas during nest defense, inhibition of VT receptors may increase aggressive behavior. The mechanism by which VT receptors modulate behavior, however, is not well understood.

The behavioral role of the VT3 receptor, which is homologous to the OT receptor in mammals, may be explained by studies using OT receptor antagonist or MT. Goodson et al. (2009) demonstrated that i.c.v. infusions of MT increase the amount of time zebra finches spend with familiar cage-mates and with large groups. I.c.v. infusions of OT antagonist, in contrast, reverse these effects on social preference. Infusions of OT antagonist into the LS likewise reduce the amount of time spent with large groups. These findings suggest that VT3, which is expressed in the LS in zebra finch (see chapter 2 Fig.2K), may be involved in modulating social preference behaviors in zebra finches.

Sex comparison and effects of testosterone (T)

Interestingly, the effects of VT and MT on behavior in songbirds appear to be sex-specific. I.c.v. infusions of V1a antagonist or VT affect aggression only in males (Goodson et al., 2004), whereas i.c.v. infusions of OT antagonist and MT affect social preference only in females (Goodson et al., 2009). One possible explanation is that there

may be a sex difference in the density of receptors in brain regions that are reached by the ligands. I looked for a sex difference in VT receptor expression in both the white-throated sparrows and the zebra finch in several brain regions involved in social behavior, but did not find an effect of sex in either species. The lack of a sex difference in zebra finch VT receptor expression was somewhat surprising as a sex comparison in [^{125}I]OVTA binding in the zebra finch LS revealed a large effect size, with males exhibiting higher levels of binding than females (see chapter 1 Fig. 3). In another nonapeptide receptor binding study, Goodson et al. (2006) conducted a sex comparison of VT-like binding in the LS in several species of songbird, revealing a large effect size, with higher levels of VT-like binding in males than in females (Goodson et al., 2006). Our finding that there are no sex differences in VT receptor expression is consistent with mammalian studies in which nonapeptide receptor binding and mRNA do not differ between males and females (Insel and Shapiro, 1992; Insel et al., 1994; Phelps and Young, 2003; Beery et al., 2008; but see Delville and Ferris, 1995).

Because many behaviors in songbirds that are affected by VT are also sensitive to gonadal steroids, I tested the effects of T on VT receptor expression. I did not, however, find any evidence for an effect of T on receptor expression for any receptor subtype in any brain region. This was somewhat surprising because Voorhuis et al. (1988) found an effect of T on VT-like binding in the canary ventral tegmental area (VTA) and RA, and I looked for an effect of T on VT receptor expression in the VTA and arcopallium, which includes RA. Furthermore, several studies in rodents demonstrate an effect of T on VP receptor binding and expression (Dubois-Dauphin et al., 1994; Delville et al., 1996;

Johnson et al., 1995; Young et al., 2000). It is possible that in birds, the effects of T on VT receptors occur during the translation of the receptor protein, resulting in increased VT-like binding, and not during the transcription of the receptor mRNA.

Although T did not have an effect on VT receptor expression, I confirmed that levels of plasma T were elevated in T treated birds compared to controls, and that T had an effect on behavior. Specifically, I found that the T increased levels of song production. I did not, however, find a relationship between song and VT receptor expression. Although a relationship between VT receptor expression and behavior in songbirds has never been described, there is some evidence for a relationship between nonapeptide receptor expression and anxiety behavior in rats (Wigger et al., 2004).

Conclusion

The studies described in this thesis provide a detailed map of VT receptors in the songbird brain. With this information, future studies can target specific receptor populations to better characterize their role in behavior. For example, Kabelik et al. (2009b) used antisense oligonucleotides to block VT release specifically in the BSTm. This type of technique could be used to block VT receptor subtypes in specific brain regions in order to detect whether a particular receptor is involved in behavior. Future studies should take advantage of the diverse social behavior found in songbirds and the growing literature on the avian VT system in order to better understand the role of VT receptors in modulating behavior.

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Abbreviations

A	arcopallium
Ad	dorsal arcopallium
APH	parahippocampal area
BSTm	bed nucleus of the stria terminalis
Cb	cerebellum
CS	superior central nucleus
DIVA	dorsal intermediate ventral anterior nucleus
DLL	lateral portion of the dorsolateral nucleus of the anterior thalamus
DMP	dorsomedial nucleus of the posterior thalamus
EW	nucleus of Edinger-Westphal
FPL	lateral forebrain bundle
GCt	midbrain central gray
GLV	lateral geniculate nucleus
HA	apical hyperpallium
Hb	habenula
HD	densocellular hyperpallium
Hp	hippocampus
HVC	proper name
ICo	intercollicular nucleus
IO	isthmo-optic nucleus
INP	intrapeduncular nucleus
IPN	interpeduncular nucleus
LA	lateral anterior nucleus of the thalamus
LLd	nucleus of the dorsal lateral lemniscus
LMAN	lateral magnocellular nucleus of the anterior nidopallium
LoC	locus coeruleus
LS	lateral septum
LSt	lateral striatum
M	mesopallium
MLd	lateral mesencephalic nucleus
MS	medial septum
MSt	medial striatum
MV	motor nucleus of the trigeminal nerve
N	nidopallium
Nc	caudal nidopallium
NCM	caudomedial nidopallium
nIV	trochlear nucleus
OMd	dorsal nucleus of the oculomotor nerve
OMv	ventral nucleus of the oculomotor nerve
PMI	paramedial internal nucleus of the thalamus
POM	medial preoptic nucleus
PPT	pedunculopontine tegmental nucleus
Pt	nucleus pretectalis
PTM	medial pretectal nucleus
PVN	paraventricular nucleus

RA	robust nucleus of arcopallium
SH	septohippocampal septum
SLu	semilunar nucleus
SNc	compact substantia nigra
SNr	reticulated substantia nigra
TeO	optic tectum
TnA	nucleus taeniae of the amygdala
VMH-lat	lateral ventromedial hypothalamus
VMH-med	medial ventromedial hypothalamus
VP	ventral pallidum
VTA	ventral tegmental area

Table 1. The Distribution of [^{125}I]-OVTA and [^{125}I]-LVA binding and VT1, VT3 and VT4 Receptor mRNA in White-throated Sparrow Brain.

Brain Region	[^{125}I]-OVTA	[^{125}I]-LVA	VT1	VT3	VT4
Ad	++/+++	+	-	++/+++	-/+
APH	+/++	-	-	+	-
BSTm	-	-	-	+/++	-/+/++
Cb	-/+	-/+	-	++	-
DLL	+/++	-	-	+/++	-
DMP	-/+	-	-	++/+++	++/+++
EW	-/+	-	-	-	+/++
GCt	-/+	-	+/++	+	+
GLV	+/++	-	-	+/++	+
Hp	-/+	-	-	+	-
ICo	-/+	-	-	+	+
IO	-/+/++	-	-	+/++	+
LA	+/++	-	+/++	+	+
LMAN	-	-	-	-	+++
LoC	-/+/++	-	-	+/++	-/+
LS	++/+++	++	-/+	+/++/+++	-/+
M	+/++	-	-	+/++	-
MS	-/++/+++	+	-	+/++/+++	-/+
MSt	-/+	-	-	-/+	+
MV	+/++	-	-	++/+++	++
N	+/++	-	+/++	+/++	-
OMd	-/+	-	-	+++	-
OMv	-/+	-	-	+++	-
PT	-	-	+++	+	-
PTM	-/+/++	-	-	-	+
PVN	-	-	-	++	-/+
RA	-/+/++/+++	-/+	-	-/+	-
SNc	-	-	+	-	+/++
TeO	+	++	-/+	+/++	++/+++
TnA	+/++/+++	-	-	-/+	-
VMH-lat	-	-	-	+	++
VMH-med	-	-	-	+	++
VTA	-	-	-	-	+/++

*Plus signs indicate relative density of VT-like binding or relative expression of VT receptor mRNA. Minus signs indicate the absence of VT-like binding or VT receptor mRNA. Slashes indicate individual variation. Because each VT receptor subtype was run in a separate assay, symbols for VT receptor mRNA pertain to levels of expression observed within each particular subtype, and not in relation to other subtypes.

Table 2. The Distribution of [^{125}I]-OVTA and VT1, VT3 and VT4 Receptor mRNA in Zebra Finch Brain.

Brain Region	[^{125}I]-OVTA	VT1	VT3	VT4
Ad	+/++/+++	-/+	-/+	-
AM	++	+	-	-
APH	-/+/++	-	+	-
Area X	-	-	-	-/+
BST	-	-	-	-/+
Cb	-/+	-	-	-
DMP	+/++/+++	-	+	+
EW	-	-	-	-
GCt	-/+	-	-	+
GLV	-/+	-	-	-
HA	+/++	+	-	-
HD	-/+	+/++	-	-/+
Hp	-	-	-	-
HVC	-/++	-	+	-
IO	-	-	-	-
LA	-/+	-/+	-	-
LMAN	-	-	-	-
LoC	+/++	-	-	+
LS	++/+++	-/+	+/++	-/+
LSt	-	+	-	-
M	-/+	+/++	-	-
MS	-/+	-/+	-	-
MSt	-/+	-	-	-/+
MV	+	-	++	-
N	+/++	-/+	+	-/+
Nc	+/++/+++	+	+	+
OMd	+	-	++/+++	-
OMv	-/+	-	++/+++	-
Pt	-	++/+++	-	-
PVN	-	-	-	-
RA	-	-	-	-
SNc	-	-	-	+
SNr	-	-/+	-	+
TeO	+/++	+	+	+/++
TnA	-/+/++	-/+	-	-
VMH-lat	-	-	-	-/+/++
VMH-med	-	-/+	-	+
VTA	-/+/++	-/+	+	+/++

*Plus signs indicate relative density of VT-like binding or relative expression of VT receptor mRNA. Minus signs indicate the absence of VT-like binding or VT receptor mRNA. Slashes indicate individual variation. Because each VT receptor subtype was run in a separate assay, symbols for VT receptor mRNA pertain to levels of expression observed within each particular subtype, and not in relation to other subtypes.

Figure Legends

Figure 1. VT1, VT3 and VT4 receptor expression and [I^{125}]OVTA and [I^{125}]LVA binding in sections in the brain of a white-throated sparrow. Anterior-posterior (AP) coordinates follow Stokes et al. (1974). Symbols represent the distribution of where receptor expression and binding were observed. Blue " x " represents [I^{125}]OVTA binding, blue " o " represents [I^{125}]LVA binding, yellow " = " represents VT1 expression, red " / " represents VT3 expression and green " o " represents VT4 expression.

Figure 2. VT1, VT3 and VT4 receptor expression and [I^{125}]OVTA binding in sections in the brain of a zebra finch. Anterior-posterior (AP) coordinates follow Nixdorf-Bergweiler and Bischof (2007). Symbols represent the distribution of where receptor expression and binding were observed. Blue " x " represents [I^{125}]OVTA binding, yellow " = " represents VT1 expression, red " / " represents VT3 expression and green " o " represents VT4 expression.

Figure 1.

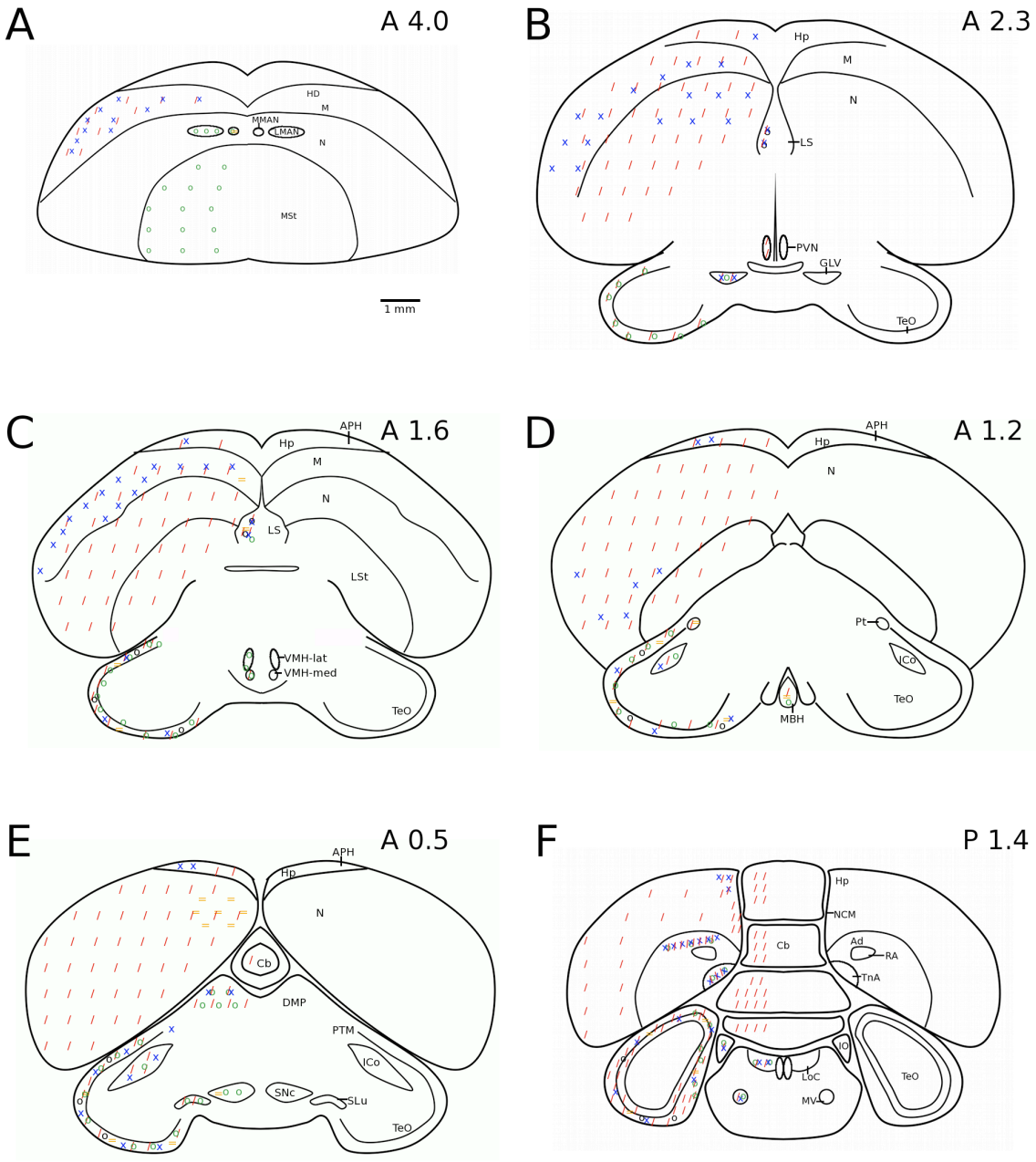
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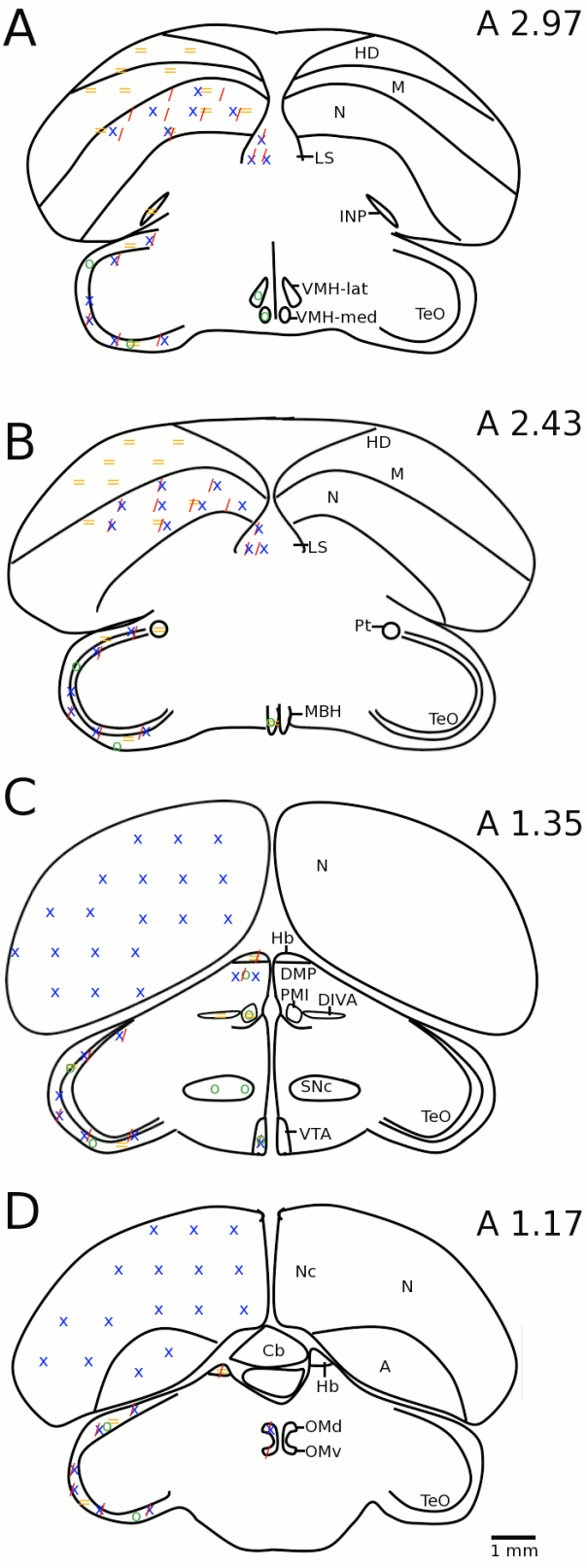
Figure 2.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