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Neural distribution of oxytocin receptors during development in zebra finches

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Abstract

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Juvenile zebra finches memorize and learn to sing the song of a single male caregiver during a complex vocal learning process. Juveniles are highly motivated to interact with their tutor, and they learn song best when they can do so. It is currently unknown what neurological mechanism may contribute to the social motivation of juvenile finches, but social motivation and affiliation of juvenile mammals may be facilitated by the nonapeptides oxytocin (OT) and vasopressin (VP). Three nonapeptide receptors, the oxytocin, vasopressin 1a, and vasopressin 2 receptors, have been proposed to facilitate the effects of OT and VP on social behavior. Variation in the neural distribution of these receptors can greatly alter their effects on behavior. Here we used qPCR to quantify mRNA expression of nonapeptide receptors in four brain regions: lateral septum(LS), caudomedial nidopallium (NCM), HVC, and dorsal arcopallium (Ad). We have shown that zebra finches express nonapeptide receptors in regions that underlie social motivation or song learning throughout the entirety of development. Expression differed according to age, but not sex, in multiple brain regions. While the distribution of nonapeptide receptors was previously known for adult finches, this was the first study to find expression of nonapeptide receptors in juvenile songbirds. Our study has provided the groundwork for the use of zebra finches as a model for understanding the relationship between the nonapeptide system and juvenile social motivation in the context of behavioral and neurological development.

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Introduction

Background

Socially rewarding stimuli enhance the motivation of juveniles to stay engaged in their social environment and actively learn social cognitive skills from caregivers. Children preferentially orient towards rewarding auditory and visual social stimuli, such as parent's voices and faces, to facilitate such learning (Chevallier et al., 2012). Caregiver vocalizations elicit attention and active responses also in juvenile male zebra finches (*Taeniopygia guttata*), which learn the song of their father during a complex learning process early in life (Houx and Ten Cate, 1998). The results of operant conditioning tasks indicate that in the absence of any unconditioned reward, juveniles find their tutor's song rewarding and will keypress to hear it (Adret, 1993). Juveniles develop a preference for the song of their father over those of other males, and learn to copy his song almost exclusively (Adret 1993; Immelmann, 1969). Cross-fostering studies have provided evidence that early exposure to social stimuli determines who the juvenile prefers to listen to and copy (Immelmann, 1969). Juveniles tend to learn from their male caregiver even if he is a heterospecific foster father and the biological father is housed in the same enclosure (Immelmann, 1969). Juveniles will continue to interact with and learn from the caregiver they have imprinted on even after fledging and once they can feed independently (Zann, 1996). In combination with evidence that juveniles learn better from live tutors than pre-recorded tutor songs (Eales 1989, Kuhl, 2006), this evidence could indicate that song facilitates learning by enhancing the motivation of juveniles to interact with their caregiver. Despite the likely contributions of social reward and social motivation to the ability of juveniles to learn song, it is still unclear what neurological mechanisms underlie the motivation of juvenile zebra finches to interact with a specific caregiver, even after fledging, and learn his song.

One proposed neurological mechanism is the nonapeptide system (Gordon et al., 2011). A recent study provided evidence that nonapeptides facilitate the formation of affiliative bonds of juvenile zebra finches with their caregivers (Baran et al., 2016). Some authors have demonstrated that species-specific social stimuli like physical touch or vocalizations of a caregiver may induce release of nonapeptides in the brain (Landgraf & Neumann, 2004; Seltzer et al., 2010). Because the peptides can be released diffusely in the brain (Landgraf & Neumann, 2004), variation in receptor distribution may greatly alter their effects on behavior (Goodson et al., 2012). This hypothesis is supported by evidence that species with different strategies for social behavior also differ with respect to receptor distribution (Goodson et al., 2012). In adult mammals and birds, nonapeptide receptors are located not only within a network of brain regions involved in social behaviors like aggression, sexual behaviors, and affiliation, but also in cortical areas that receive signals from this social behavior network (Goodson, 2005; Bielsky & Young, 2004). These downstream cortical areas contribute to social recognition through memory formation and may regulate the activity of the social behavior network in a top-down manner (Bielsky & Young, 2004).

The distribution of nonapeptide receptors during development varies not only by species but also by age (Vaidyanathan et al., 2016). Evidence from mice, rats, and voles has revealed that receptors are not always expressed at constant levels throughout maturation, and that transient expression likely corresponds to species-specific sensitive periods for neural growth and behavioral development (Hammock & Levitt, 2013; Vaidyanathan & Hammock, 2016; Tribollet et al. 1989, Barberis and Tribollet, 1996). Limited work, however, has directly tested whether periods of transient nonapeptide receptor expression overlap with sensitive periods for behavioral development and whether receptors are transiently expressed in brain regions that

physically mature in response to developmentally relevant environmental stimuli (Vaidyanathan & Hammock 2016). To our knowledge, no study has been conducted on the relationship between the nonapeptide receptor system and the motivation to learn complex social skills over extended periods during development. Therefore, a primary aim of this study was to map the neural distribution of nonapeptide receptors throughout the entire course of development in juvenile zebra finches, a species for which the distribution of these receptors in early life is currently unknown.

The zebra finch is a powerful model species for examining the relationships between the social motivation of juveniles to interact with caregivers during social learning and neurological development because the timelines for both have been extensively studied (Nordeen and Nordeen, 1990; Bottjer et al., 1985; Scharff & Nottebohm, 1991; Iyengar et al., 1999; Mooney, 2009). Song learning involves two phases: one for sensory acquisition and another for sensorimotor learning (Figure 1). The sensory phase of learning begins early in development, around day 15 post hatch (p15) and continues to p65 (Böhner, 1990). During this phase both males and females memorize their male caregiver's song and develop a preference for it (Böhner, 1990, Riebel et al., 2002). Even though lasting memories of song are not formed before p15, birds can respond to vocal stimuli by begging immediately upon hatching (Arnold, 1975; Muller & Smith 1978). During the sensorimotor phase, which overlaps with the sensory phase and lasts from around p35 to p90 (Immelmann, 1969; Johnson, 2002), the juvenile males begin to copy their father's song. During this period, juveniles remain motivated to learn and will repeatedly rehearse their song (Johnson, 2002). Females do not sing, but they develop a preference for their father's song (Riebel et al., 2002).

Hypothesis

Broadly, we hypothesized that nonapeptide receptors would be expressed, in brain regions underlying social motivation and song learning in juvenile zebra finches, during two phases of song learning. Because males sing and females do not, we hypothesized further that receptor expression would differ between males and females during the sensorimotor phase of song learning.

Experimental Approach

To more clearly understand the relationship between nonapeptide receptor distribution and the timeline of song learning in juvenile zebra finches, we measured the mRNA expression levels of three nonapeptide receptors known to be expressed in the songbird brain, from age p5 to p95. We used quantitative-real time polymerase chain reaction (qPCR) to quantify mRNA in four regions of interest: the lateral septum (LS), the caudomedial nidopallium (NCM), HVC, and the dorsal arcopallium (Ad). We selected these regions on the basis of prior literature suggesting their potential roles in social recognition or song learning (described below).

Nonapeptide Receptors:

Each vertebrate species typically has two nonapeptides. Mammals typically have oxytocin and vasopressin, while birds typically possess the homologous peptides mesotocin and vasotocin (Acher & Chauvet, 1995). Mammals possess four nonapeptide receptors, three of which have been studied in the context of social behavior: the oxytocin receptor (OTR), vasopressin receptor 1a (V1a), and vasopressin receptor 2 (V2) (Albers, 2015). Each receptor has a unique distribution within the brain and has unique binding affinities for oxytocin and

vasopressin (O'Connell & Hoffmann, 2011; Goodson et al., 2012). Birds possess a homologous set of nonapeptide receptors that also have unique neural distributions and unique binding affinities for either mesotocin or vasotocin (Leung et al., 2009; Goodson et al., 2012).

Of oxytocin and vasopressin receptors, oxytocin receptors have more frequently been proposed to underlie social motivation (Gordon et. al, 2011). In juvenile rats, mice, and prairie voles, oxytocin receptors are expressed in a variety of brain regions involved in social recognition and memory during the early stages of development (Vaidyanathan and Hammock, 2016). The avian homolog of this receptor, the oxytocin-like (OT-like) receptor, resembles the mammalian OTR but is predicted to bind both mesotocin and vasotocin with roughly equal affinity (Baeyens and Cornett, 2006). The OT-like receptor has been suggested to play a role in affiliation and pair-bonding in adult zebra finches (Goodson et al., 2012)

The mammalian V1a receptor has been studied in the context of social behavior most frequently out of any of the vasopressin receptors (Albers 2015; Goodson et al., 2012). Multiple studies have suggested that the V1a may contribute to pair-bonding, affiliation, and social recognition, as well as vocal communication (Bielsky & Young, 2004; Albers, 2015; Campbell et al., 2009; Goodson & Bass, 2001). Campbell et al., (2009) compared the distribution of V1a receptors in two species of singing mice, and found higher densities of V1a-like receptors in the vocal structures of the species that relies more heavily on vocal communication (Campbell et al., 2009). The homolog in songbirds, the V1a-like receptor, has also been proposed to be involved in affiliation, aggression, and vocal communication (Goodson et al., 2012, Goodson et al, 2009)

In comparison with the OTR and V1a, the mammalian V2 receptor has been studied less frequently in the context of social behavior (Albers, 2015). Juvenile rats express the V2 receptor in brain regions that underlie memory (Kato et al., 1995). Although the function of the V2-like

receptor in birds has not been investigated in detail, it may mediate the effects of vasotocin in brain regions that underlie social behavior (Goodson et al., 2012, Leung et al., 2009). The V1a-like and V2-like receptors have both been predicted to bind vasotocin with a higher affinity than mesotocin (Baeyens & Cornett, 2006).

Regions of Interest:

Lateral Septum: In adult zebra finches and some species of mammals, nonapeptide receptors in the LS mediate the effects of both vasopressin-like and oxytocin-like neuromodulators on social recognition and other social behaviors (Bielsky & Young, 2004, Goodson et al., 2009). Nonapeptide receptor expression or oxytocin binding have been found in the LS of rats, mice, and voles during both the juvenile period and adulthood (Wang & Young, 1997; Tribollet et al., 1989; Hammock & Levitt 2013; Vaidyanathan & Hammock, 2016). All three receptors are expressed in the LS of adult zebra finches (Leung et al., 2011).

Caudomedial nidopallium: NCM, which is homologous to the mammalian secondary auditory cortex (Jarvis et al., 2013), is proposed to be a site of tutor song memory storage (Bolhuis & Moorman, 2015). It shows greater electrophysiological activity in response to the tutor song than to conspecific songs (Moorman et al., 2011), and therefore could facilitate differentiation of the tutor song from the songs of other males. Lesions of NCM diminish the preferences of adult males for tutor song, but do not alter preferences for other sounds, like female vocalizations (Moorman et al., 2011). In adult zebra finches, only the OT-like and V2-like receptors are expressed in NCM (Leung et al., 2011).

HVC: HVC has been proposed to be similar to sub populations of neurons in layer two and three of the mammalian motor cortex that are associated with vocal motor output, and is

classically considered a vocal pre-motor region in songbirds (Jarvis et al., 2004). It is likely involved in both the sensory and motor phases of song learning (Bolhuis & Moorman, 2015; Theunissen & Doupe, 1998). While only limited expression of the OT-like receptor was detected in HVC in adult zebra finches (Leung et al. 2011), preliminary data from our lab have indicated that the OT-like receptor is expressed in the HVC during the sixth week of development (Maney & Rodriguez-Saltos, 2016).

Dorsal Arcopallium: It is unclear whether Ad has a homologous region in mammals, but it shares features with the output layers of the mammalian neocortex, layers V and VI (Jarvis et al., 2013), in that it sends outputs to striatal and thalamic areas (Dugas-Ford et al., 2012). It has been argued that one function of Ad is to generate a comparison between the structures of memorized songs and newly generated ones to influence motor output (Bottjer & Altenau, 2010). Lesions of Ad may interfere with retention of the tutor song memory over time or disrupt the ability of finches to match their own song to template songs stored in memory (Bottjer & Altenau, 2010). Adult zebra finches express only the OT-like and V2-like receptors in Ad (Leung et al., 2011).

Methods

Animals

This study was conducted with the approval of Emory University Institutional Animal Care and Use Committee (# DAR-2003144-052618BN). We collected juveniles from a zebra finch breeding colony at Emory University (Atlanta, GA). The colony is housed in a mixed-sex aviary on long photoperiod (14h light, 10 h dark) where the birds were given food and water ad libitum. All birds were free to engage in social interaction, and parents had constant access to their offspring. Any birds with obvious signs of social stress were excluded.

We collected ninety-six birds in total. The youngest birds sacrificed were five days old (+/- 1 day), and each subsequent group was 10 days older than the last (e.g., p5, p15, p25, p35, p45, p55, p65, p75, p85, and p95 days). Five males and five females were collected for each age group except for post-hatch 65 day old females (p65F; n=4) and post-hatch 95 day old males (p95M; n=3). Because zebra finches can be reliably sexed by plumage and type of gonad at p45, birds of at least that age were sexed by plumage and the presence of an ovary or testis. Younger birds were sexed using PCR analysis of a small liver sample with the P2 and P8 primers developed by Griffiths et al. (1998).

Brain Sectioning

All birds were rapidly decapitated after being anesthetized with isoflurane. The brains of p5 birds were left in the skull because they are fragile and easily damaged if removed. The brains of all older birds were dissected from the skull. After removal, the brains were flash-frozen in powdered dry ice and stored at -80^{o}C until sectioning on a Leica CM1860 cryostat at -12^{o}C (Leica Biosystems). All brains were sectioned along a transverse plane corresponding to Stokes

et al., (1974). Alternating 300µm and 60µm sections were thaw-mounted on Superfrost (Fisher Scientific) and Superfrost Plus (Fisher Scientific) slides respectively and were returned to storage at -80^{o}C until the 300µm sections were punched and the 60µm sections were stained. To minimize variation in RNA degradation during storage, brains were cut and punched in an order that limited the difference in storage time to seven days between any two brains, longer and shorter storage periods were balanced by age and sex groups.

Nissl Staining

60µm sections were fixed to slides by dipping in 4% paraformaldehyde solution for 35 minutes, followed by two five-minute rinses in 0.1 M PBS. After dehydrating in 70%, 95%, and 100% ethanol, slides were stained with 0.75 M toluidine blue in 100% EtOH. Excess stain was removed with brief dips in 70%, 95%, and 100% ethanol before the sections were cleared with xylenes and cover slipped in DPX mountant.

Tissue Collection

Tissue was sampled under a dissecting microscope (Leica Biosystems) from four brain regions: Ad, HVC, NCM, LS. Because the sizes and locations of these nuclei differ slightly with age, sex, and angle of sectioning, adjacent 60µm sections were Nissl stained to provide a map of each section. For each tissue sample of Ad and HVC, three side-by-side 0.5mm punches were collected in two adjacent 300µm brain sections in the same hemisphere. A single 1mm punch was collected from NCM in the same hemisphere of two adjacent sections. For each bird and each of these three brain regions, the hemisphere for which the caudal border of each region was most clearly visible in the Nissl stained sections was selected to punch. This was done to

minimize the possibility of obtaining tissue from outside the region of interest. Two side-by side 0.5mm punches were collected from LS in two adjacent sections, directly along the midline. For the brains of p5 chicks, each sample of Ad, HVC, and NCM consisted of two sets of punches, one from each hemisphere in the same brain section. For Ad and HVC, two side by side 0.5mm punches were obtained from each hemisphere. For NCM, a single 1mm punch was obtained from each hemisphere. For the lateral septum, a single 0.5mm punch was obtained directly on the midline in two adjacent sections. Brain punches were re-frozen at -80^{o}C for a maximum of 2 days before RNA isolation.

RNA isolation and cDNA synthesis

Each tissue sample was homogenized with a rotor stator homogenizer after the addition of Qiazol (Qiagen, Hilden, Germany). Samples were further homogenized on Qiashredder columns according to the specifications of the accompanying protocol (Qiagen). An Allprep DNA/RNA Micro kit (Qiagen) was then used to extract both total DNA and total RNA from each sample following the manufacturers' instructions. RNA and DNA concentrations of each sample were quantified using a NanoDrop Lite Spectrophotometer (ThermoFisher Scientific). All RNA samples were stored at -80^{o}C until cDNA synthesis.

RNA for each sample was then reverse transcribed into cDNA using a Transcriptor First Strand cDNA Synthesis Kit with random hexamer primers (Roche). Between 200ng and 500ng of RNA was used for each 20µl cDNA reaction depending on the amount of RNA extracted during isolation. 400ng was transcribed for most samples, but when less than 400ng of RNA was extracted, the entire amount of extracted RNA was used. For each brain region, samples from all the birds were equally divided onto two cDNA plates that contained 49 samples balanced for age

and sex along with negative and positive control samples. cDNA was stored at -20^{o}C until real time quantitative polymerase chain reaction (RT-qPCR).

Quantification of Receptor Expression

To quantify expression of the receptors, we performed qPCR on a Roche LightCycler 480 Real-Time qPCR system (Roche) using primers that were custom designed using Roche universal probe library system assay design software and Genbank. Hydrolysis probes were selected from the Roche Universal Probe Library (Roche; Table 1). Each sample was run in triplicate in 10µl reactions containing 2.5µl of diluted cDNA, 1.3µl of water, 5.0µl of 2x Probes Master (Roche), 0.5µl of forward and reverse primer each, and 0.2µl of hydrolysis probe. Prior to qPCR, cDNA was diluted in PCR grade water. In the qPCR reactions used to quantify the mRNA expression of both reference genes, GADPH and PPIA, cDNA was diluted 1:10. In the reactions used to quantify expression of OT-like receptor mRNA and V1a-like receptor mRNA, cDNA was diluted at 1:5. In the reactions used to quantify V2-like receptor mRNA expression, cDNA was diluted 1:3. Two standard curves of cDNA from male and female zebra finches were also placed on every plate to measure reaction efficiency. Five stepwise 1:5 dilutions were made from a sample containing 50 ng of cDNA. Cycling conditions were 95 °C for 10 min, 45 cycles of 95 °C for 10 sec, 60 °C for 30 sec, and 72 °C for 10 sec.

Data Analysis

LightCycler 480 software (Roche) was used to calculate crossing point (Cp) values for each sample using the Absolute Quantitative/2nd Derivative Max setting. LightCycler 480 software (Roche) also calculated the efficiency (E) of each qPCR reaction based on the starting

concentration of each dilution in the standard curve and its Cp value. The software subsequently calculated the absolute quantity of RNA in each sample using the formula 'RNA Quantity=E^-Cp' where 'E' is the efficiency of the reaction and 'Cp' is the mean Cp value for each triplicate.

For two plates, which contained the V1a-like receptor gene samples for NCM and LS for all birds, the standard curve could not be calculated properly due to technical error during plate set-up. An external standard curve that consisted of all the data points in the four working standard curves from the two other V1a-like receptor gene plates was used in place of the non-working standard curves. The positive control sample of the non-working plates was also placed onto the standard curve to factor a working sample from each plate into calculations of efficiency.

Because a coefficient of variance of 21.8% for each triplicate of Cp values represents the maximal threshold with which a two-fold change in RNA quantity can be detected with 95% confidence, outliers were removed from triplicates yielding a coefficient of variance above 21.8% to minimize variance within triplicates (Bookout et al., 2006). A modified Dixon's q test was used to determine which replicate of each triplicate was an outlier (Dean and Dixon, 1951). The difference between the largest Cp value and the median Cp value was compared to the difference between the smallest Cp value and the median Cp value. If the larger of these two differences was twice the value of the smaller, then the replicate with the larger difference was removed. A maximum of one point was removed from each triplicate. Outliers were a sign of technical error of sample preparation within each triplicate.

The RNA concentrations of the target genes VT1 (V2-like receptor), VT3 (OT-like receptor), and VT4 (V1a-like receptor), as calculated by the Lightcycler 480 software (Roche), were normalized against those of the concentrations of the reference genes, GADPH and PPIA.

Normalization was done to account for variations between samples in the amount of RNA extracted or efficiency of transcription into cDNA. For each gene of interest, mRNA concentrations were normalized with each reference gene separately using the equation (gene of interest concentration/reference gene concentration). The geometric mean of the two normalized concentration values was taken to obtain the final normalized RNA concentrations that were used for all subsequent statistical comparisons.

Gene expression was analyzed in SPSS software (Version 22.0, IBMcorp, 2013) using two linear mixed models(LMM) that included the entire data set of RNA concentrations. We selected the LMM because it can account for missing data points, unlike other multivariate models such as MANOVA (Krueger and Tian, 2004). The dependent variable in both models was RNA expression level. Subject was also included as random factor in both. In the first model (referred to as LMM_{age}), the independent variables were age, sex, brain region, gene and all possible interactions of these variables. *Post-hoc* LMMs were then used to test for the effects of age, sex, and brain region within each gene, and for the effects of age and sex within each brain region and gene combination. Independent t-tests were used to test for pairwise differences between age groups. Time points that had high levels of expression were determined to be peaks if the expression levels differed significantly from any of the subsequent two or previous two time points.

For the second LMM containing the whole data set (referred to as LMM_{phase}), we separated birds based on the sensitive phases for song learning. The procedures used for this LMM were identical to those of the first, but the independent variable of age was substituted for learning phase. We divided song learning into three periods: 1) the sensory learning period from p5 to p25, 2) the early sensorimotor period from p35 to p55, and 3) the late sensorimotor period

from p65 to p95. Our selection of these groupings was based on previous literature suggesting the start of the sensorimotor phase to be p35 and the end of sensory phase to be p65 (Johnson, 2002; Eales 1989). Thus, what we call the early sensorimotor period encompasses the overlap between end of the sensory phase of learning and the beginning of the sensorimotor phase (Figure 3). What we call the sensory and late sensorimotor phases mostly correspond to sensory and motor learning, respectively (Figure 3). This terminology is consistent with previous literature that distinguishes between song learning phases in their analysis (Nick & Konishi, 2005).

Results

In the LMM_{age}, we found significant effects of gene, brain region, an age by region interaction, and a gene by region interaction (Table 2). In the LMM_{phase}, we found significant effects of gene, brain region, learning phase, a gene by region interaction, a learning phase by gene interaction, and a learning phase by region by gene interaction (Table 2). Because the gene significantly affected expression levels in both models, we conducted *post-hoc* LMMs for all three genes using two separate models which included either age or learning phase, respectively, in the group of independent variables. For all three genes, *post-hoc* tests revealed a significant effect of both age and learning phase (Table 3). Expression levels depended on brain region in both models, so subsequent LMMs including either age or learning phase were conducted for each gene and brain region combination (Table 4, 5, 6).

1) OT-like Receptor

We found OT-like receptor expression in all four brain regions of males and females at all stages of development. LMMs within each brain region revealed significant effects of age and learning phase in HVC and of age in NCM (Table 4).

For LS, no significant effect of age or learning phase was found (Table 4).

For NCM, there was a peak in expression at age p65, at which point expression was significantly different from both p55 (t_{17}=-3.09, p=.01; Figure 4B) and p85 (t_{16}=2.56, p=.02; Figure 4B).

For HVC, there was a peak in expression at age p65, at which point expression was significantly different from both p45 (t_{16}=-2.51, p=.02; Figure 4C) and p55 (t_{16}=-2.89, p=.01; Figure 4C). Expression during the early sensorimotor period also

differed from that in the sensory (t_{57}=2.52, p=.01; Figure 5A) and late sensorimotor (t_{64}=-4.04, p<.001; Figure 5A) periods.

For Ad, no significant effect of age or learning phase was found (Table 4).

2)V1a-like receptor:

We found V1a expression in all four brain regions of males and females at all stages of development. LMMs within each brain region revealed a significant effect of age in HVC and NCM (Table 5). We also found a significant effect of critical period in HVC, NCM, and LS (Table 5).

For LS, expression during the sensory phase was significantly different from that during the late sensorimotor phase (t_{61}=-2.01, p=.49; Figure 5B).

For NCM, pairwise t-tests comparing individual age groups revealed a peak in expression at p5. Expression differed significantly from that at p15 (t_{18}= 3.25 p<.01; Figure 6B) and p25(t_{18}= 2.39 p= 0.03; Figure 6B). There was also a nearly significant peak at p75, at which point expression almost differed from that at p55 (t_{18}= -1.89 p=.07; Figure 6B). In the model that included the variable of learning phase, expression differed between the sensory and early sensorimotor phases (t_{58}=2.81, p=.02; Figure 5B).

For HVC, pairwise t-tests revealed a peak in expression at p15. Expression at p15 differed from p25 (t_{18}=2.07 p=.05; Figure 6C) and p35 (t_{18}=2.07 p=.05; Figure 6C). Although the peak in expression occurred at p15, expression was also high at p5 and differed from p25 (t_{17}=2.7, p=.02; Figure6C) at that point. During the sensory period, expression differed significantly from that of both the early

sensorimotor (t_{56}=2.47, p=.02; Figure 5B) and late sensorimotor (t_{64}=2.74, p=.01; Figure 5B) periods.

For Ad, no significant effect of age or learning phase was found (Table 5).

3)V2-like receptor:

We found V1a expression in all four brain regions of males and females at all stages of development. The early pattern of V2-like receptor expression was different from that of the other receptors because there was little quantifiable expression at p5 in any of the regions. LMMs within each region revealed a significant effect of age and song learning period on expression in LS.

For LS, pairwise t-tests comparing individual age groups revealed a peak in expression at p25. Expression differed significantly from that at p5 (t_{17}= -2.36 p= 0.03; Figure 7A). The average expression levels at p35 were high and resembled a peak, but no significant differences were found between expression at this age and that of adjacent age groups (p35 vs. p15; t_{17}= 1.98, p= 0.06; Figure 7A). Expression was also significantly different between the sensory and early sensorimotor periods (t_{52}=-2.67, p= 0.02; Figure 5C), and between the sensory and late sensorimotor periods (t_{55}=-2.48, p= 0.01; Figure 5C).

For NCM, no significant effect of age or learning phase was found (Table 6).

For HVC, no significant effect of age or learning phase was found (Table 6).

For Ad, no significant effect of age or learning phase was found (Table 6).

Discussion

In the present study, we describe the patterns of OT-like receptor, V1a-like receptor, and V2–like receptor RNA expression levels at multiple time points over the course of development in male and female zebra finches. During the sensory and sensorimotor phases of song learning, respectively, young birds are socially motivated to interact with their caregivers and actively learn song (Adret, 1993, Johnson et al., 2002). Males and females develop a preference for their caregiver's song and will key-press to hear it in the absence of any additional unconditioned reward (Adret, 1993). Juvenile males also relentlessly practice their song during the sensorimotor phase of learning and sing undirected bouts of song, not directed towards any other individual, at much higher rates than adult males (Johnson et al., 2002). Although the neurological mechanisms underlying the social motivation of juvenile finches are not known in detail, there is strong evidence that nonapeptides play a role in the social recognition and social motivation of juvenile mammals early in development (Vaidyanathan & Hammock, 2016; Gordon et al., 2011). Perinatal disruptions of nonapeptide receptors in the entire brain of juvenile mammals can disrupt social recognition and social motivation. (Bielsky & Young, 2004; Ross & Young, 2009). Ross & Young (2009) argued that, in mice, administration of an oxytocin antagonist early in life resulted in reduced motivation of juveniles to return to caregivers after a period of separation. Administration of nonapeptide receptor antagonists has been found to reduce affiliative interactions with caregivers also in zebra finches (Baran et al., 2016). In this study, we found that all three nonapeptide receptors are expressed throughout development in four brain regions that underlie social recognition and song learning in zebra finches (Goodson et al., 2009; Mooney et al., 2009; Bolhuis & Moorman, 2015; Bottjer & Altenau, 2010). Our findings grant viability to

the hypothesis that nonapeptide receptor expression could facilitate social recognition and social motivation to engage in active song learning from a tutor.

Lateral Septum:

Multiple authors have provided evidence that the LS facilitates social recognition, social affiliation, and social memory in adults of a variety of species (Goodson & Bass, 2001). Direct manipulations of the nonapeptide system in the LS of adult mammals alters social recognition (Bielsky & Young, 2004). In adult zebra finches, antagonizing OT-like receptors in the LS results in reduced affiliative behaviors that are normally indicative of pair-bonding activity (Goodson et al., 2009). Given that nonapeptide receptor activity in the LS of adult zebra finches contributes to affiliative behavior, activity at these receptors in the LS of juveniles could also facilitate affiliative behaviors. We found that nonapeptide receptors are expressed in this region throughout the entire course of development. Importantly, expression occurred during the early juvenile period at a time when zebra finches are learning to recognize and are memorizing their tutor's song (Immelmann, 1969). By p35, zebra finches can form a strong enough memory of their tutor's song that they can learn to produce a copy of that song without any further exposure to their tutor during the sensory learning phase (Böhner,1990). V2-like receptor expression peaked around p25 and p35, while both the OT-like receptor and the V1a-like receptor were consistently expressed early in development. Nonapeptide receptor expression in the LS of juvenile zebra finches early in development is consistent with findings that juvenile mammals express these receptors in the LS during early life (Tribollet et al., 1989; Vaidyanathan & Hammock, 2016). If nonapeptides function similarly in the LS of juveniles and adults,

expression of the OT-like receptor and the V1a receptor in LS could facilitate either social recognition or affiliation.

We also found that expression of the OT-like receptor and the V1a like receptor in the lateral septum depended on the phase of learning. Expression was highest in the late sensorimotor period. Young zebra finches approach sexual maturity and may begin to form pairs during this time (Zann, 1996). Around p65, juveniles gradually begin to attend more to the vocalizations of other conspecifics than those of parents (Mulard et al., 2010). It is not surprising that nonapeptide receptors were highly expressed in the LS late in development because nonapeptidergic activity in this region underlies pair bonding and affiliation during adulthood (Lim and Young, 2004, Goodson et al., 2009).

Caudomedial Nidopallium:

Multiple authors have provided evidence that NCM is responsive to playbacks of the tutor song, and could be a storage site for tutor song memory (Mooney, 2009; Moorman et al., 2011). Lesions of NCM during adulthood have been shown to diminish preferences for the tutor's song (Gobes & Bolhuis, 2007). Gobes and Bolhuis suggest that activity in NCM may underlie the formation of a preference for tutor song during development. Given the contribution of nonapeptides to social recognition and social motivation during development (Ross & Young, 2009 Bielsky & Young, 2004), nonapeptide receptors in NCM of juvenile finches could potentially facilitate the social recognition of or motivation to hear tutor song. Our findings support this possibility because we found expression of nonapeptide receptors in NCM throughout development. Expression was not completely stable over time; there were peaks early and late in development. There was a peak in V1a-like receptor expression at p5, at which age

juvenile zebra finches are still in the nest and are heavily dependent on their caregivers (Zann, 1996). For both zebra finches and other species of birds, juveniles are capable of imprinting on caregiver vocalizations upon or before hatching (Zann, 1996; Zajonic et al., 1975). This evidence indicates that juveniles are receptive to auditory stimuli and begin to form affiliative bonds with their caregivers at a time when V1a-like receptor expression is high in NCM. There were also peaks in expression during the late sensorimotor period at p65 and p75, which correspond to early stages of sexual maturity (Zann, 1996, Mulard et al., 2010). Even though juvenile finches show a reduced preference for the tutor's song in the late juvenile period, they will continue to show a relatively strong preference for it into adulthood (Gobes & Bolhuis, 2010). Future research should address whether nonapeptide activity in NCM could be related to the maintained recognition of and preference for tutor song late in development.

HVC:

While HVC is typically thought of as a premotor nucleus critical for the motor component of song learning (Bottjer et al., 1985), evidence suggests that HVC is involved in song perception (Nick & Konishi, 2005). Early in the sensorimotor period, HVC cells are responsive to the tutor's song (Nick & Konishi, 2005). The region may contribute to song learning during development in part by facilitating the neural response to tutor song early in life. We found that nonapeptide receptors were expressed throughout development in HVC. As we found for NCM, in HVC there were peaks in expression early and late in development. There was an early peak in V1a-like expression at p15, which corresponds to a time when HVC cells are proliferating (Bottjer et al., 1985). Some authors have proposed that the onset of HVC maturation occurs around p15 (Bottjer et al., 1985). More recent evidence from *in situ*

hybridization strongly suggests that HVC cells have already begun to differentiate by p5; mRNA expression of Trk-b, a gene involved in cell differentiation, is strikingly localized in the HVC nucleus at that age (Chen et al., 2005). It is unclear how the observed peak in V1a-like receptor expression during the time of HVC cell differentiation may contribute to song learning because little is known about HVC's contribution to behavior so early in development.

We also found peaks in nonapeptide receptor expression during the late sensorimotor period. During this time, HVC cells become less responsive to the tutor song and more responsive to the bird's own song (Nick & Konishi, 2005). The late peak in nonapeptide receptors, therefore, occurs during a time when the contribution of HVC to sensory representations of the tutor song is declining. While males are motivated to practice their song at high rates during the late sensorimotor period (Johnson et al., 2002), it is unlikely that nonapeptide receptor expression in HVC is related to song production or a neural response to the bird's own song because we found no difference in nonapeptide receptor expression between males and females. At the least, this finding indicates that receptor expression in HVC late in development is not caused by song production in males.

Dorsal Arcopallium:

Like HVC, Ad has also been associated with motor learning in zebra finches. Lesions of Ad during the juvenile period do not cause immediate deficits in song production, but over time lesioned birds lose the ability to sing a precise copy of the tutor's song. (Bottjer & Altenau, 2010). Lesions of Ad could disrupt memories of tutor song or alter the birds' abilities to match their own songs to stored song memories. Once again, our finding that all three receptors are expressed throughout development in Ad is consistent with evidence that nonapeptide receptors

are expressed in brain regions involved in social memory and vocal communication (Vaidyanathan et al., 2016, Campbell et al., 2009). We found no peaks in expression, or any effect of learning phase on expression in Ad. There was constant expression throughout the early and late sensorimotor periods during the time when Ad may contribute to sensorimotor learning of song (Bottjer & Altenau, 2010).

Other considerations:

It should be considered that the variability between individuals was high within many age groups for each gene and brain region. Because a primary aim of this study was to quantify gene expression in the brains of juvenile zebra finches over the entire course of development, we reduced the sample size within each age group in favor of measuring expression at more developmental time points. Small sample sizes and high variability amongst individuals potentially reduced our power to detect differences in receptor expression between the sexes at individual time points. Despite this shortcoming, our findings are consistent with the results of other studies that have revealed high individual variation in the expression of genes for nonapeptides and their receptors. (Bielsky & Young, 2004, Vaidyanathan & Hammock, 2016). Expression of genes for both the receptors and ligands varies among individual adult zebra finches in multiple brain regions (Leung et al 2010, Leung et al., 2011). Individual variation in the expression of these genes predicts variation in affiliative behaviors, such as maternal attachment and pair bonding, in some species of rodents (Donaldson & Young, 2008). Variation in song learning abilities between birds may, accordingly, be a cause or potential effect of our observed variation in receptor expression.

To our knowledge, this is the first study to describe either the presence or locations of nonapeptide receptors in the brains of juvenile songbirds. Our findings are consistent with the results of a recent study that provided evidence for the role of nonapeptides in affiliative behaviors of juvenile zebra finches towards their parents (Baran et al., 2016). In that study, juvenile finches were treated with an intracerebroventricular vasotocin receptor antagonist during the first week of life. Their preference to spend time with either their parents or unfamiliar conspecifics was measured multiple times from p30 to p86. The authors found that the antagonist-treated group failed to show an affiliative preference to spend time their parents. In retrospect, the results of this study have important implications for our understanding of the role of nonapeptides in juvenile zebra finch behavior, but a limitation of this study was that the authors chose to administer a nonapeptide receptor antagonist without any evidence that juvenile zebra finches express nonapeptide receptors in the brain. Because we have provided evidence that these receptors are present in juvenile finch brains throughout development, and because we have located the receptors within individual brain regions, future experiments can be designed to locally manipulate the nonapeptidergic system in these brain regions.

Manipulations of receptors in regions where the receptors are known to be expressed at particular developmental time points could be a more precise approach for understanding the relationships between the neurological components of the nonapeptide system and developmentally relevant social behaviors. This knowledge could enhance our understanding of human developmental disorders that are characterized by disruptions in both the nonapeptide system and social learning. Autism spectrum disorder (ASD), for example, can cause significant deficits in social learning early in life, and these deficits may be the result of disrupted social reward and motivation (Chevallier et al., 2012). The role of nonapeptides in ASD is supported by

evidence that individuals diagnosed with autism have reduced transcription of oxytocin receptor mRNA (Gregory et al., 2009). Our study has provided the groundwork for the use of zebra finches as a model for understanding the relationship between the nonapeptide system and juvenile social motivation as connected to behavioral and neurological development. Because neurological components of the nonapeptide system can be manipulated and studied within the context of the well-described song learning process of juvenile finches, this model could improve our understanding of deficits in social motivation and social learning in humans, particularly in the context of neurodevelopmental disorders like ASD.

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Tables and Figures

Table 1. Reference and Target Genes quantified with qPCR

Gene	Hydrolysis Probe No.	Gene Accession No.
GAPDH	76	NM_001198610.1
PPIA	38	NM_001245462.1
OT-like receptor	4	JN594029.1
V1a-like receptor	107	JN594032.1
V2-like receptor	89	JN594025.1

Table 2. Results of whole LMM analyses

	LMM_{age} [b]			LMM_{phase} [c]		
Variable	F stat	Df (num,denom)	p value	F stat	Df (num,denom)	p value
Gene	**214.35**	**2, 398.94**	**<.001**	**120.29**	**2, 1016**	**<.001**
Sex	0.07	1, 105.31	.79	0.068	1, 96	.795
Region	**19.63**	**3, 461.40**	**<.001**	**19.46**	**3, 1023**	**<.001**
Time[a]	1.52	9, 105.23	.15	**6.60**	**2, 95**	**.002**
Gene*Sex	1.07	2, 398.94	.34	0.652	2, 1016	.521
Gene*Region	**41.43**	**6, 390.02**	**<.001**	**26.148**	**6, 1016**	**.000**
Gene*Time[a]	**3.06**	**18, 398.71**	**<.001**	**3.35**	**4, 1016**	**.010**
Sex*Region	0.84	3, 461.40	.48	0.763	3, 1023	.515
Sex*Time[a]	0.83	9, 105.23	.59	0.957	2, 95	.388
Region*Time[a]	1.39	27, 460.65	.09	1.642	6, 1023	.132
Gene*Sex*Region	0.39	6, 390.02	.89	0.293	6, 1016	.941
Gene*Sex*Time[a]	0.59	18, 398.71	.91	0.080	4, 1016	.988
Gene*Region*Time[a]	**1.75**	**54, 393.22**	**<.002**	**2.186**	**12, 1016**	**.011**
Sex*Region*Time[a]	0.80	27, 460.65	.75	0.534	6, 1023	.783
Gene*Sex*Region*Time[a]	0.82	54, 393.22	.82	0.255	12, 1016	.995

a: time refers to either age or learning phase depending on the model; b: linear mixed model including age as an independent variable;
c: linear mixed model including learning phase as an independent variable

Table 3. Results of LMM analyses within gene

		LMM_{age} [b]			LMM_{phase} [c]		
Gene	Variable	F stat	Df (num,denom)	p value	F stat	Df (num,denom)	p value
OT-like Receptor	**Time[a]**	2.50	9, 378	.10	**7.53**	**2, 378**	**.001**
	Sex	0.27	1, 378	.60	0.33	3, 378	.57
	Region	**29.38**	**3, 378**	**<.001**	**23.88**	**1, 378**	**<.001**
	Sex*Time[a]	1.04	9, 378	.407	0.54	2, 378	.59
	Region*Time[a]	1.19	27, 378	.239	**2.13**	**6, 378**	**.05**
	Sex*Region	0.69	3, 378	.560	0.78	3, 378	.51
	Sex*Region*Time[a]	0.85	27, 378	.684	.684	6, 378	.97
V1a-like receptor	**Time[a]**	**2.07**	**9, 378**	**.031**	**3.60**	**2, 378**	**.03**
	Sex	0.03	1, 378	.87	.066	1, 378	.80
	Region	**36.66**	**3, 378**	**<.001**	**28.54**	**3, 378**	**<.001**
	Sex*Time[a]	0.41	9, 378	.93	0.48	2, 378	.62
	Region*Time[a]	**1.67**	**27, 378**	**.02**	**2.23**	**6, 378**	**.04**
	Sex*Region	0.15	3, 378	.93	0.19	3, 378	.91
	Sex*Region*Time[a]	0.85	27, 378	.69	0.45	6, 378	.85
V2-like receptor	**Time[a]**	**3.91**	**9, 353**	**<.001**	**3.85**	**2, 353**	**.022**
	Sex	3.63	1, 353	.058	2.50	1, 353	.115
	Region	**5.95**	**3, 353**	**.001**	**5.35**	**3, 353**	**.001**
	Sex*Time[a]	*1.54*	9, 353	.132	0.46	2, 353	.632
	Region*Time[a]	0.60	27, 353	.948	0.36	6, 353	.905
	Sex*Region	0.33	3, 353	.808	0.20	3, 353	.899
	Sex*Region*Time[a]	0.30	27, 353	1.0	0.41	6, 353	.837

a: time refers to either age or learning phase depending on the model; b: linear mixed model including age as an independent variable; c: linear mixed model including learning phase as an independent variable

Table 4. Results of LMM analysis for the OT-like receptor

		LMM_{age} [b]			LMM_{phase} [c]		
Brain region	Variable	F stat	Df (num,denom)	p value	F stat	Df (num,denom)	p value
LS	Time[a]	0.79	9, 92	.63	2.50	1, 92	.09
	Sex	0.04	1, 92	.83	0.05	2, 92	.82
	Sex*Time[a]	0.90	9, 92	.53	0.08	2, 92	.92
NCM	**Time[a]**	**2.44**	**9, 95**	**.02**	2.65	1, 95	.08
	Sex	1.73	1, 95	.20	1.88	2, 95	.17
	Sex*Time[a]	0.80	9, 95	.62	0.10	2, 95	.90
HVC	**Time[a]**	2.60	9, 95	.10	**7.96**	**1, 95**	**.001**
	Sex	0.66	1, 95	.42	0.72	2, 95	.40
	Sex*Time[a]	0.64	9, 95	.76	0.32	2, 95	.73
Ad	Time[a]	1.50	9, 96	.14	2.54	1, 96	.08
	Sex	0.94	1, 96	.34	0.92	2, 96	.24
	Sex*Time[a]	1.57	9, 96	.14	2.25	2, 96	.11

a: time refers to either age or learning phase depending on the model; b: linear mixed model including age as an independent variable; c: linear mixed model including learning phase as an independent variable

Table 5. Results of LMM analysis for the V1a-like receptor

		LMM_{age} [b]			LMM_{phase} [c]		
Brain region	Variable	F stat	Df (num,denom)	p value	F stat	Df (num,denom)	p value
LS	**Time[a]**	1.61	9, 92	.12	**3.26**	**1, 92**	**.04**
	Sex	0.01	1, 92	.92	0.04	2, 92	.85
	Sex*Time[a]	0.82	9, 92	.60	0.15	2, 92	.86
NCM	**Time[a]**	**4.69**	**9, 96**	**<.001**	**3.66**	**1, 96**	**.03**
	Sex	0.31	1, 96	.58	0.39	2, 96	.53
	Sex*Time[a]	0.53	9, 96	.85	0.27	2, 96	.76
HVC	**Time[a]**	**3.63**	**9, 95**	**.001**	**5.95**	**1, 95**	**.004**
	Sex	1.31	1, 95	.25	1.12	2, 95	.29
	Sex*Time[a]	1.29	9, 95	.25	0.31	2, 95	.74
Ad	Time[a]	0.74	9, 95	.67	0.72	1, 95	.49
	Sex	0.002	1, 95	.97	0.03	2, 95	.86
	Sex*Time[a]	0.626	9, 95	.77	0.89	2, 95	.41

a: time refers to either age or learning phase depending on the model; b: linear mixed model including age as an independent variable; c: linear mixed model including learning phase as an independent variable

Table 6. Results of LMM analysis for the V2-like receptor

		LMM_{age} [b]			LMM_{phase} [c]		
Brain region	Variable	F stat	Df (num,denom)	p value	F stat	Df (num,denom)	p value
LS	**Time[a]**	**3.40**	**9, 82**	**.001**	**3.06**	**1, 82**	**.05**
	Sex	0.33	1, 82	.57	0.28	2, 82	.60
	Sex*Time[a]	**2.10**	**9, 82**	**.04**	0.46	2, 82	.63
NCM	Time[a]	0.71	9, 87	.70	0.84	1, 87	.43
	Sex	0.26	1, 87	.61	0.14	2, 87	.71
	Sex*Time[a]	1.06	9, 87	.40	0.03	2, 87	.97
HVC	Time[a]	1.92	9, 95	.06	0.64	1, 95	.53
	Sex	0.93	1, 95	.34	0.45	2, 95	.50
	Sex*Time[a]	0.45	9, 95	.90	0.28	2, 95	.76
Ad	Time[a]	1.13	9, 89	.34	1.48	1, 89	.23
	Sex	1.41	1, 89	.24	1.02	2, 89	.32
	Sex*Time[a]	0.66	9, 89	.75	0.70	2, 89	.504

a: time refers to either age or learning phase depending on the model; b: linear mixed model including age as an independent variable; c: linear mixed model including learning phase as an independent variable

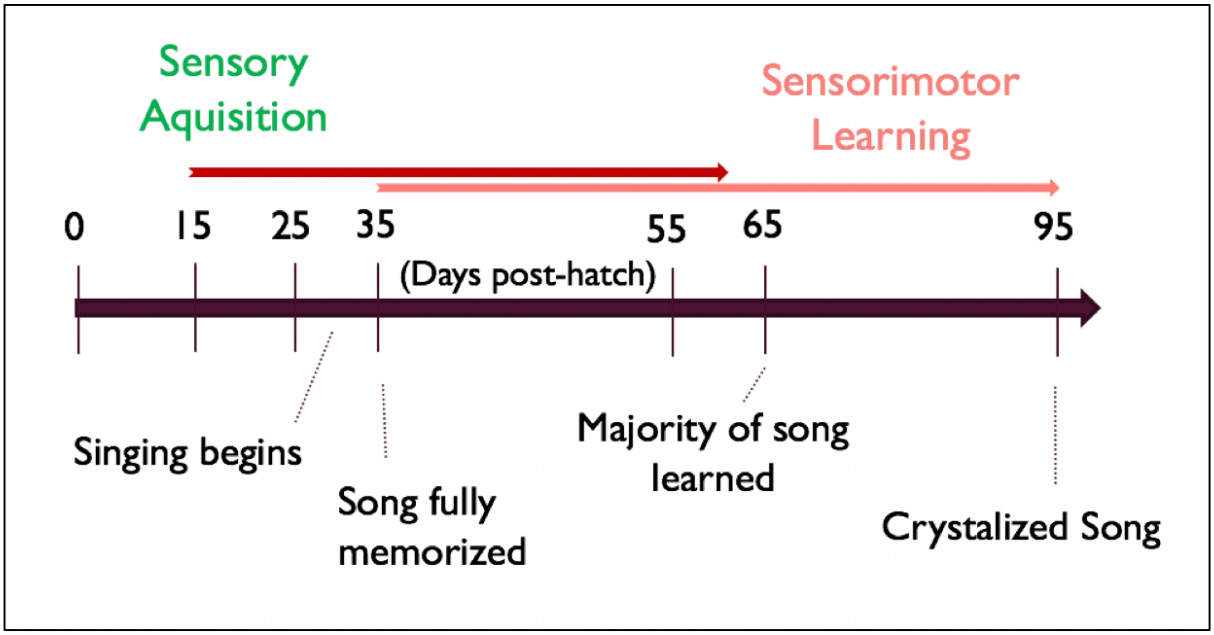

Figure 1. **Timeline of song learning in zebra finches.** Birds are capable of memorizing tutor song by post hatch day 35 (p35) but new song elements heard up to day p65 can be memorized and incorporated into song (Eales, 1989). Birds begin to sing subsong between p30 and p35 (Johnson, 2002). The height of behavioral plasticity in the sensorimotor period occurs between p50 and p60 as the bird starts to develop a more adult-like song structure (Fehér et al., 2016; Johnson et al., 2002). Analysis of acoustic features indicates that the majority of structural components, like syllables, are learned by p65, while most of the additional spectral qualities are refined by p80 (Fehér et al., 2016). Birds sing a fully "crystallized" adult song by age p90 or p100 (Immelmann, 1969).

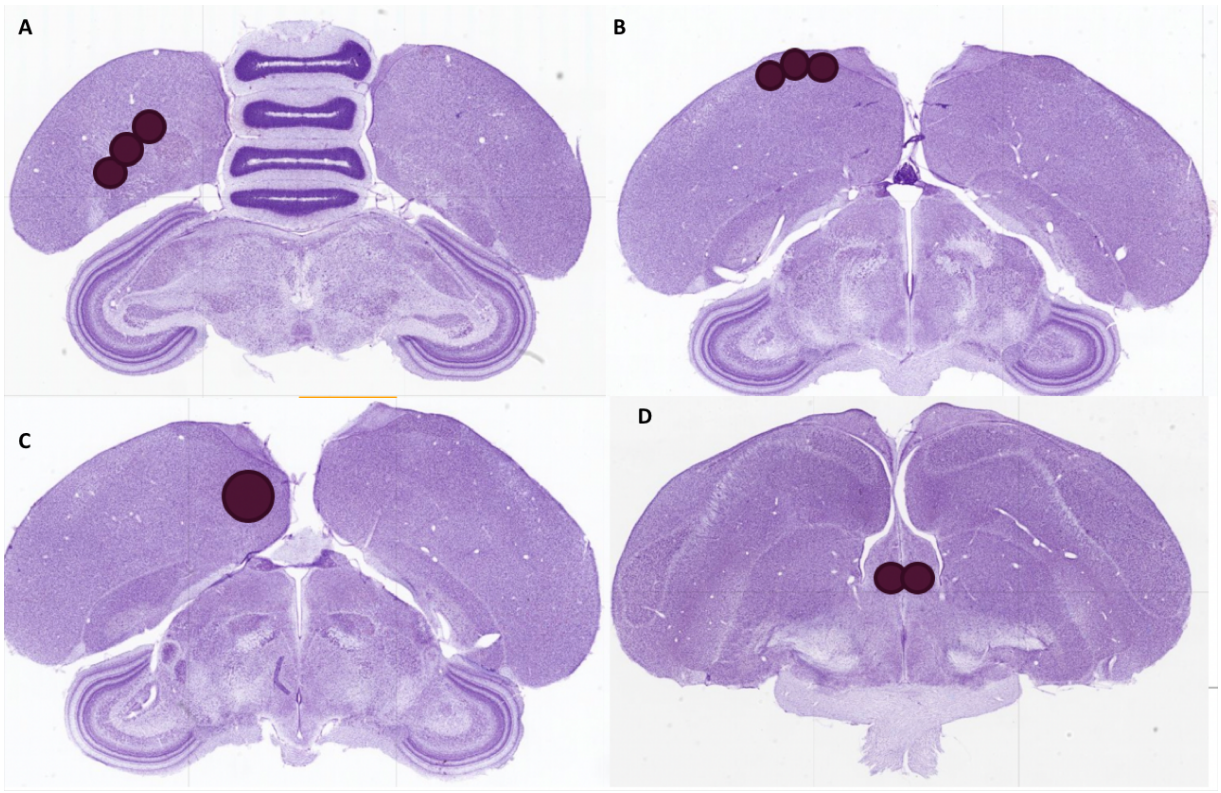

***Figure 2*. Examples of locations of brain punches**. Images of four representative Nissl-stained sections, each containing a region of interest, are shown. Circles indicate the locations of micropunches. The placement of side-by-side 0.5mm punches is shown for the regions Ad (A), HVC (B), LS (D). The location of a single 1mm punch is shown for NCM (C). Images are from zebrafinch.brainarchitecture.org (Karten et al., 2008).

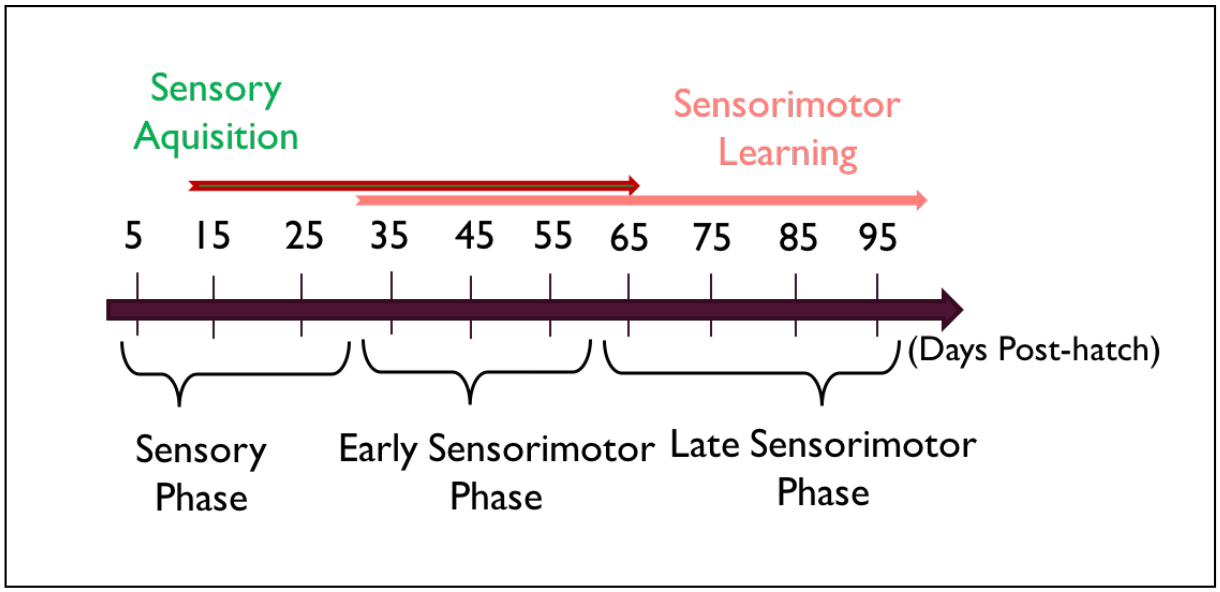

Figure 3. **Phases of song learning used in data analysis.** Song learning consists of two overlapping phases (Eales 1989). In our study, the age groups p5, p15, and p25 were included in the sensory phase, p35, p45, and p55 were included in the early sensorimotor phase, and p65, p75, p85, and p95 were included in the late sensorimotor phase.

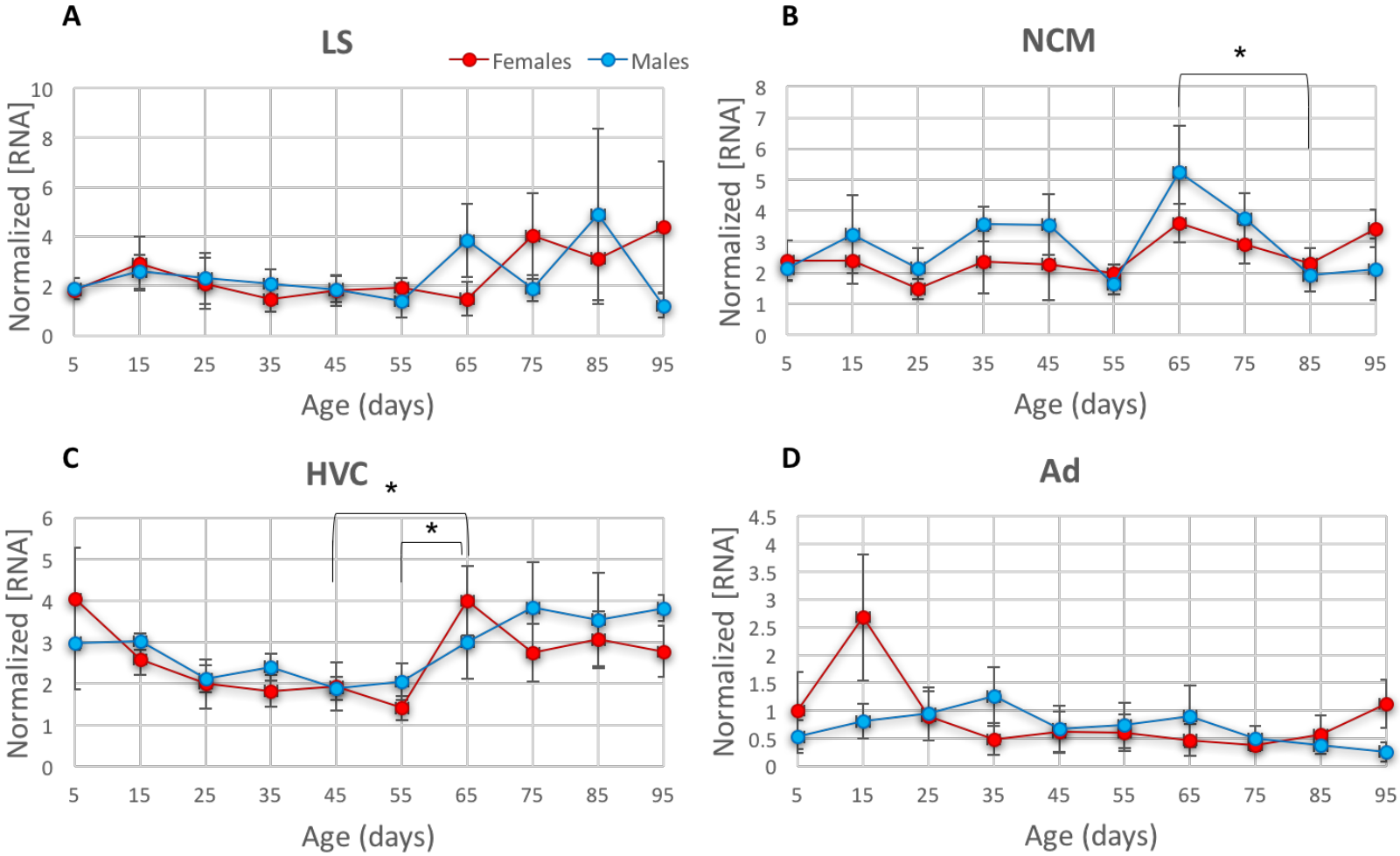

Figure 4. **OT-like receptor expression in males and females of each age group**. Mean normalized concentrations of OT-like RNA (mRNA copies/ng) in males and females from each age group are plotted for each brain region: Ad (A), HVC (B), NCM (C), and LS (D). Mean expression values +/- SEM for females are plotted in red, and males are plotted in blue. *p<.05

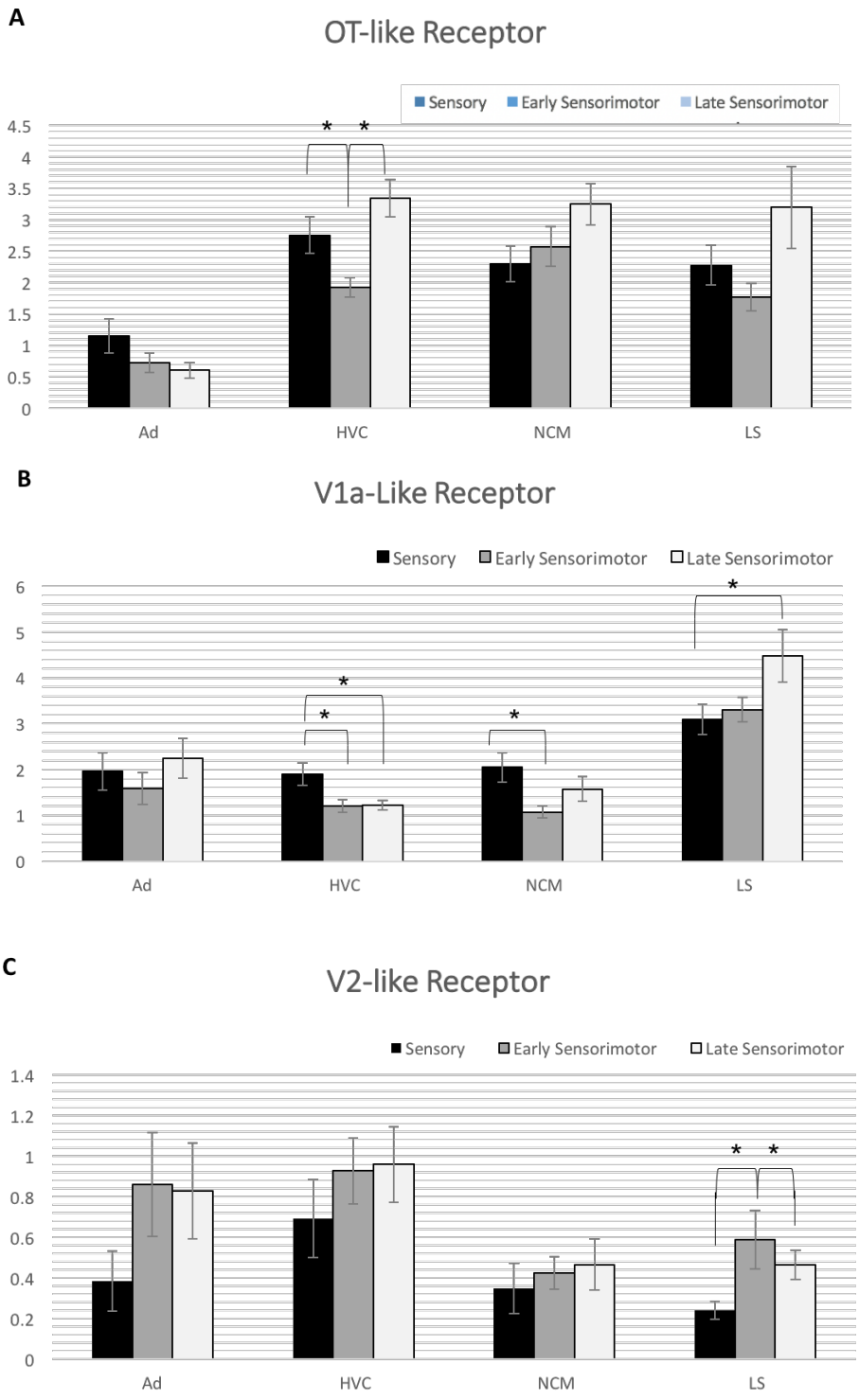

Figure 5. **Expression of nonapeptide receptor RNA during the phases of song learning.** Mean values of normalized RNA concentrations (RNA copy number/ng RNA) for the OT-like receptor (A), V1a-like receptor (B), and V2-like receptor (C) during each phase of song learning. Data from males and females are pooled. *p<.05, $^{+}$ p<.10

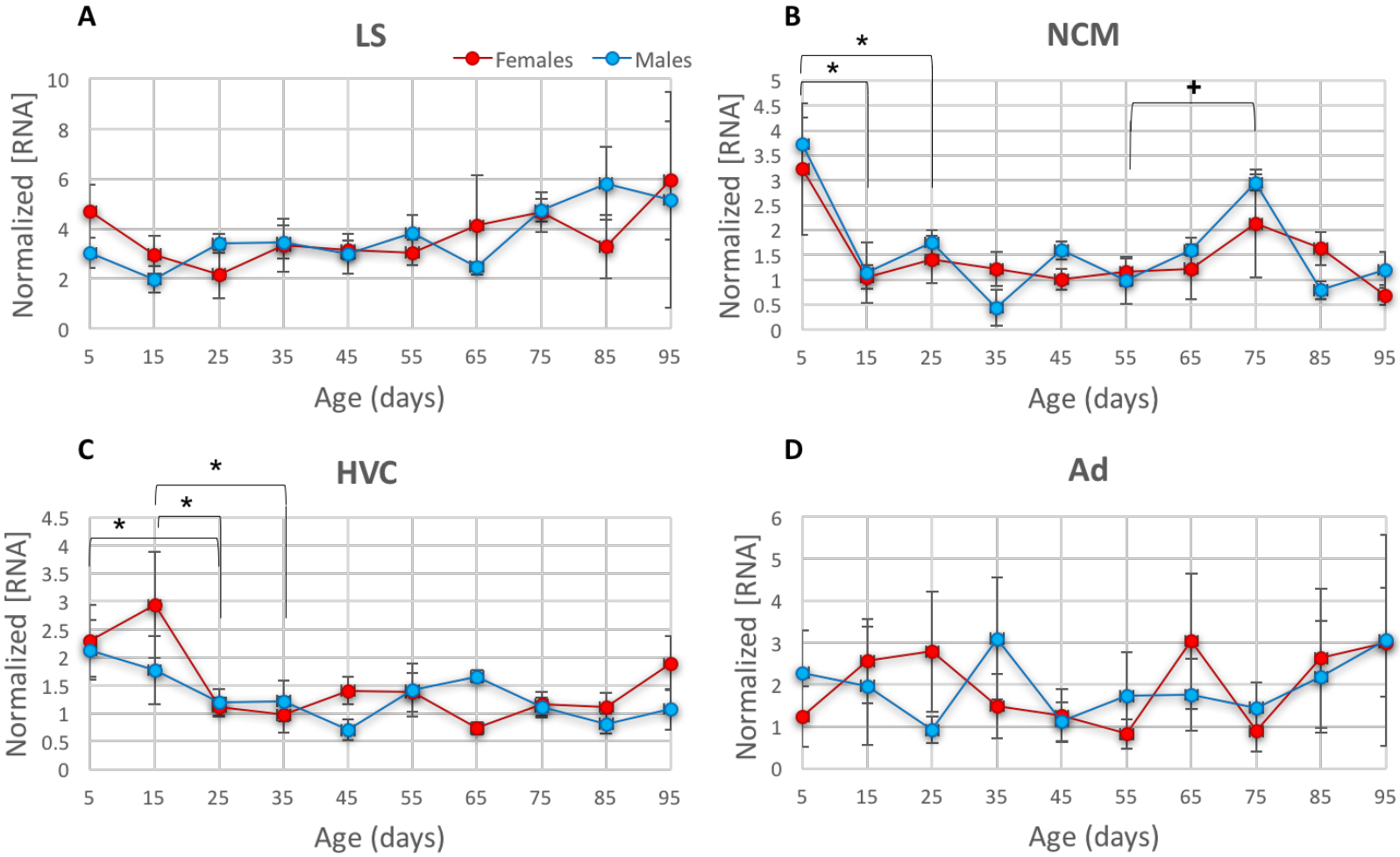

Figure 6. **V1a-like receptor expression in males and females of each age group**. Mean normalized concentrations of OT-like RNA (mRNA copies/ng) in males and females from each age group are plotted for each brain region: Ad (A), HVC (B), NCM (C), and LS (D). Mean expression values +/- SEM for females are plotted in red, and males are plotted in blue. *p<.05, $^{+}$p<.10

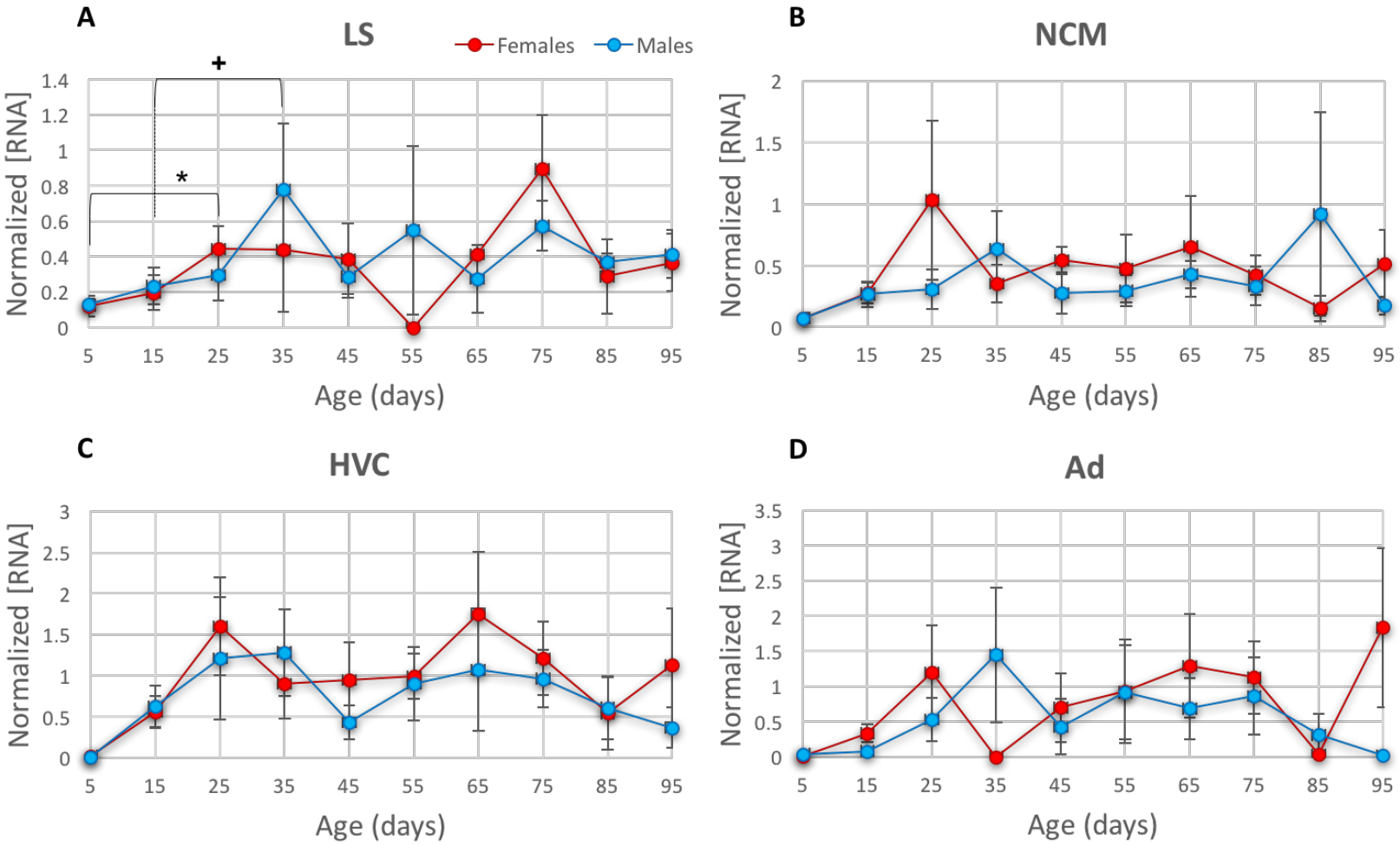

Figure 7. **V2-like receptor expression in males and females of each age group**. Mean normalized concentrations of OT-like RNA (mRNA copies/ng) in males and females from each age group are plotted for each brain region: Ad (A), HVC (B), NCM (C), and LS (D). Mean expression values +/- SEM for females are plotted in red, and males are plotted in blue. *p<.05 $^{+}$p<.10