ABSTRACT

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Autism spectrum disorder is associated with deficits in three main behavioral areas: social behaviors, repetitive behaviors, and language abnormalities. Accumulating evidence suggests a potential role for oxytocin signaling in the etiology of autism. Oxytocin signaling is integral to complex social behaviors such as fear, social recognition, and monogamous pair bonding initiation (Wang and Aragona 2004; Kirsch, Esslinger et al. 2005; Choleris, Little et al. 2007). Furthermore, evidence of a causal relationship between altered oxytocin levels and repetitive behaviors exists both humans and animals (Drago, Pederson et al. 1986; Hollander, Novotny et al. 2003). However, limited research exists to document a role for oxytocin and oxytocin related peptides in communication related behavior either in humans or animal models. Oxytocin immunoreactive neurons have been found in brain areas associated with the vocal behaviors of both the mustached bat and the plainfin midshipman fish and increased plasma levels of oxytocin have been linked with the production of sound in humans (Goodson, Evans et al. 2003; Grape, Sandgren et al. 2003; Prasada Rao and Kanwal 2004). Since song birds learn a form of vocal communication, the zebra finch represents a promising animal model for the study of such peptides to determine their role in vocal learning and development and for evaluating effects of exogenously administrated oxytocin on vocal development. As an avian species, zebra finches utilize mesotocin rather than oxytocin signaling (these nonapeptides differ by a single amino

acid). Since previous studies suggest a feasible role for oxytocin related peptides in vocal development we developed the following hypotheses: 1) that the mesotocin receptor is distributed in song regions within the male zebra finch brain responsible for both the production of and the learning of song; 2) that the early exposure to peripheral oxytocin would alter normal male zebra finch vocal development; 3) the alteration of zebra finch vocal development would be associated with a decrease in mesotocin receptors within song regions that persists until adulthood. Specific aim 1 was to determine the localization of mestocin receptors within the CNS of an adult male zebra finch. Both traditional cloning techniques and the identification of coding sequences in-silico were employed to gain knowledge of the cDNA sequences corresponding to the mRNA encoding of proteins integral to the mesotocin signaling within the zebra finch which allowed for the development of nucleic acid probes and an antibody to study expression of the zebra finch mesotocin receptor. In sections of fixed zebra finch brain tissue, mesotocin receptor expression patterns at various stages of vocal development: 10 days, 25- [auditory learning], 50- [subsong], 75- [plastic song], and 100-days of age [crystallized song] were determined. Expression of the mesotocin receptor was detected within areas associated with either song learning (Area X, lMAN (lateral magnocelluar nucleus of the anterior nidopallium), song production (HVC (used as a proper noun), RA (robust nucleus of the archopallium), or auditory inputs (L2) at 25, 50, 75, and 100 days of age with peak receptor expression occurring at 50 days. In-situ hybridization experiments were also done to assess mesotocin receptor mRNA expression at 25, 50, 75, and 100 days and results indicated significant expression of mesotocin mRNA in the vocal motor song region HVC. This distinct pattern of staining of the mesotocin receptor, which changes as a function of age corresponding to noted periods of vocal learning in brain areas previously determined to be critical for various

aspects of song learning and production, suggests a feasible role for mesotocin signaling in either the production of song, in song learning or both. Specific aim 2 was to determine if exposure to exogenous oxytocin could alter vocal development. Nests of zebra finch hatchlings were injected with varying doses of oxytocin on days 5-9 post hatch. Young male birds were allowed to learn a song from an established adult male tutor and their resulting songs were recorded, analyzed, and compared to those of the control treated group. The overall quality of the song learned (as expressed by song stereotypy scores) and mean note duration were significantly decreased as a function of treatment in both oxytocin dose groups (30 μg/kg, 300 μg/kg) when compared to that of the vehicle treated group. Our results clearly show that exogenous peripheral exposure to oxytocin is capable of producing alterations in song learning in the zebra finch and this may be the result of oxytocin effects on song learning and/or the production of song as a form of social communication. Specific aim 3 determined if the exogenous exposure to oxytocin would persistently alter the density of mesotocin receptors within song regions in treated birds when compared to that of the control treated group. No significant difference was found to exist in the mean optical density of mesotocin receptor immunohistochemical staining in the four song regions examined (vocal motor regions HVC and RA, and song learning regions lMAN and Area X) between the vehicle treated control group and the oxytocin treated birds. These findings suggest that the alteration in normal zebra finch vocal development oxytocin is not through a persistent alteration in mesotocin receptor densities in song control regions. In conclusion, we have shown that the oxytocin related peptide mesotocin may be critical to either song learning, the auditory perception of song, or the vocal production of song since its receptors are located in song control regions previously demonstrated to be critical for these functions. Furthermore, we have demonstrated that the peripheral administration of oxytocin to a young

zebra finch hatchling is capable of producing an alteration in the production of the final adult song which could be through a dysfunction in one or all of the various processes of song learning (such as the vocalization of song, the auditory perception of song, or the memorization and learning of song) but that this alteration is not by a long term down-regulation of the mesotocin receptor in song control regions. These results highlight a potential relationship between oxytocin exposure and the alteration of vocal development which is a deficit associated with autism spectrum disorders.

OXYTOCIN MODULATION OF ZEBRA FINCH VOCAL DEVELOPMENT.

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LIST OF ABREVIATIONS

ADDM	Autism and Developmental Disabilities Monitoring
APRE	acute-phase response elements
Area X	Area X within medial striatum
ASD	autism spectrum disorders
Asn	asparagine
Asp	aspartic acid
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CNS	central nervous system
CSF	cerebral spinal fluid
DAG	diacylglycerol
DBRPC	double-blind, randomized, placebo-controlled
DLM	medial portion of the dorsolateral nucleus of the thalamus
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders IV Edition
GABA	gamma-aminobutyric acid
GPCR	G-protein coupled receptor
HEK	human embryonic kidney
ICV	intracerebroventricular
IP	intraperitoneal
IP3	inositol triphosphate
kDa	kilodalton

lMAN	lateral magnocelluar nucleus of the anterior nidopallium
MDMA	3,4-methylenedioxymethamphetamine
mRNA	messenger RNA
NMDA	N-methyl-D-aspartate
OT	oxytocin
OXTR	oxytocin receptor
PDD-NOS	Pervasive Developmental Disorder Not Otherwise Specified
PGE2	prostaglandin E2
PVN	paraventricular nucleus of the hypothalamus
RA	robust nucleus of the archopallium
Tukey's HSD	Tukey's honestly significant difference test
SC	subcutaneous
SNPs	single nucleotide polymorphisms
SON	supraoptic nucleus

CHAPTER ONE: INTRODUCTION

Autism Spectrum Disorders

Definition

As defined by the *Diagnostic and Statistical Manual of Mental Disorders IV Edition (DSM-IV)* autism spectrum disorders (ASD) are a group of neurodevelopmental disorders defined as pervasive developmental disorders that include austism, Asperger's disorder, and pervasive developmental disorders not otherwise specified, and individuals suffering from these disorders exhibit the following abnormal behaviorial characteristics "1) limited reciprocal social interactions, 2) disordered and non-ordered verbal and nonverbal communication, and 3) restricted, repetitive behaviors and circumscribed interests" [(Volkmar and Klin 2005) as cited in (Tager-Flusberg and Caronna 2007)]. ASD is typically diagnosed in the toddler years when children begin to to show symptoms of the abnormal behaviors in the three areas such as abnormal communication with others, unusual playtime routines, and a lack of social bonding with parental figures or other children (Tager-Flusberg and Caronna 2007). As of December 2009, the Centers for Disease Control and Prevention estimated in their ADDM autism prevalence report that the diagnosis of ASD had increased to 1 in every 110 births in America affecting 1 in 70 boys (Rice December 18, 2009). The Autism Society reports that 1.5 million Americans currently live with the effects of ASD and an estimated lifetime cost ranging from $3.5 to $5 million to care for a child with ASD (Society 2010). Asperger's disorder is generally characterized as lacking the delay in early language development typically seen in autism, but with patients having marked dysfunction in social skills and restricted interests. These children use words that are from a more advanced vocabulary than other children and their intellectual abilities can range from comparable to that of their peers to brilliance. In contrast, it is estimated

that at least half of all children with autism have intellectual capabilities defined as mental retardation or scoring below 70-75 on standardized IQ tests (Klin, McPartland et al. 2005). The third disfunction of the three behaviorial disorders grouped under the umbrella of ASD, PDD-NOS, is typically diagnosed when individuals demonstrate impairments in the triad of symptoms associated with ASD but they do not meet the full diagnoistic criteria for either Autism or Aspergers as defined in the DSM-IV (Association 2000). Please refer to Table 1.1 for the diagnostic criteria of autism, Asperger's Disorder, and PDD-NOS (Association 2000).

Abnormal Social Skills

Alterations in social skills when compared to that of their age matched peers is one of the defining characteristics of ASD. These children avoid social contact even with their own parents, typically using the parent as a means to get what they desire (e.g. food, toys) and their behavior is characterized by severe outbursts such as tantrums when forced to interact with others. They sometimes appear to be fearful of new people and new situations. Most or all forms of social interactions are impaired in these individuals with a lack of eye gaze to speakers, abnormal body stances, little to no interest in developing social bonds such as friendship, and limited apparent social give and take. Therefore, all of the aspects discussed early in the formation of social bonds are hindered (Miles, McCathren et al. 1993-2003 Aug 27 [updated 2010 Apr 13]). ASD sufferers do not engage in the initial approach of another individual and one of the DSM-IV diagnostic criteria for ASD includes "a lack of spontaneous seeking to share enjoyment, interests, or achievements with other people" (Association 2000). Please see Table 1.1 for more detail. Proper processing of social cues is vital to social bonding and autistic individuals are frequently described as unable to process facial expressions such as frowns or smiles and other non-verbal cues (body posturing, eye gazes, and touch) to ascertain another

person's emotional state (Lim and Young 2006).

Communication Deficits

The dysfunction in language and communication is another core symptom in the diagnostic criteria of ASD with children whose communication skills vary from impaired to being completely nonverbal. Autistic toddlers have delays in learning to speak and display a significant decrease in vocalizations compared to their peers, and the communications that are given by affected individuals are often used as a means to control their surroundings in the form of demands for food, toys, and other items and as well as complaints (Wetherby 1986; Mitchell, Brian et al. 2006). The vocalizations that are used can be characterized as odd and may include sounds such as moans, screeches, hums, grunts, or the echoing of sounds and phrases spoken which is referred to as echolalia [(Kanner 1943) as cited in (Rapin and Dunn 2003)]. A decrease in the use of words previously learned by a toddler between years one and two is seen in one fourth of children diagnoised with autism. This change in speech is frequently accompanied by the child becoming socially reclusive or a shift in the child's disposition (Luyster, Richler et al. 2005). Non-verbal communication is also impaired in affected individuals as autistic children use a decreased number of gestures and eye gaze to either communicate their wants or to understand meaning and this differs from the non-verbal communication skills and abilites of children affected with other forms of communication disorders (Mitchell, Brian et al. 2006). Children with ASD are also unable to interpret the tone (e.g, pleasant, angry) of social interaction and communication from non-verbal cues such as the expression on a person's face (grinning, grimacing, scowling), the posture of the speaker, or the tone of the speaker's voice (Loveland and Tunali 1991). This lack of non-verbal and altered verbal communication follows people suffering from ASD into adulthood where these

difficulties can impede interactions with others. Recent prospective studies indicate 60% - 75% of adults with autism experience poor or very poor outcomes when they were followed throughout their lives (Newschaffer, Croen et al. 2007).

Oxytocin

Oxytocin-Related Ligands

Oxytocin (OT) is a neurohypophysial hormone and was named after the "quick birth" for its classical physiological role in the generation of uterine contractions during labor and delivery [(Dale 1906) as cited in (Gimpl and Fahrenholz 2001)]. Oxytocin is a member of the nonapeptide superfamily and is characterized by a disulfide bridge between cystiene residues 1 and 6 which results in a six-amino acid cyclic portion and a three residue terminal portion [(Du Vigneaud, Ressler et al. 1953) as cited in (Gimpl and Fahrenholz 2001)]. The nonapeptides can be classified into two families: the OT family and the vasopressin family and representatives of both familes can be found most of all of species classified as vertebrates (Archer, Chauvet et al. 1995). Oxytocin peptides have been associated with reproductive functions and social behaviors. As a member of the avian family of vertebrates, zebra finches produce mesotocin instead of OT, but the difference between the two peptides is a single amino acid substitution of an isoleucine for the leucine present in OT at the eighth residue. See Table 1.2. There are limited published reports in the scientific literature detailing the physiological actions of the nonapeptide mesotocin although it can be found in a great number of animals.

Gene Structure

Oxytocin and vasopression are located on the same chromosome but are transcribed in opposite directions in order to produce their gene products [(Gainer 1998) as cited in (Gimpl and Fahrenholz 2001)]. Please see Figure 1.1 for more detail. The general structure of the gene is

three exons with the first encoding a translocation signal which targets the nascent protein to the rough endoplasmic reticulum, the nonapeptide hormone itself, the tripeptide processing signal (GKR) that acts as the site of cleavage of the peptide from its carrier molecule neurophysin by a dibasic endopeptidase, and the first 9 residues of neurophysin. The second and third exons encode the remaining portion of neurophysin and in humans it has been determined that these genes are located on chromosome 20p13 (Rao, Loffler et al. 1992). Neurophysin acts as a carrier molecule ensuring the proper packaginig of OT within the neurosecretory vesicles and it has been determined to exist in a 1:1 ratio with OT within the neurosecretory granules of the posterior pituitary (Rose, Wu et al. 1996). The bond between OT and neurophysin (pKa ~6.4) is via multiple hydrogen bonds that are stronger in the acidic environment in the granules of neurons (pH ~5.5) than in the plasma (pH 7.4) and thus the two proteins disassociate upon release into the bloodstream (Velikson, Cohen et al. 1998).

Oxytocin Receptor

Homology

The high degree of homology amongst members of the OT family of nonapeptides is also mirrored in the high degree of homology for the corresponding receptors. Published reports indicate that the toad mesotocin receptor shares a 70% identity with the amino acid sequence of mammalian OT receptors, while the isotocin receptor of the teleost fish has a 66% identity (Hausmann, Meyerhof et al. 1995; Akundova, Getmanova et al. 1996). Gimpl and Fahrenholz (2001), reported that oxytocin and vasopressin receptors contain sequence homologies with each other (approximately 50% for the V1 vasopressin subtype and 40% for the V2 vasopressin subtype) with the highest areas of homology contained within the extracellular loops and the transmembrane helices and with the lowest being found in the termini of the proteins (Kimura,

Tanizawa et al. 1992; Seibold, Brabet et al. 1992; Thibonnier, Auzan et al. 1994; Gimpl and Fahrenholz 2001).

Structure

The oxytocin (OT) receptor is a standard Gq G-coupled protein receptor, and it is structurally similar to other members of the rhodopsin-type G-protein superfamily with 7 transmembrane spanning α-helices, an extracellular NH_2 terminal domain, and an intracellular COOH-terminal domain as demonstrated in Figure 1.2 (Kimura, Tanizawa et al. 1992). The OT receptor's molecular mass is calculated to be 44 kDa based on cDNA sequences (Kimura 1998; Breton, Chellil et al. 2001). Previously published western blot data demonstrate labeled bands of higher molecular weights in the brushtail possum (60kDa), the rat (65kDa), the macaque monkey (55kDa) and human (66kDa) when using OT receptor antibodies (Frayne J 1998; Whittington K 2004; Fink JW 2005; Feng J 2009). In their review of the oxytocin receptor system Gerald Gimpl and Faulk Fahrenholz (2001), therorized that the differing molecular masses might be attributed to differences in glycoslylation since it has been determined that the OT receptor has potential glycoslylation sites in the NH_2 terminal domain though the number of glycoslations can vary from species to species (two for the mouse and rat and three for the human, pig, sheep, and other mammals) and from location to location within a specific species (Muller, Soloff et al. 1989; Kojro, Hackenberg et al. 1991; Gimpl and Fahrenholz 2001).

Cell Signaling and Receptor Properties

As a Gq coupled receptor, the OT receptor facilitates its actions upon binding of an agonist by the stimulation of phospholipase C, the subsequent production of inositol triphosphate (IP3) and diacylglycerol (DAG) (Kimura, Tanizawa et al. 1992; Ku, Qian et al. 1995). The increase in these two secondary messengers leads to the stimulation of protein kinase C whose

phosphorylation targets vary from tissue to tissue and a rise in intra-celluar calcium (Anwer and Sanborn 1989). The rise in intra-cellular calcium can lead to the initiation of multiple intracellular events depending on the location of the OT receptor. For example, in smooth muscle cells, the increase in calcium intiates the formation of the calcium-calmodulin complex which causes the activation of myosin light-chain kinase activity that generates smooth muscle contraction (Sanborn, Dodge et al. 1998). In neurons, it is thought that the increasing levels of intra-cellular calcium modulates neuronal firing patterns and generate neurotransmitter release and protein synthesis (Gimpl and Fahrenholz 2001). See figure 1.3 for an illustration of signaling events.

Receptor and Signaling System Regulation

Agonist stimulation may cause a rapid desensitization of the OT receptor as it does with most of the other GPCRs. Gimpl and Fahrenholz (2001), reported in their review of the oxytocin receptor system that they observed a greater than 60% internalization of OT receptors expressed in HEK 293 fibroblasts within 5 to 10 minutes of exposure to an agonist [unpublished data as reported in (Gimpl and Fahrenholz 2001)]. Furthermore it was observed by Gimpl and colleagues that the population of internalized OT receptors was not recycled back to the surface of the cell upon agonist withdrawl [unpublished data as reported in (Gimpl and Fahrenholz 2001)]. The internalization of these receptors occurs mainly as a result of a clathrin-dependant pathway (Gimpl, Burger et al. 2000). Figure 1.4 represents a possible pathway for OT receptor desensitization. Gimpl and Fahrenolz in their review (2001), suggest that the OT receptor may contain potential serine phosphorylation sites to act as a retention signal for the internalized receptor as a similar retention system has been shown to exist in the V2 (vasopressin) receptor (Innamorati, Sadeghi et al. 1998; Gimpl and Fahrenholz 2001). Persistent stimulation of OT

receptors by OT not only alters the number of receptors at the cell surface, but it has been demonstrated to alter the ability of the receptor to bind to OT as demonstrated by an experiment where human myometrial cells that were exposed to OT for 20 hours displayed a reduction of the ability of OT to bind to the OT receptor by tenfold. Furthermore in this experiment while the total number of OT receptors was not altered by 48 hours of incubation with OT, OT receptor mRNA was reduced which the authors hypothesized may be due to either transcriptional suppression or destabilization of OT receptor mRNA (Phaneuf, Asboth et al. 1997). In another experiment HEK 293 cells treated with micromolar concentrations of OT for 18 hours displayed a reduction of 50% of the initial binding capacity determined for the OT receptors (Jasper, Harrell et al. 1996).

Spatial Receptor Distribution within the CNS

The distribution of OT receptors within the central nervous system varies from species to species. In the rat, OT receptors are expressed in several brain regions including the olfactory system, cortical areas, the limbic system, basal ganglia, the thalamus, the hypothalamus, the brain stem, and the spinal cord and there was no difference in the expression pattern between males or females (Barberis and Tribollet 1996). The diversity of central OT receptor expression patterns in mammals may reflect the great divergence of social behaviors associated with each species. An example of this is the differing patterns of central OT receptor distribution found between monomogous prarie voles and the more promiscuous montane voles where high levels of OT receptors were found to be localized in areas asoociated with reward such as the nucleus accumbens and the ventral palladium in the monomogous prarie voles when compared to that of the montane voles (Wang and Aragona 2004). The guinea pig, the hamster, the rabbit, and the marmoset all lack OT receptors within the ventral subiculum of the hippocampus, but there is a

high density of these receptors in rat brains (Tribollet, Dubois-Dauphin et al. 1992). Human brain contains a large number of OT binding sites within the pars compacta of the substantia nigra unlike other species investigated thus far suggesting a role for OT in motor functions (Mazurek, Beal et al. 1987). In studies performed in the rat by Yoshimura and colleagues, (1993) it was determined that OT receptor mRNA was detectable in many but not all of the areas previously demonstrated to contain OT receptors (Yoshimura, Kiyama et al. 1993). For example mRNA levels were unquantifiable in areas of the olfactory system, the limbic system, the thalamus and hypothalamus, and the brain stem where high densities of OT binding have been demonstrated (Yoshimura, Kiyama et al. 1993). The regulation of central OT receptors can be complex and also varies amongst mammals. OT receptors within the brain have been found to be regulated in the rat by estrogen, testosterone, progesterone, and glucocortiocoids (Schumacher, Coirini et al. 1990; Tribollet, Audigier et al. 1990; Coirini, Schumacher et al. 1991; Insel, Young et al. 1993; Patchev, Schlosser et al. 1993; McCarthy 1995).

Physiological Functions

Peripheral Effects

Physiological functions of the OT signaling system can be divided into two areas based on the physical location of the hormone: peripheral effects and central effects. The pregnant uterus is where OT serves as one of the most potent uterotonic agents and a synthetic analog of OT known as pitocin is used clinically to initiate and maintain uterine contractions during parturition (Brunton, Parker et al. 2008). In the human uterus the OT receptor increases by 200 times at the onset of labor and delivery when compared to that of the non-gravid uterus (Fuchs, Fields et al. 1984). This increase in receptors allows OT to stimulate contractions at plasma concentrations that are ineffective in the non-gravid uterus, and following parturition these levels

of OT receptors and receptor mRNA are quickly reduced (Zingg, Rozen et al. 1995). Another well known role of OT in the periphery of females is its role in the elicitation of milk from the mammary gland. The stimulation the nipple by the suckling of an infant causes sensory nerve impulses that journey from the breast to the hypothalamus where they cause the production of action potentials that result in a release of of OT into the plasma. From the plasma, the OT is carried back to the myoepithelial cells in the female breast thus causing contraction and subsequent flow of milk from the nipple (Jones, Robinson et al. 1983).

Central Effects

Increasing evidence in the literature supports a link between OT receptor signaling within the central nervous system and social behaviors such as social bonding, memory and learning, and emotional responses (Carter, Williams et al. 1992; Heinrichs, Meinlschmidt et al. 2004; Kirsch, Esslinger et al. 2005; Carter, Grippo et al. 2008; Kemp and Guastella 2010). Social bonding is composed of several conceptual levels of behavior including an initial approach and affiliation, the proper recognition of social cues, and the formation of a social bond (Lim and Young 2006). Social approach and motivation can be measured in animal models by latency to approach and it was determined that OT knockout mice pups display an increased latency to seek out their mother (Young, Winslow et al. 1997). Subcutaneous injections in the Mongolian gerbil or central infusion in the rat of OT increases social contact time between animals (Witt, Winslow et al. 1992; Razzoli, Cushing et al. 2003). Within primates an increase in OT concentrations in the CSF has been positively correlated with the social and gregarious bonnet monkey when compared to that of the generally asocial pigtail macaque (Rosenblum, Smith et al. 2002). Evidence is accumulating for a role of OT in the prosocial effects of MDMA (Ecstasy) in the rat. For example, pretreatment with tocinoic acid (an OT receptor antagonist) significantly attenuates

the facilitation of social interaction produced by MDMA, but has no effect on social behavior when given alone (Thompson, Callaghan et al. 2007).

The recognition of proper social cues depends predominantly on olfactory cues in animals and visual cues in humans. Oxytocin knockout mice are more prone to fail to recognize other mice after repeated social encounters than are wild type mice as determined by Ferguson and co-workers (2000). In these experiments the knock out animals maintained a level of olfactory investigation comparable to what was seen with the introduction of a novel conspecific to mice that they had previously been exposed to, but they did not have alterations in their ability to locate hidden food, habituate to an acoustic startle, or learn spatial cues for a water maze (Ferguson, Young et al. 2000). These behavioral differences between the oxytocin knock out mice and their wild type counterparts are eliminated following OT administration (Winslow and Insel 2002). It was also discovered that microparticle delivery of antisense DNA directed against specific sequences of the mRNA of the mouse OT receptor in the medial amygdala blocks normal social recognition in female mice as well (Choleris, Little et al. 2007). The amygdala in humans is proposed to be responsible for processing basic emotional behavior as it has been shown that the visualization of fearful faces activates this nucleus, as well as for potentiating complex social behaviors since a decrease in amygdala activation is linked to hypersociability and aggression while a increase in activation is correlated with social avoidance and phobias (Kirsch, Esslinger et al. 2005). When compared with placebo, intranasal application of OT reduces activation and coupling of the amygdala to brainstem regions implicated in the manifestation of fear (Kirsch, Esslinger et al. 2005). Another study measured the levels of circulating OT in participants of a sequential anonymous "trust game" with monetary payoffs where trust and trustworthiness are measured and it was determined that OT levels were higher

in subjects who were the recipient of trust and with individuals who displayed trustworthy behavior when compared to other participants (Zak, Kurzban et al. 2005).

Social bonding is well studied in both the prairie voles that form monogamous bonds between mates and sheep that form intensely selective bond with only their own lamb (Lim and Young 2006). Mating in prairie voles facilitates the formation of a monogamous pair bond between mates in contrast to another member of the vole family the Montane vole which are solitary creatures that do not exhibit social bonding and frequently abandon their young 2 weeks after birth. Both species have similar distributions of the OT peptide within the CNS, but they display distinct distribution patterns of OT receptors within the brain with the monogamous prairie voles exhibiting higher levels of OT receptors in regions associated with reward such as the nucleus accumbens and the ventral palliduim (Insel and Shapiro 1992; Lim and Young 2006). Central infusion of OT was found to induce pair bonding in both male and female prairie voles in the absence of mating and this pair bond formation could be prevented by ICV infusion of an OT receptor antagonist into the nucleus accumbens despite repeated mating bouts between the animals (Lim and Young 2006). Sheep are unique animal models of attachment since a ewe forms a selective bond with her own lamb which is a result of hormone priming with estradiol and progesterone followed by vagino-cervical stimulations that are hypothesized to result in an increase in OT within the mother's brain (Kendrick, Levy et al. 1991). The role of oxytocin in this bond formation was demonstrated by experiments conducted by Levy and collegues where it was determined that anesthesia with an epidural blocked the physical sensations of labor and delivery for the ewe resulting in a blockade of mother-infant bond formation which could be overcome by exogenous ICV administration of OT (Levy, Kendrick et al. 1992).

Oxytocin signaling is implicated in various aspects of learning and memory where it can either

enhance or impair memory formation and its actions appear to depend to some degree on the context of the memory (i.e. social memory versus explicit memory) (Gulpinar and Yegen 2004). It has been theorized that OT has an amnestic function in labor and delivery allowing a woman to forget the pain of giving birth and OT has been found to reduce the activity of NMDA receptors whose substrates are highly implicated in learning and memory in cultured neurons (Caruso, Agnello et al. 1993; Evans 1997). In a double-blind, randomized, placebo-controlled (DBRPC) study, Heinrichs et al. (2004), investigated the effects of intranasal OT on memory formation in three tests of memory function in 38 adult males. Tests included a word stem completion test and a cued recall test amoung others and the researchers used two types of word stimuli: reproductive and neutral. OT impaired recall performance in the cued recall test regardless of word meaning. However treatment with OT was found to selectively impair the generation of associated target words with reproductive meanings in the implicit conceptual test (category-cued semantic association test) but had no effect on generation of neutral words (Heinrichs, Meinlschmidt et al. 2004). In contrast to the previously detailed study, several studies in humans have determined that OT enhances the formation of social memories (Guastella, Mitchell et al. 2008; Rimmele, Hediger et al. 2009). Intranasal administration of OT was found to specifically improve memory recall of faces but not for nonsocial stimuli and in another DBRPC study OT-administered participants were more likely to recognize happy faces compared to angry and neutral faces than the placebo-administered participants (Rimmele, Hediger et al. 2009). Hurelmann and colleagues in 2010 confirmed the effect of OT on the encoding of social memories where intranasal OT improved the learning performance of male volunteers when social stimuli were used and OT also raised emotional empathy responses to both positive and negative stimuli similar to those found in women who were untreated. Two

patients with previous injuries that damaged the amygdala bilaterally did not display an enhancement in either the learning or empathy tests but they did perform normally on nonsocial reinforced learning and cognitive empathy (Hurlemann, Patin et al. 2010). These findings suggest that OT may function as an important facilitator for the formation of social memories and in particular positive social experiences and these behavioral actions may be dependent on a functional amygdala.

Little study of the OT receptor system in animal models capable of vocal development has occurred and one of the core symptoms of ASD is an alteration in language and communication. However it has been noted that oxytocin knock-out mice pups displayed a reduction in ultrasonic vocalizations upon separation from their mothers (Winslow, Hearn et al. 2000). Oxytocin immunoreactive neurons have been found in auditory cortices of the mustached bat and densely distributed in the ventral telencephalon and areas within the hypothalamus and brainstem encompassing all sites involved in vocal-acoustic integration of the plain midshipman fish (a species of fish who have adapted their airbladder for sound production) (Goodson, Evans et al. 2003; Prasada Rao and Kanwal 2004). The distribution of the OT receptor was recently investigated in two species of singing mice using autoradiography and moderate expression was found in the medial preoptic area, an area linked with vocalization. Furthermore vocalizations have been determined to increase plasma OT levels in humans. Grape and colleagues (2003), found a significant elevation in OT in both amateur and professional singers following a singing lesson (Grape, Sandgren et al. 2003). A recent study investigated the serum levels of OT in children who were comforted by their mothers by either complete contact, speech-only, or no contact following a social stressor. Children who received the full complement of comforting measures including physical and vocal contact were found to have the highest levels of OT and

displayed the quickest return to baseline levels of salivary cortisol (a biological stress marker), and similar results were found for the vocal comfort group when compared to the no contact group indicating that both physical touch and vocalizations may be capable of inducing the release of OT (Association 2000; Seltzer, Ziegler et al. 2010).

Oxytocin and the Etiology of ASD

The etiology of ASD remains unclear and several hypotheses exists in the literature that include infection and immune dysfunctions, endocrine factors, factors such as xenobiotic and prescription medication exposures during pregnancy, and alterations in neurotransmitters and neuropeptides as possible causes for the development of ASD. Since the characteristics typifying ASD include alterations in social behaviors it is logical to investigate OT and OT receptor signaling due to its key role in social behaviors. Furthermore, males are five times more likely to be diagnosed with autism than females are, and this sex difference in diagnosis suggests that a sexually dimorphic peptide such as OT may be a critical factor in the development of the disease (Carter 2007). Oxytocin levels have been determined to be different in some but not all of the species assayed, but in those species that differences were seen, it was determined that OT is higher in females (Pfaff 2002; Yamamoto, Cushing et al. 2004). As stated previously both OT and its receptor are subjected to estrogen-sensitive synthesis. It has also been demonstrated that significant differences in plasma OT levels and in circulating forms (bioactive form versus the form with several C-terminal peptide extensions) were found when age-matched autistic children were compared with non-autistic children (Modahl, Greene et al. 1998; Greene, Fein et al. 2001). Animal and human clinical data have further implicated a causal relationship between altered OT levels and repetitive behaviors which as stated earlier can be characteristic of patients with autism (Drago, Pederson et al. 1986; Hollander, Novotny et al. 2003). Although these studies

base their results on peripheral instead of central OT levels, they do provide support for the involvement of OT in autism.

Genetic Abnormalities within the Oxytocin Receptor

While only 10% of autism cases can be attributed to a single gene disorder, multiple genotyping studies have revealed that OT receptor (OXTR) gene mutations are correlated with the diagnosis of autism within Chinese Han, Japanese, and American Caucasian populations (Wu, Jia et al. 2005; Jacob, Brune et al. 2007; Liu, Kawamura et al. 2010). Approximately 30 single nucleotide polymorphisms (SNPs) are present in the OT receptor gene in humans and the study by Wu and colleagues (2005), identified a correlation between four SNPs in the OT receptor and autism in a sample of Han Chinese families (Wu, Jia et al. 2005). A combined analysis of data from the Autism Genetic Resource Exchange and Finnish autism samples further implicated alterations within the 3p24-46 locus and linkage to ASD (Ylisaukko-oja, Alarcon et al. 2006). Lerer and colleagues (2008), completed a comprehensive study of all of the 18 previously implicated SNPs spanning the entire OT receptor gene region and it was found a mathematical linkage between OXTR (oxytocin receptor) gene polymorphisms with IQ and Vineland Adaptive Behavior Scales scores and ASD with a particular single haplotype being showing a significant association with ASD (Lerer, Levi et al. 2008). Polymorphisms of the OXTR gene were also significantly associated with affect, loneliness, and non-verbal intelligence in normal human subjects (Lucht, Barnow et al. 2009). Epigenetic regulation of the OXTR has also been demonstrated in human patients suffering with ASD. An analysis of the genome of multiplex autism families assessed OXTR gene expression within the temporal cortex and demonstrated that genomic deletion containing the OT receptor gene previously implicated in ASD was present in an autism proband and his mother who exhibited many symptoms of obsessive-compulsive disorder but not in an

affected sibling. The sibling however was found to have aberrant gene silencing by DNA methylation. This led the authors to compare the DNA methylation status in the temporal cortex of affected patients compared to control samples, and a positive correlation was found between an increase in DNA methylation and subsequent OXTR mRNA decreased expression and the ASD phenotype (Gregory, Connelly et al. 2009).

CD38

CD38 is a transmembrane glycoprotein with ADP-ribosyl cyclase activity that catalyses the formation of Ca^{2+} signaling molecules. It is theorized to be involved in the regulation of the secretion of OT, and this was determined with the use of CD38 knockout mice that displayed altered maternal and social behaviors, had fewer ultrasonic vocalizations upon separation from their mother, and lowered levels of peripheral OT (Bartz and McInnes 2007; Jin, Liu et al. 2007; Liu, Lopatina et al. 2008). The lack of CD38 disrupted depolarization-induced OT secretion and reduced calcium levels in axon terminals of oxytocinergic neurons within knockout mice brains. Replacement of OT subcutaneously or by lentiviral-vector-mediated delivery of human CD38 to the hypothalamus of the mice rescued social memory and maternal care (Jin, Liu et al. 2007). To determine if an association exists between CD38 and autism, Munesue and colleagues (2010), analyzed 10 SNPs of CD38 by re-sequencing in Japanese and Caucasian cases and two SNPs showed significant associations with individuals designated as high-functioning autistic (IQ > 70) within the US but not Japanese samples. Another mutation of CD38 which causes an arginine to be replaced with tryptophan was found to be associated with ASD in Japanese patients. Interestingly the fathers and brothers in families who carried this tryptophan mutation were either diagnosed with ASD or displayed ASD traits and had lower plasma OT than those without the mutation (Munesue, Yokoyama et al. 2010).

Exogenous Oxytocin Hypothesis

Another hypothesis set forth by Hollander and colleagues (2007), is that excessive amounts of OT, particularly the large amounts used in the induction of labor, could contribute to the development of ASD by the persistent down-regulation of the OT receptor (Rojas Walh 2007). As stated previously, there is evidence of OT receptor internalization following agonist stimulation and an alteration of receptor densities, even a transient one, may lead to a dysfunction at a critical period of newborn development (Phaneuf, Asboth et al. 1998; Phaneuf, Rodriguez Linares et al. 2000; Robinson, Schumann et al. 2003). In several studies OT appears to have a dose-dependent effect on social behaviors where lower doses facilitate a behavior such as social recognition, but higher doses disrupt social memory (Heinrichs, Meinlschmidt et al. 2004; Rimmele, Hediger et al. 2009; Bardou, Leprince et al. 2010). Administration of OT was found to first increase the firing rate within the central and medial amygdaloid nuclei within the rat, but when repeated doses were given receptors were desensitized and the firing rate decreased (Terenzi and Ingram 2005). Alterations in OT sensitivity within the central nervous system have been documented when OT was given to men suffering from early-parental-separation experience and this evidence indicates that early disturbances to the OT receptor signaling system may have long-term effects within human beings (Meinlschmidt and Heim 2007).

Oxytocin and the Blood-brain Barrier

Proposed mechanisms for the ability of peripheral OT to cross the blood-brain barrier include the existence of a possible carrier-mediated transport mechanism for OT similar to the one that exists for vasopressin (Durham, Banks et al. 1991; Zlokovic, Banks et al. 1992), or the formation of active fragments that can cross the blood-brain barrier that have been previously demonstrated to affect memory processes (Bohus, Ader et al. 1972; de Wied, Diamant et al. 1993). Intranasal

OT is more than likely directly transported to the cerebrospinal fluid from the nasal mucosa where it is then transported across the ependyma into the brain parenchyma (Bittencourt and Sawchenko 2000). It is further hypothesized that small amounts of exogenous OT can cross the blood brain barrier once a certain plasma concentration is achieved as reported by Ring and colleagues (2006). Their work demonstrated that peripherally administered OT (although a substantially higher concentration was required) could produce comparable anxiolytic effects in mice to that observed following ICV administration of OT, and the effects of the peripherally given OT could be reversed with a central infusion of an OT receptor antagonist (Ring, Malberg et al. 2006). Furthermore intraperitoneal injections of OT (0.05 mg/kg and 1.0 mg/kg) were able to alter spontaneous behaviors such as horizontal and vertical activity and grooming of Wistar rats. The lower dose of OT caused an increase in locomotor activity and grooming suggestive of an anxiolytic effect while the higher dose reduced both locomotion and rearing behaviors. Both of these results indicate that the nonapeptide OT is indeed capable of crossing the blood brain barrier (Klenerova, Krejci et al. 2009).

Treatment of ASD with Oxytocin

Given the evidence of an alteration in OT signaling in patients suffering from ASD, OT is under current investigation as a potential treatment for some of the core symptoms of autism. A recent review of novel treatments for ASD (2009), gave OT a grade recommendation of B (grades consisted of A through D with A having the support of 2 randomized controlled studies and D having inconsistent or inclusive studies) for off-label use in treating affected patients designating that OT was proven effective by at least one prospective randomized controlled study (Rossignol 2009). An infusion of oxytocin produced a significant reduction in both the severity of six autism-associated repetitive behaviors tested (need to know, repeating, ordering, need to tell/ask,

self-injury, and touching) and the number of different types of behaviors displayed in 15 adults diagnosed with autism or Asperger's disorder when compared to that of an infusion of placebo (Hollander, Novotny et al. 2003). Intravenous OT treatment increased the processing and retention of social cognition in a randomized, placebo-controlled, double-blind crossover investigation in adults diagnosed with an autistic spectrum disorder. In this experimental design 15 adults who met the diagnostic criteria for ASD of the DSM-IV (See table 2 for more details) and the Autism Diagnostic Interview-Revised were given either placebo or synthetic OT infusions over a 4-hour period and then asked to describe emotional mood of the speaker (happy, indifferent, angry, or sad) of neutral sentences such as "The boy went to the store" and "Fish can jump out of the water" (Hollander, Bartz et al. 2007). A randomized controlled trial of intranasal OT by Guastella and colleagues (2009), showed a beneficial effect of oxytocin in the reduction of anxieties and symptoms present in five participants diagnosed with social anxiety disorder (Guastella, Howard et al. 2009). The Guastella laboratory followed this study with a double-blind, randomized, placebo controlled study of intranasally administered OT with 16 male autistic youth who exhibited significant improvement in performance on the Reading of the Mind in the Eyes Task which assesses the ability to read emotions from the eyes of subtle affective facial expressions (Guastella, Einfeld et al. 2010). Furthermore, social behaviors such as stronger social interactions, increased feelings of trust, and an increase in gaze time on the socially informative regions of the face have been shown to be increased in autistic patients following intranasal OT administration (Andari, Duhamel et al. 2010).

The Zebra Finch as an Animal Model of Vocal Development

General Parallels to Human Learning

The zebra finch is a proposed animal model of vocal development since male zebra finches learn

a song pattern in a manner similar to human language acquisition and the neurobiology of both song learning and production has been extensively studied (Scharff and Nottebohm 1991; Doupe and Kuhl 1999). Therefore, functional relationships can be delineated between brain circuitry, neural features, and song behavior. There are several parallels that exist between the process of learning a particular song pattern in young birds and the development of language in young humans. Foremost, both species must learn their respective forms of communication from the sounds of the adults of the species which is a rather unique characteristic (Doupe and Kuhl 1999). Babies and birds also must have the ability to participate in hearing and in the vocal motor production of sound in order to learn communication; a deficit in either area results in absent or abnormal language (Doupe and Kuhl 1999). Finally there are points of time in the lives of both the zebra finch and the human infant where vocal development is optimal indicating significant ability of the brain to adapt and change during the life span of both species (Doupe and Kuhl 1999).

Zebra FinchVocal Development

Male zebra finches learn song that consists of a series of notes of a specific frequency and order during juvenile development through early adulthood as depicted in Figure 1.5. The female zebra finch does not learn to produce a song, but does recognize specific song patterns produced by different males. Once males learn a specific song pattern, it does not change (Immelmann 1969; Arnold 1975; Bohner, Chaiken et al. 1990). There are at least two distinct developmental stages for song acquisition: auditory phase and sensory-motor phase (See Figure 1.5). Young males first listen and memorize the song of an adult male in the first stage, and an absence of an adult tutor causes the formation of an abnormal song termed “isolate” songs. This isolate song can be modified in adulthood if the zebra finches undergo social interaction with live tutors

where they begin to either modify or eliminate many of the phonologically abnormal notes of the isolate song (Morrison and Nottebohm 1993). In the second stage of vocal learning the bird begins to "practice" this memorized song and initially the song is highly variable (sub-song), but these vocalizations are slowly refined (plastic song) to match the song memorized during the auditory phase to produce a final stereotyped adult song. This refinement period requires sensorimotor learning or vocal practice where the bird must actively sing and compare its song to the memorized template. Birds must be able to hear themselves practice to produce a normal song both as a juvenile and as an adult. Nordeen and Nordeen determined that adult birds only retained 36% of their songs following bilateral cochlear removal compared to the 90% of the song retained by the control birds 16 weeks after surgery (Nordeen and Nordeen 1992).

Human Vocal Development

Human language development is similar to that of the zebra finch. At birth infants can discriminate all phonetic sounds in languages spoken throughout the world, but a recent study noted distinct differences between the cries of German and French newborns. These data suggest that the surrounding speech prosody that human fetuses are exposed to while in the uterus may influence vocalizations and possibly later vocal learning (Mampe, Friederici et al. 2009). The first vocalizations of the human infant are highly variable and are termed as babbling with infants producing first vowel like sounds and then vowel-consonant combinations (dada, mama, gaga). Infants begin to recognize language specific sound combinations at approximately 9 months of age and they continually practice sounds until their first words are spoken at about a year (Doupe and Kuhl 1999). Human babies however are readily able to effectively communicate months before they are able to speak words by using a variety of non-vocal communications such as various tones of cries and reaching, pushing away, or pointing to an

object, and they often understand many more words and phrases than they speak (Tomasello and Kruger 1992). Receptive and expressive vocabulary grows at a steady pace from 12 to 18 months, and there is an enormous expanse in both words known and used in the human toddler from 18 months until their second birthday (Fenson, Dale et al. 1994). As with the zebra finches, auditory feedback is important for the maintenance of vocal communication as speech skills will rapidly deteriorate if a child is deafened before the age of puberty [(Konishi 1963; Konishi 1965) as cited in (Doupe and Kuhl 1999), (Waldstein 1990)].

Neural Substrates for Vocal Learning within the Zebra Finch

The neuronal circuitry underlying song learning and production within the zebra finch is contained within several discrete interconnected brain regions that include prominent striatal (Area X), thalamic (DLM), and telencephalic (lMAN, HVC, RA) components. Please see figure 1.6 for an illustration. Each of these regions performs different functions in both song learning and production, and is typically grouped as such. Area X (Area X within medial striatum), DLM (medial portion of the dorsolateral nucleus of the thalamus), and lMAN (Lateral magnocelluar nucleus of the anterior nidopallium) compose what is thought of as the "song learning pathway" which may be described as a circuit that loops from the higher forebrain song control areas through the basal ganglia to the thalamus and back to the forebrain song nuclei in the zebra finch (Aronov, Andalman et al. 2008). A lesion in a juvenile bird of Area X will result in a disordered song that never crystallizes and a lesion of lMAN will cause a premature crystalization of an immature song (Bottjer, Miesner et al. 1984; Sohrabji, Nordeen et al. 1990; Scharff and Nottebohm 1991). However a lesion of Area X or lMAN in adults following song crystallization does not affect song production (Scharff and Nottebohm 1991). RA (Robust nucleus of the arcopallium) and HVC are components of the "vocal production pathway"

(Simpson and Vicario 1990; Vicario 1991). HVC is described as containing a central pattern generator for song and it projects to RA which is involved in the control of vocal motor nuclei (Vu, Mazurek et al. 1994; Wild, Li et al. 1997). A lesion in RA will abolish song production at any age of the animal. It is of note that CNS regions associated with song control undergo substantial changes in neuronal number and connectivity and it is proposed that these changes coincide with vocal learning (Bottjer and Johnson 1997). For example Bottjer and colleagues (1985), noted the volumes of HVC, RA, and Area X increased dramatically in adult males compared to that of female birds between the ages of 12 to 53 days corresponding to when the animals are actively learning song (Bottjer, Glaessner et al. 1985). Field L2 is the recipient of thalamic auditory inputs and projects to a series of secondary auditory areas and eventually to the HVC, and it is theorized that early processing of sounds could occur in these area (Doupe and Kuhl 1999).

Neural Substrates for Vocal Learning in Humans

In both humans and song birds the brain is lateralized which results in areas responsible for language and speech usually residing in the left hemisphere of the brain with the right hemisphere containing areas in control of language only a small percentage of the time. The two major areas of the human brain that are commonly recognized as being responsible for language are Broca's area, hypothesized to be partially responsible for language production, and Wernicke's area which contributes to language processing but it is known that the entire perisylavian cortical area as well as the posterior parieto-temporal cortex are integral for the proper production of language (Davis, Zhang et al. 1996). Broca's area is located in the posterior frontal cortex and lesions in this area cause difficulties in the expression of language with affected individuals producing slow speech that stops and starts and rarely contains proper

grammatical syntax. These individuals generally retain knowledge of vocabulary and will have limited difficulties naming objects, but are unable to deduce meanings from the syntax of sentences (Ojemann 1991; Binder 1997). A lesion in Wernicke's area in the posterior temporal lobe causes deficits in the understanding of speech. These individuals produce grammatically correct sentences but they can include nonsensical words, and generally do not demonstrate an understanding of words spoken to them (Ojemann 1991; Binder 1997). Language is not limited to the cortex but also includes the cerebellum which appears to be critical in the production of rate and rhythm of speech. The cerebellum is strongly activated during the initial learning phase of vocal tasks, but its activation decreases with practice which may indicate a particular role for it in the production of nonautomatic speech (Petersen, van Mier et al. 1998) .

Purpose of Current Study

The focus of this study is to investigate a possible role for the OT related peptide, mesotocin, in an animal capable of vocal development, namely the male zebra finch. First, the full coding sequence of the zebra finch mesotocin receptor, the avian homolog of the human OT receptor, will be elucidated. As previously stated, mesotocin is a nonapeptide hormone that differs in sequence from OT by a single amino acid substitution. The mesotocin receptor had been previously cloned from several species, and the chicken receptor shares 83% amino acid identity with the human OT receptor. Secondly, the information gained by the cloning of the receptor will be used to generate nucleic acid and antibody probes to study mesotocin receptor distribution since limited knowledge exists of receptor distributions within the CNS of animals who are capable of vocal learning. Other receptors involved in vocal development, such as the CB1 cannabinoid receptor, are localized in CNS song regions such as lMAN, Area X, HVC, RA, etc. (Soderstrom and Tian 2006). The polyclonal antibody generated will allow the

determination of mesotocin receptor expression patterns at various stages of vocal development: 25- [auditory learning], 50- [subsong], 75- [plastic song], and 100-days of age [crystallized song]. In-situ hybridization experiments will be completed to assess mesotocin receptor mRNA patterns. Immunoblotting will allow for the delineation of the molecular mass of the mesotocin receptor in the zebra finch as well as the distribution pattern in selected tissues, and this will be followed by northern blotting to determine mesotocin receptor mRNA message size and distribution. Finally, in order to investigate the possibility that exposure to OT-related peptides may persistently alter vocal development, oxytocin will be injected into zebra finch hatchlings as fledglings for five days and followed for changes in vocal learning by recording the songs they produce and comparing them to those produced by vehicle treated birds. The brains of these animals then will undergo immunohistochemical analysis to determine whether a persistant change in mesotocin receptor expression pattern and density occurred as a result of the early OT exposure. Results will show a potential role for the oxytocin related peptide in song learning, the auditory perception of song, or the vocal production of song if its receptors are located in song control regions previously demonstrated to be critical for these functions. We will also demonstrate whether the peripheral administration of oxytocin to a young zebra finch hatchling will produce an alteration in the production of the final adult song. Taken together the results of this project may indicate the potential for a relationship between exogenous oxytocin exposure and the alteration of vocal development that is one of the characteristic triad of deficits associated with autism spectrum disorders.

Table 1.1 Diagnostic criteria for the diagnosis each of the disorders grouped under the Autism Spectrum disorder (Autsim, Asperger's, and PPD-NOS) as taken from the *Diagnostic and Statistical Manual of Mental Disorders: DSM IV. (Association 2000)*

A) Autistic Disorder

(I) A total of six (or more) items from (A), (B), and (C), with at least two from (A), and one each from (B) and (C)

(A) qualitative impairment in social interaction, as manifested by at least two of the following:

1. marked impairments in the use of multiple nonverbal behaviors such as eye-to-eye gaze, facial expression, body posture, and gestures to regulate social interaction
2. failure to develop peer relationships appropriate to developmental level
3. a lack of spontaneous seeking to share enjoyment, interests, or achievements with other people, (e.g., by a lack of showing, bringing, or pointing out objects of interest to other people)
4. lack of social or emotional reciprocity (note: in the description, it gives the following as examples: not actively participating in simple social play or games, preferring solitary activities, or involving others in activities only as tools or "mechanical" aids)

(B) qualitative impairments in communication as manifested by at least one of the following:

1. delay in, or total lack of, the development of spoken language (not accompanied by an attempt to compensate through alternative modes of communication such as gesture or mime)
2. in individuals with adequate speech, marked impairment in the ability to initiate or sustain a conversation with others
3. stereotyped and repetitive use of language or idiosyncratic language
4. lack of varied, spontaneous make-believe play or social imitative play appropriate to developmental level

(C) restricted repetitive and stereotyped patterns of behavior, interests and activities, as manifested by at least two of the following:

1. encompassing preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal either in intensity or focus
2. apparently inflexible adherence to specific, nonfunctional routines or rituals
3. stereotyped and repetitive motor mannerisms (e.g hand or finger flapping or twisting, or complex whole-body movements)
4. persistent preoccupation with parts of objects

(II) Delays or abnormal functioning in at least one of the following areas, with onset prior to age 3 years:

(A) social interaction
(B) language as used in social communication
(C) symbolic or imaginative play

(III) The disturbance is not better accounted for by Rett's Disorder or Childhood Disintegrative Disorder

B) Asperger's Disorder

(I) Qualitative impairment in social interaction, as manifested by at least two of the following:

(A) marked impairments in the use of multiple nonverbal behaviors such as eye-to-eye gaze, facial expression, body posture, and gestures to regulate social interaction

(B) failure to develop peer relationships appropriate to developmental level

(C) a lack of spontaneous seeking to share enjoyment, interest or achievements with other people, (e.g.. by a lack of showing, bringing, or pointing out objects of interest to other people)

(D) lack of social or emotional reciprocity

(II) Restricted repetitive & stereotyped patterns of behavior, interests and activities, as manifested by at least one of the following:

(A) encompassing preoccupation with one or more stereotyped and restricted patterns of interest that is abnormal either in intensity or focus

(B) apparently inflexible adherence to specific, nonfunctional routines or rituals

(C) stereotyped and repetitive motor mannerisms (e.g. hand or finger flapping or twisting, or complex whole-body movements)

(D) persistent preoccupation with parts of objects

(III) The disturbance causes clinically significant impairments in social, occupational, or other important areas of functioning.

(IV) There is no clinically significant general delay in language (E.G. single words used by age 2 years, communicative phrases used by age 3 years)

(V) There is no clinically significant delay in cognitive development or in the development of age-appropriate self help skills, adaptive behavior (other than in social interaction) and curiosity about the environment in childhood.

(VI) Criteria are not met for another specific Pervasive Developmental Disorder or Schizophrenia."

C) Pervasive Developmental Disorder Not Otherwise Specified

The essential features of PDD-NOS are severe and pervasive impairment in the development of reciprocal social interaction or verbal and nonverbal communication skills; and stereotyped behaviors, interests, and activities. The criteria for Autistic Disorder are not met because of late age onset; atypical and/or sub- threshold symptomotology are present.

Table 1.2 Structures of the oxytocin/vasopressin (OT/VP) superfamily as adapted from (Kanda, Satake et al. 2005).

Nonapeptide	Structure	Organism
Vertebrate OT Family		
Oxytocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-(NH2)	Mammals
Mesotocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Ile-Gly-(NH2)	Marsupial, Non-mammalian vertebrates
Isotocin	Cys-Tyr-Ile-Ser-Asn-Cys-Pro-Ile-Gly-(NH2)	Bony Fish
Vertebrate VP Family		
Vasopressin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-(NH2)	Mammals
Vasotocin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-(NH2)	Non-mammalian vertebrates, Jawless fish
Invertebrate OT/VP Family		
Lys-conopressin	Cys-Phe-Ile-Arg-Asn-Cys-Pro-Lys-Gly-(NH2)	Leech, geography cone, imperial cone,
Arg-conopressin	Cys-Ile-Ile-Arg-Asn-Cys-Pro-Arg-Gly-(NH2)	Striped cone
Annetocin	Cys-Phe-Val-Arg-Asn-Cys-Pro-Thr-Gly-(NH2)	Earthworm
Cephalotocin	Cys-Tyr-Phe-Arg-Asp-Cys-Pro-Ile-Gly-(NH2)	Octopus
Octopressin	Cys-Phe-Trp-Thr-Ser-Cys-Pro-Ile-Gly-(NH2)	Octopus

Figure 1.1 Organization of the mammalian Oxytocin/Vasopressin Gene as adapted from (Gimpl and Fahrenholz 2001).

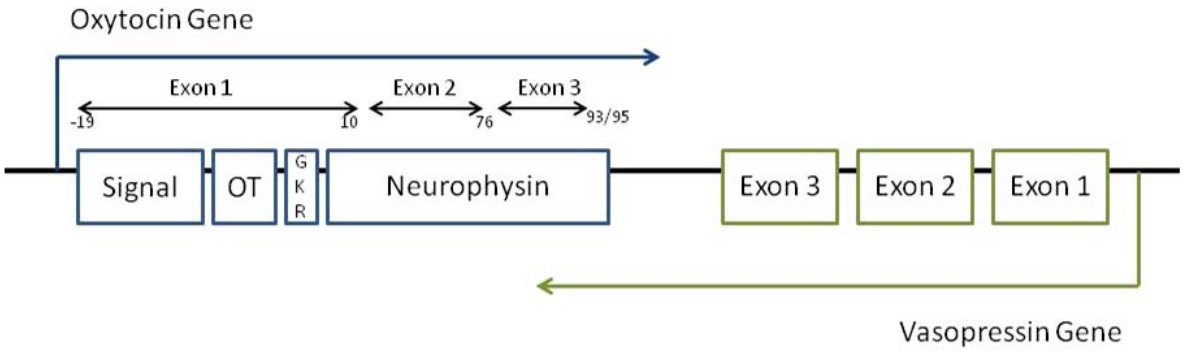

Oxytocin Gene
Exon 1
Exon 2
Exon 3
-19
10
76
93/95
Signal
OT
G
K
R
Neurophysin
Exon 3
Exon 2
Exon 1
Vasopressin Gene

Figure 1.2 Model of a typical oxytocin receptor indicating regions that are thought to be involved in ligand-binding and signal transduction events. A solid line indicates domains of the protein that interact with the linear C-terminal tripeptidic of oxytocin while the cyclic portion of oxytocin interacts with the region highlighted with dashes. The area highlighted with dots designates the conserved residues of the protein required for coupling to Gq. Adapted from (Gimpl and Fahrenholz 2001).

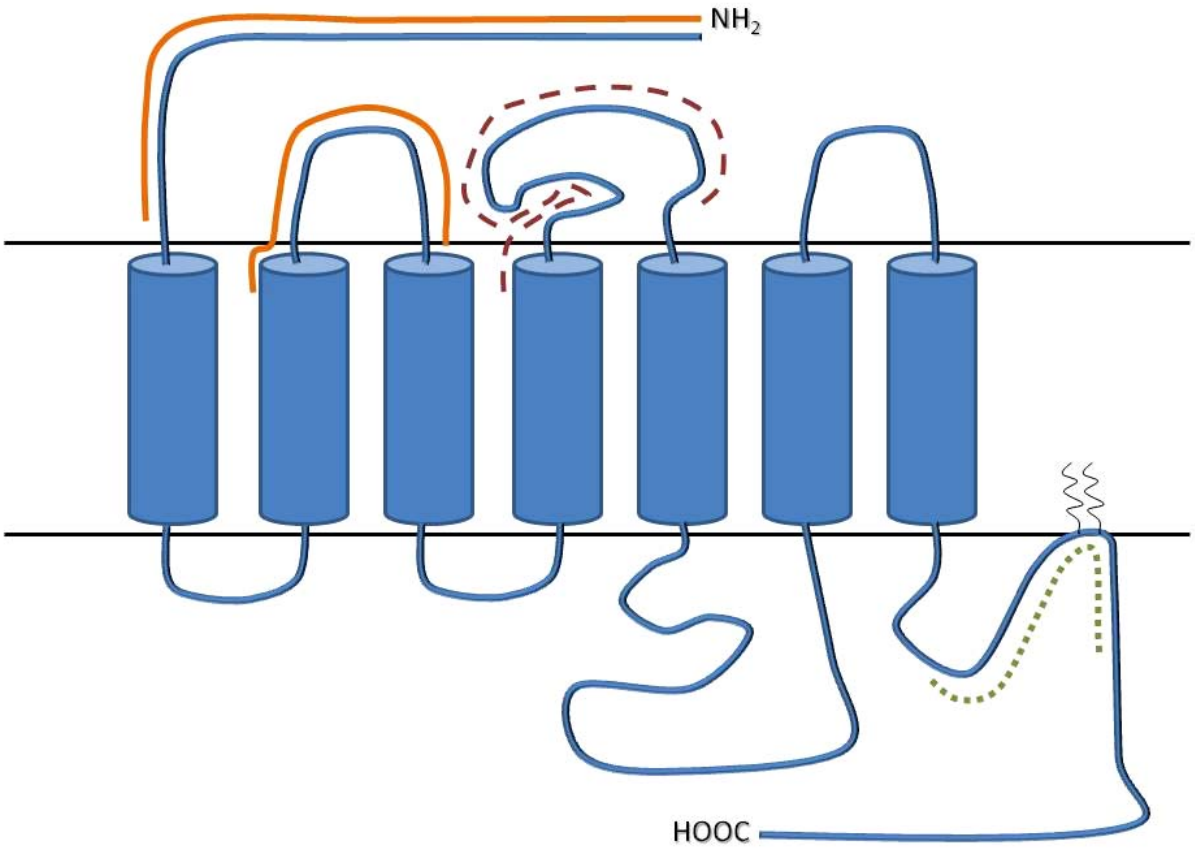
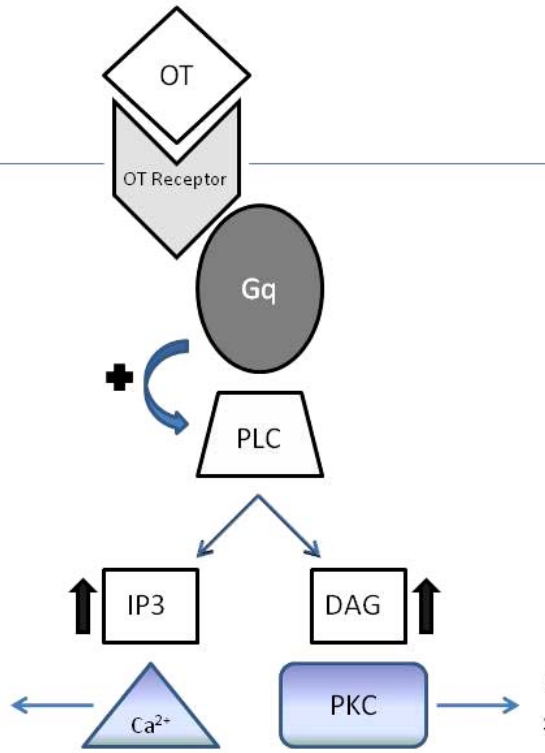

NH2
HOOC

Figure 1.3 Oxytocin receptor signaling pathway.



Smooth Muscle – Activation of myosin light-chain kinase activity via Ca2+/calmodulin

Neurons – release of neurotransmitters and protein synthesis

Phosphorylates tissue specific proteins

Figure 1.4 Oxytocin receptor desensitization. Proposed pathway for agonist induced desentization of the oxytocin receptor.

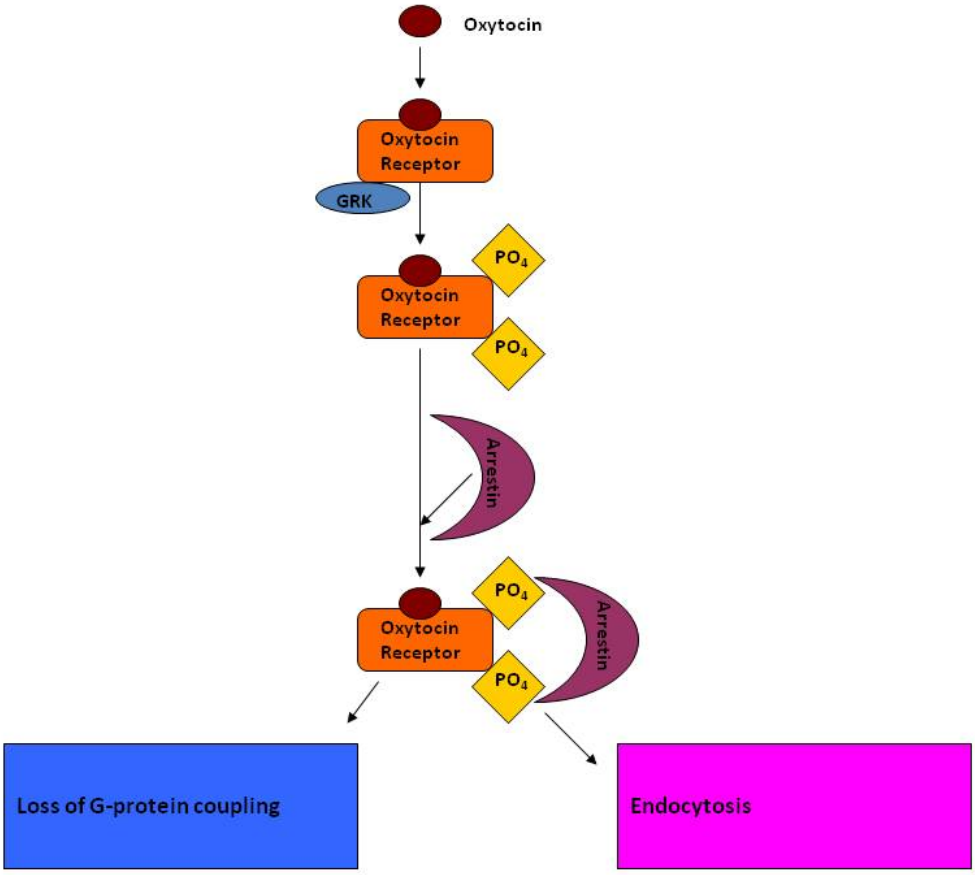

Oxytocin
Oxytocin Receptor
GRK
PO4
Oxytocin Receptor
PO4
Arrestin
PO4
Oxytocin Receptor
Arrestin
PO4
Loss of G-protein coupling
Endocytosis

Figure 1.5 Timeline and Sonogram examples of zebra finch vocal development (Soderstrom and Tian 2004) A. After hatching, zebra finches remain in the nest several weeks. At 20 days of age they fledge from the nest but remain dependant on their parents for food until approximately 35 days of age. Auditory learning begins around the time the birds fledge from the nest and continues to at least 35 days. Sensory-motor learning can begin as early as day 30 and continues to early adulthood. B. and C. Examples of subsong and plastic songs respectively are shown. Once the animal has learned his song the vocal pattern produced throughout his lifetime is defined as stereotyped (Panel D) and cannot be altered.

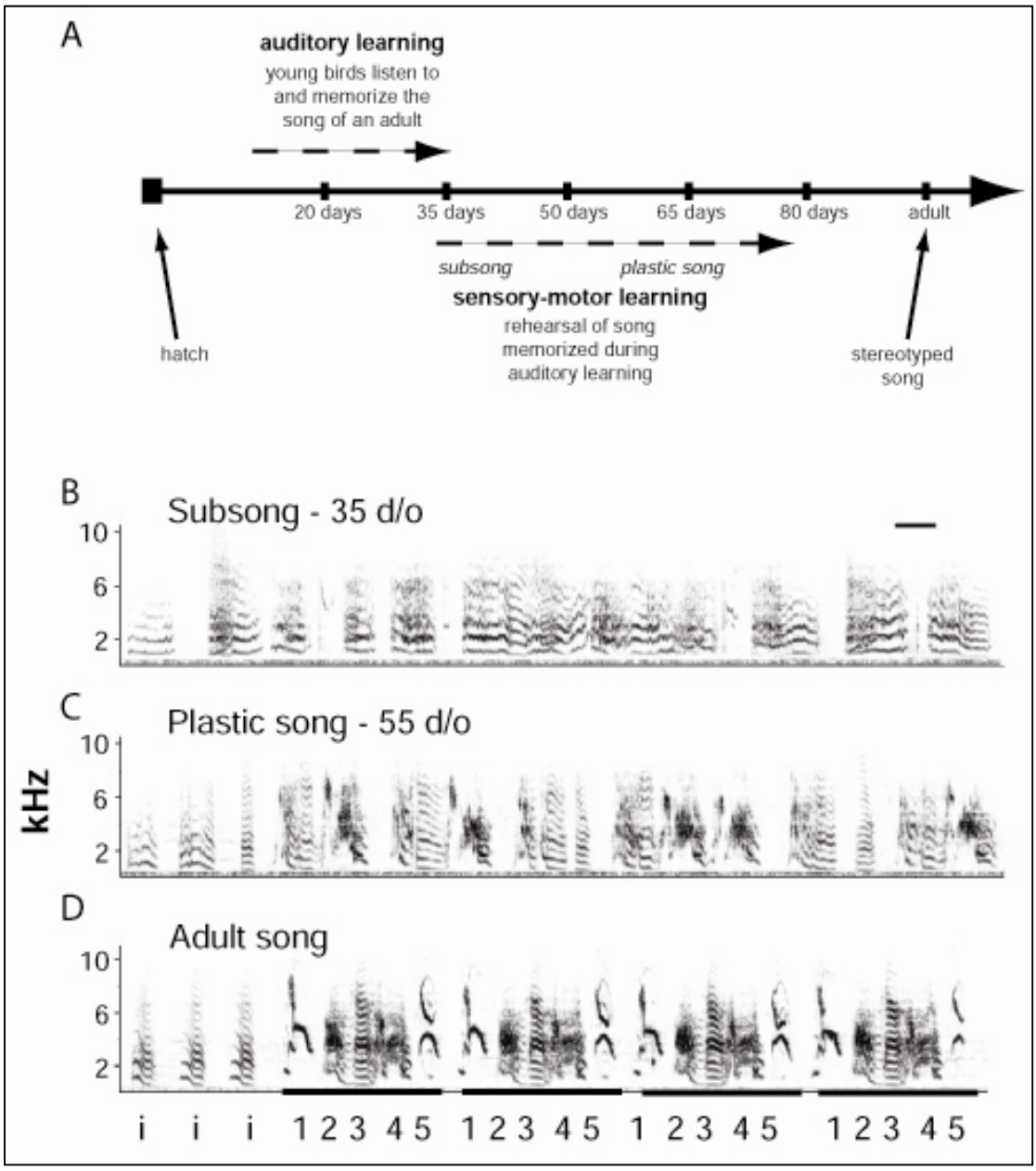
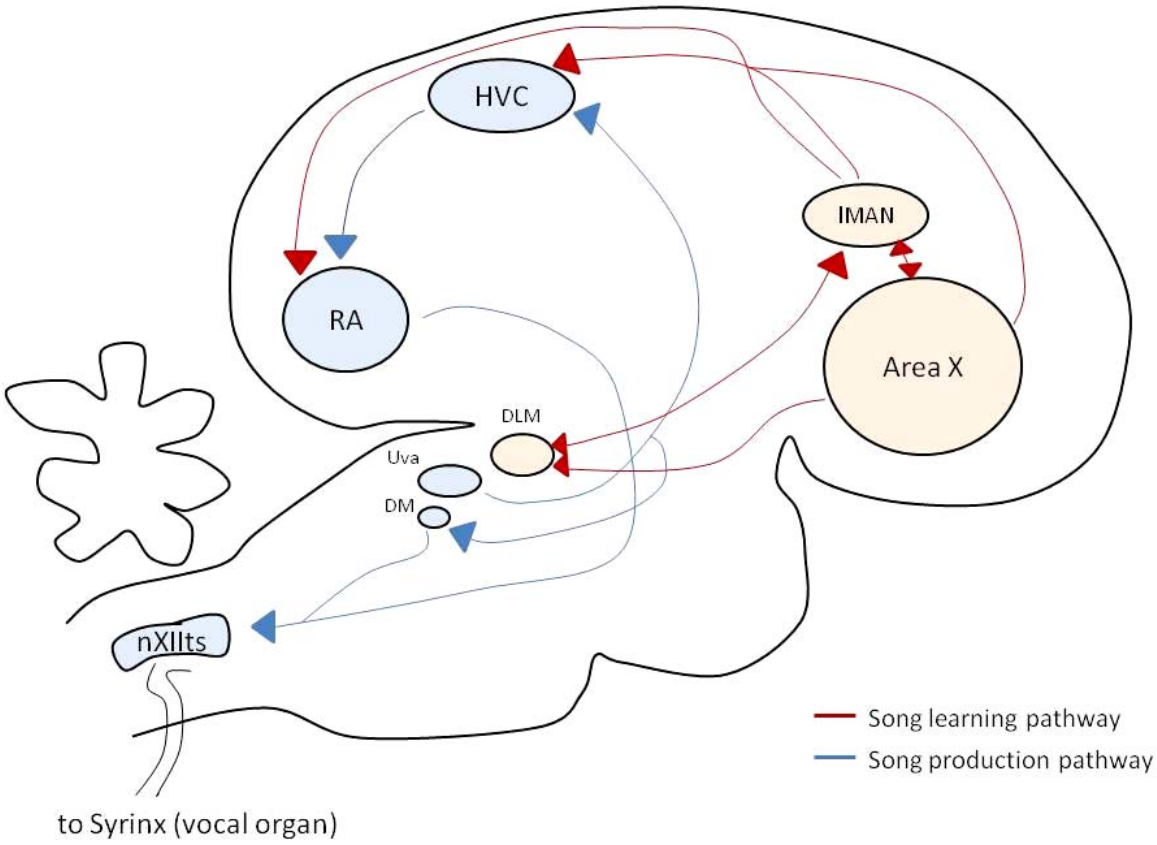

A
auditory learning
young birds listen to and memorize the song of an adult
20 days
35 days
50 days
65 days
80 days
adult
subsong
plastic song
sensory-motor learning
rehearsal of song memorized during auditory learning
hatch
stereotyped song
B
Subsong - 35 d/o
C
Plastic song - 55 d/o
kHz
D
Adult song
10
6
2
i i i 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 1 2 3 4 5

Figure 1.6 Parsaggital view of the zebra finch CNS demonstrating the relative positions of the major song regions and their axonal connections. The song regions implicated in vocal learning and their connections are highlighted in red, while the song regions necessary for the motor production of song and their connections are highlighted in blue. Abbreviations: Area X (area X of striatum); DLM (medial portion of the dorsolateral nucleus of the thalamus); DM (dorsomedical nucleus of the intercoillicular complex); HVC (higher vocal center); lMAN (lateral magnocellular nucleus of the anterior neostriatum); RA (robust nucleus of the archistriatum); nXIIts (hypoglossal nucleus, tracheosyringeal portion).


HVC
IMAN
RA
Area X
DLM
Uva
DM
nXIIts
Song learning pathway
Song production pathway
to Syrinx (vocal organ)

CHAPTER TWO: MATERIALS AND METHODS

Materials and Chemicals

Except where otherwise noted, all materials and reagents were purchased from Fisher Scientific (Fair Lawn, NJ), Sigma (St. Louis, MO) or Invitrogen (Carslbad, CA). Immunochemicals were purchased from Vector (Burlingame, CA). Oxytocin (Bachem, Torrance, CA) was dissolved in vehicle consisting of 1X phosphate buffered saline.

Animals

Male zebra finches used in these experiments were bred in our aviary and housed in flight aviaries. These aviaries were maintained at the ambient temperature of 78ºF, and birds were given free access to mixed seeds (SunSeed VitaFinch), grit, water, and cuttlebone. The light-dark cycle was maintained at LD 14:10 hours. The male birds were removed from the care of their parents, raised in groups by surrogate females, and tutored by the same adult male until 50 days of age. Following tutor exposure, the animals were placed in visual but not auditory isolation until their use in an experiment unless otherwise noted. All experimental procedures employing animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Brody School of Medicine at East Carolina University and were conducted in accordance with the guidelines for the humane use of animals in research (NIH "Public Health Service Policy on Humane Care and Use of Laboratory Animals" [revised 2002]). Every effort was made to reduce the use of animals to the minimum number required to achieve sufficient statistical power.

Traditional Cloning: 3'RACE (Rapid Amplification of cDNA Ends)

Isolation of Total RNA with Trizol

A zebra finch was obtained from our aviary and was anesthetized with a 50mcl injection of Equithesin (42.5 mg chloral hydrate, 95.7 mg pentobarbital sodium, 21.3 mg magnesium sulfate solubilized in 1 mL containing 44.3% propylene glycol, 11.5% ethanol, and 44.2% distilled water) and placed into a dark container to allow anesthesia to take effect. The brain was isolated and homogenized with a Fisher Science Powergen 1000 (Fair Lawn, NJ) in 1 mL of trizol per 50-100 mg of tissue. Following a 5 min incubation at room temperature (15ºC-30ºC), 0.2 mL of chloroform per 1 mL of trizol was added. Samples were capped, mixed vigorously for 15 seconds, and incubated at room temperature for 2-3 minutes. Samples were then centrifuged for 15 minutes at 4ºC at 12,000 x g. The aqueous phase containing the RNA was then transferred into a fresh tube and 0.5 mL of isopropyl alcohol per 1 mL of trizol used was added and incubated at room temperature for 10 minutes. Samples were then centrifuged at 4ºC at 12,000 x g for 10 minutes, the supernatant was removed, and the resulting pellet was washed 75% ethanol (1 mL per 1 mL trizol used). The samples were centrifuged a final time at 4ºC at 7,500 x g for 5 minutes, the supernatant was removed, and the pellet was air-dried for 5 minutes. If long-term storage was required, then the RNA pellet would be stored in the 75% ethanol at -20ºC. Otherwise the pellet was then dissolved in RNase free water by pipetting several times and incubating at 55Cº for 10 minutes.

First Strand cDNA synthesis

Invitrogen Superscript 1º Strand Synthesis System for RT-PCR (Carlsbad, CA)

The following components were mixed and briefly centrifuged:

Component	Amount per Sample

Total RNA	2.5 µg
Oligo dT primer (dT_{20})	50 µM
dNTP mixture (10 mM each dATP, dCTP, dGTP, dTTP)	10 mM
DEPC-Tx Water	q.s. to 10 µL

Samples were then incubated at 65ºC for 5 minutes and then placed on ice for 2 minutes. Reaction mixtures were prepared in order of the following: 10X RT Buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl) 25 mM $MgCl_2$, 0.1 M DTT, 1 unit RNase Out, and then 9 µL of the reaction mixture were added to each sample tube (RNA/primer mixture). The reactions were then mixed gently, spun briefly, and incubated at 25ºC for 2 minutes. Superscript II Reverse Transcriptase (50 units) was then added to each tube. Tubes were mixed and incubated at 25ºC for 10 minutes before they were transferred to 42ºC to incubate for an additional 50 minutes. Reactions were terminated by 15 minute incubation at 70ºC, chilled on ice, and collected by centrifugation. One unit of RNase H (1µL) was added to each tube and the reactions were then incubated at 37ºC for 20 minutes.

3' RACE PCR Strategy

The following reactions were set up in sterile 0.5 mL PCR tube:

Component	Amount per Sample
Diluted 1ºcDNA (1:10)	2.0 µL
10XPCR Buffer (200 mM Tris HCl (pH 8.4), 500 mM KCl)	5.0 µL
Oligo dT primer (dT_{17})	10 µM
Gene specific primer	10 µM
dNTP mixture (10 mM each dATP, dCTP, dGTP, dTTP)	10 mM
Taq DNA Polymerase	1 unit

DEPC-Tx Water	q.s. to 50µL

The gene specific primer used was a degenerate zebra finch mesotocin receptor primer ATG-GAATTC-TT(T/C)TT(T/C)ATGAA(A/G)C). The samples then underwent 20 of the following PCR cycles in a Biorad iCycler thermocycler (Hercules, CA): 94ºC for 30 seconds, 58ºC for 30 seconds, 72ºC for 1 minute and a final cycle of 72ºC for 2 minutes. Samples were held at 4ºC at the conclusion of the amplification. PCR products were visualized on an ethidium bromide stained 1.5% agarose gel which was subjected to electrophoresis in 1X TBE (0.1 M Tris; 90 mM Borate; 2.5 mM EDTA; pH 8.3) at 95volts for approximately 45 minutes.

TA Cloning of PCR Products

Invitrogen TA Cloning Kit (Carlsbad, CA)

A. Ligation

The following ligation reaction was set up and incubated overnight at 14ºC:

Component	Amount per Sample
pCR®II, linearized	2.0 µL
Ligase Buffer (60 mM Tris-HCl (pH 7.5), 60 mM $MgCl_2$, 50 mM NaCl, 1 mg/ml bovine serum albumin, 70 mM β-mercaptoethanol, 1 mM ATP, 20 mM dithiothreitol, 10 mM spermidine)	1.0 µL
PCR reaction	2.0 µL
Distilled Water	q.s. to 10 µL

B. Transformation

Bacterial plates containing 100 µg/mL of ampicillin were prepared with LB Broth and Agar, spread with 40 µL of 40 mg/mL of x-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside),

and the liquid was allowed to soak into the plates. Ligation reactions were briefly centrifuged and placed on ice. One vial of frozen One Shot® TOP10 Chemically Competent E. coli was thawed on ice for each ligation. A 2 µL aliquot of the ligation reaction was added to a vial of cells and gently mixed with the tip of the pipette. The vials were then incubated on ice for 30 minutes. The cells were heat shocked by placement into a 42ºC water bath for 30 seconds and placed on ice immediately after. SOC Media® (250 µL) was added to each tube and the tubes were allowed to shake for 1 hour at 37Cº. An aliquot of 50 µL was spread on each prepared bacterial plate, liquid was allowed to absorb, and the inverted plates were placed at 37ºC for 18 hours and at least 2 hours at 4ºC to allow for color shift development. Six positive colonies (white in color) were chosen at random from each plate and each single colony was grown overnight at 37ºC in 1.5 mL of LB Broth containing 100 µg/mL of ampicillin.

Plasmid Quick Preparation

Cultures were poured into 1.5 mL centrifuge tubes and spun for 30 seconds at 5,000 x g at 4ºC to pellet bacteria. Supernatant was decanted and pellet was re-suspended in 100 µL of lysis solution (50% glucose, 10 mM EDTA, 50 mM Tris-HCl (pH 8.0)). 200 µL of alkaline SDS solution (1% SDS, 20 mM NaOH) was added. Reactions were vortexed and incubated on ice for 5 minutes. 150 µL of a high salt solution (3.0 M Potassium Acetate, pH to 5.5 with glacial acetic acid) was added, the mixture was vortexed, and reactions were incubated on ice for 10 minutes. Reactions were then spun for 10 minutes at 20,000 x g at 4ºC and approximately 400 µL of supernatant was removed and added to 800 µL of ice cold ethanol. Reactions were vortexed, incubated on ice for 10 minutes, and spun at 20,000 x g at 4ºC for 10 minutes. The supernatant was removed and the resulting DNA pellet was washed with 70% ethanol and allowed to dry. The pellet was re-suspended in 10 mM Tris pH 8.0 with 1:1000 RNase and allowed to incubate

at room temperature for 30 minutes to remove RNA from the samples. The resulting DNA was quantified with a Nanodrop ND-1000 (Thermo Scientific, Wilminton, DE) and DNA was digested with EcoR1 (New England Biolabs (NEB), Ipswich, MA) to determine insert size.

Restriction Digestion Mapping

A master mix containing the following was set up per reaction desired:

Component	Amount per Sample
10X Reaction Buffer (100 mM Tris-HCl, 50 mM NaCl	1.0 µL
10 mM $MgCl_2$, 0.025 % Triton X-100, pH 7.5)	
EcoR1or ApaI Enzyme	0.25 µL
Distilled water	6.75 µL

2 µL of DNA isolated previously with the plasmid preparation was added to each reaction and reactions were allowed to proceed overnight at 37ºC. Results were visualized on on an ethidium bromide stained 1.5% agarose gel which was subjected to electrophoresis in 1X TBE (0.1M Tris; 90 mM Borate; 2.5 mM EDTA; pH 8.3) at 95volts for approximately 45 minutes.

Sequencing of Results

Plasmid preparations were sent to the Genomics Core Facility located in the Department of Biology at East Carolina University which utilized the 3130 Genetic Anyalyzer (Applied Biosystems™, Carlsbad, CA) and BigDyeTerminator v3.1 chemistry (Applied Biosystems™, Carlsbad, CA) to produce sequence data. The sequence data obtained was subjected to homology searches using NCBI's BLAST (Basic Local Alignment Search Tool) program for verification.

In-Silico Cloning

Once the shotgun zebra finch sequence became available for BLAST, the 492 base pairs of the receptor previously cloned were used for a homology search. Results of this search included the entire mesotocin receptor sequence including the ATG start codon. The cDNA sequence information using BLAST searches of cDNA and translated protein sequences from other mammals of the mesotocin pre-propeptide including neurophysin, the mesotocin receptor, and zebra finch oxytocinase-like protein were obtained in this manner. All alignments shown were produced using the ClustalW2 program found at http://www.ebi.ac.uk/Tools/clustalw2/index.html. Alignments included sequence information on the protein of interest from *gallus gallus*, *homo sapiens*, and *rattus norvegicus*.

Receptor mRNA Analysis

RNA Separation by an Agarose Gel with Formaldehyde

An agarose gel was prepared by adding 3.0 g of agarose to 144 mL of sterile water. The mixture was heated in the microwave and cooled to touch. 20 mL of 10X MOP Electrophoresis Buffer (0.2 M MOPS (pH 7.0), 20 mM sodium acetate, 10 mM EDTA (pH 8.0)) and 18 mL of deionized formaldehyde was added and the resulting gel was cast with a 3 mm comb. The gel was allowed to set and then was covered with saran wrap. The RNA samples were prepared by setting up the following components in a 1.5 mL microcentrifuge tube:

Component	Amount per Sample
RNA (10 µg/µL)	2.0 µL
10X MOP Electrophoresis Buffer	2.0 µL
Deionized formaldehyde	4.0 µL
Formamide	10.0 µL
Ethidium Bromide (200 µg/mL)	1.0 µL

The samples were then incubated at 55ºC for 60 minutes, chilled on ice for 10 minutes, and centrifuged for 5 seconds before the addition of 2 µL of 10X Formaldehyde Gel-loading Buffer (50% glycerol in DEPC-Tx H_2O [1:1000 dillution of diethyl pyrocarbonate in distilled water which has been autoclaved and allowed to cool], 10 mM EDTA (pH 8.0), 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol) on ice. The gel was set up for electrophoresis and ran for 5 minutes at 50 volts in 1X MOP Electrophoresis Buffer. Samples were loaded and the gel was run for approximately four hours at 4ºC at 35 volts. Integrity of the RNA was checked by visualization of the three distinct ribosomal bands at 28S, 18S, and 5S over a Fisher Scientific UV transilluminator.

Transfer of RNA Agarose Gel

RNA agarose gel was rinsed in DEPC-Tx H_2O and soaked for 20 minutes in 5 gel volumes of 0.05 N NaOH and for 40 minutes in 10 gel volumes of autoclaved 20X SSC (3.0 M NaCl, 0.2 M sodium citrate in DEPC-Tx H_2O, pH 7.4) at room temperature. Gel was then cut with at scalpel for orientation purposes. A nitrocellulose membrane was floated in sterile water until it was wet and then was immersed in transfer buffer (10X SSC) for 5 minutes. A capillary transfer was then set up with a paper towels, Whatman® 3M filter paper, the RNA gel, and the nitrocellulose membrane as illustrated in Figure 2.1 and the transfer was allowed to proceed overnight. The membrane was then transferred to a glass tray where it was agitated very slowly in 300 mL of 6XSSC for 5 minutes at room temperature. The membrane was then allowed to air dry and the RNA was crosslinked to the membrane using a Spectrolinker™ 1000 UV crosslinker (Westbury, NY) and the standard crosslinking protocol on the machine. Membranes were stored wrapped in aluminum foil at room temperature until use.

Creation of a DNA Template for cDNA Probe Synthesis

The following reactions were set up in sterile 0.5 mL PCR tube:

Component	Amount per Sample
Diluted 1ºcDNA (1:10)	2.0 µL
10XPCR Buffer (200 mM Tris HCl (pH 8.4), 500 mM KCl)	5.0 µL
Forward primer = 5'-GGAATGTTCGCTTCCACCTA-3'	10 µM
Reverse primer = 5'- CATCAGGTCGTGGAAGAGGT-3	10 µM
dNTP mixture (10 mM each dATP, dCTP, dGTP, dTTP)	10 mM
Taq DNA Polymerase	2.5 units
DEPC-Tx Water	q.s. to 50 µL

The samples then underwent 35 of the following PCR cycles in a Biorad iCycler thermocycler (Hercules, CA): 94ºC for 30 seconds, 58ºC for 30 seconds, 72ºC for 1 minute and a final cycle of 72ºC for 2 minutes. Samples were held at 4ºC at the conclusion of the amplification. PCR products were visualized on an ethidium bromide stained 1.5% agarose gel which was subjected to electrophoresis in 1X TBE (0.1 M Tris; 90 mM Borate; 2.5 mM EDTA; pH 8.3) at 95volts for approximately 45 minutes.

PCR Purification

NucleoTrap® Gel Extraction and PCR Purification kit (Clontech, Mountain View, CA) was used to remove PCR contaminants from PCR samples. The final volume of the PCR reaction was adjusted to 100 µL with TE (10 mM Tris, 1 mM EDTA, pH 8.0). NucleoTrap suspension was vortexed well and 10 µL was added to the PCR reaction along with 400 µL of NT2 Buffer. The reactions were incubated at room temperature for 10 minutes and were vortexed every 3 minutes during the incubation period. The reactions were then centrifuged at 10,000 x g for 30 seconds at room temperature and the supernatant was discarded. 400 µL of NT2 Buffer was added, the

samples were vortexed and spun at 10,000 x g for 30 seconds, and the supernatant was discarded. 500 µL of Buffer NT3 was added, the samples were vortexed and spun at 10,000 x g for 30 seconds, and the supernatant was discarded. This step was repeated once more and the resulting pellet was air dried for 10 minutes at room temperature. 20 µL of distilled water was added to the pellet, the mixture was vortexed, and the DNA was eluted by incubating the samples for 10 minutes at room temperature (vortexing every 2 minutes). The samples were then centrifuged at 10,000 x g for 30 seconds and the supernatant containing the purified DNA fragment was transferred to a 1.5 mL centrifuge tube. The resulting DNA was quantified with a Nanodrop ND-1000 (Thermo Scientific, Wilminton, DE).

Probe Synthesis with Klenow Enzyme

Set up the following reaction components to create a DNA mixture:

Component	Amount per Sample
DNA (50 ng/µL)	2.0 µL
Random Hexamers (3 µg/µL)	2.5µL
Distilled water	34.0 µL

DNA mixture was boiled for 5 minutes, chilled in an ice bath for 5 minutes, and centrifuged to collect samples for 30 seconds at 4ºC before remaining on ice until use. Set up the following reaction components to create a reaction mixture:

Component	Amount per Sample
NEB Buffer 2 (10 mM Tris-HCl, 50 mM NaCl 10 mM $MgCl_2$, 1 mM Dithiothreitol pH 7.9) (Ipswich, MA)	5 µL
BSA (10 mg/mL) (NEB, Ipswich, MA)	0.5 µL

dNTP-C (10 mM each dATP, dGTP, dTTP)	0.5 µL
dCTP, [$^{\alpha\text{-}33P}$]- 3000Ci/mmol (Perkin Elmer, Waltham, MA)	5.0 µL
Klenow (exo-) (NEB, Ipswich, MA)	1.0 µL

Add reaction mixture to DNA mixture and incubate at 37ºC for 2 hours. Unincorporated nucleotides were removed with a Sephadex G-50 spun column and 100 µL of denatured salmon sperm DNA (10 mg/mL) was added. The purified probe was boiled for 5 minutes, chilled on ice for 5 minutes, and then added to either a RNA blot (Northern Blots), DNA blot (Southern Blots) or tissue sections (*In-situ* hybridization). Incorporated radioactivity was counted by the use of a Packard TriCarb Liquid Scintillation Analyzer 2200CA (GMI, Inc., Ramsey, MN) which counted 1 µL of cDNA probe in 5 mL of scintillation fluid.

Southern Blotting

A fragment of DNA from the mesotocin receptor (648 base pair fragment used as the template for probe synthesis) and one fragment of zebra finch cannabinoid receptor (~1 kb) were amplified by PCR and ran on a 1.5% agarose gel along with a plasmid harboring the 492 base pair fragment cloned by 3'RACE. This agarose gel was transferred via capillary transfer (see Figure 2.1 for an illustration). The resulting nitrocellulose membrane was then subjected to a denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 minutes at room temperature and a neutralizing solution (0.5 M Tris-Cl (pH 7.4), 1.5 M NaCl) for 5 minutes by laying the membrane on top of three pieces of saturated Whatman™ 3M filter paper with either solution. The membrane was then allowed to air dry and the DNA was crosslinked to the membrane using a Spectrolinker™ 1000 UV crosslinker (Westbury, NY) and the standard crosslinking protocol on the machine. The dried blot was wet with 2X SSC (0.3 M Sodium Chloride, 30 mM Sodium Citrate) and then prehybridized with 5 mL of ULTRAhyb® Ultrasensitive Hybridization Buffer

(Applied Biosystems™, Carlsbad, CA) in a heat sealed bag for 1 hour at 42ºC. Denatured cDNA probe (see above) was added to the bag at $1x10^6$ CPM, the bag was re-sealed, and the blot hybridized overnight at 42ºC. The blot was then washed with 2X SSC, 0.1% SDS two times at room temperature for 10 minutes each wash. The blot was further washed with a 0.2XSSC, 0.1% SDS at 65ºC for 15 minutes. The blot was then dried and exposed to film overnight.

Northern Blotting

A nitrocellulose membrane that was previously crosslinked with RNA from a RNA agarose gel (see above) was wet with 2X SSC at room temperature and incubated with 15 mL of pre-hybridization solution (50% formamide, 0.8 M NaCl, 20 mM PIPES (pH 6.5), 0.5% SDS, 100 µg/ml sonicated herring sperm DNA) for 2 hours at 65ºC in a heat sealed bag. Single stranded cDNA probe (see above for preparation details) equaling to $1x10^6$ CPM was added to the bag, the bag was re-sealed, and the blot hybridized overnight at 42ºC. The membrane was then removed and washed in the following solutions for 10 minutes for each wash at 65ºC: 1X SSC / 0.1% SDS, 0.5X SSC / 0.1% SDS, 0.5X SSC / 0.1% SDS. The blot was then allowed to air dry before being exposed to film overnight.

Receptor Protein Analysis

Mesotocin Receptor Antibody Production

A zebra finch specific anti-mesotocin receptor antibody was developed using the deduced amino acid sequence from the cDNA cloned and the identification of a suitable epitope with the help of Spring Valley Laboratories, Inc (Woodbine, MA). The resulting antisera was raised in rabbit and targeted a 16-amino-acid region within the intracellular loop portion of the zebra finch mesotocin receptor (LLSSSTRYLKSRPAC) which encompassed residues Lys 347 – Cys 361. The predicted epitope was TRYLKS.

Tissue Homogenization

An animal was anesthetized as described previously and tissues were rapidly dissected on ice. Organs were weighed and placed into a glass test tube containing 25 volumes of ice cold homogenization buffer (0.32 M sucrose, 5 mM Tris pH 7.4). Tissues were homogenized with a Teflon grinder. Homogenates were centrifuged at 4ºC for 15 minutes; the supernatant was removed while samples were on ice; and the pellets were discarded. Supernatants were centrifuged at 20,000 x g for 1 hour at 4ºC, and the resulting pellet was either re-suspended with a glass and Teflon grinder in 50 mM Tris, 5 mM EDTA (pH 7.4) or frozen at -80ºC for long term storage.

Protein Concentration Determination

Pierce® BCA protein assay kit was used for spectrophotometric protein determination (Rockford, IL). Protein or standard samples were mixed with Pierce® BCA reagent A/B solutions and absorbance was measured in duplicate using the Synergy HT spectrophotometer (South Coast Metro, CA) at a wavelength of 562 nm. The sample absorbance was calculated and protein concentrations extrapolated from a standard curve generated with data from serially diluted pre-determined standard samples of bovine serum albumin.

Western Blotting

Preliminary experiments were performed to optimize conditions of the optimal primary and secondary antibody concentrations for the immunoblot procedure. Protein samples containing 35 µg of total protein were prepared for immunoblotting by placing into 1XSDS loading buffer (63 mM Tris HCl, 10% Glycerol, 2% SDS, 0.0025% Bromophenol Blue (pH 6.8)) and heating for 7 minutes at 100ºC. Samples were then briefly centrifuged to collect and loaded onto a 5% stacking gel (4.3% acrylamide mix (37.5:1 acrylamide to bisacrylamide), 125 mM Tris-HCl (pH

6.8), 0.1% SDS, 0.01% ammonium persulfate, 0.1% TEMED (Tetramethylethylenediamine)) and a 12% resolving gel (12% acrylamide mix (37.5:1 acrylamide to bisacrylamide), 375 mM Tris-HCl (pH 8.8), 0.1% SDS, 0.01% ammonium persulfate, 0.1% TEMED (Tetramethylethylenediamine)) for Tris-glycine SDS-Polyacrylamide gel electrophoresis to fractionate proteins by size using a Mini-PROTEAN II Cell ran at 95V (Bio-Rad, Hercules, CA) in running buffer (25 mM Tris Base, 250 mM glycine, 0.1% SDS (pH 8.3)). The proteins were then transferred to PDVF (polyvinylidene fluoride) membranes presoaked in transfer buffer (24 mM Tris Base, 192 mM glycine, 20% methanol) using a Mini-PROTEAN II Cell ran at 95 volts (Bio-Rad, Hercules, CA). Membranes were blocked for 3 hours in 1XTBST (50 mM Tris.HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) containing 10% casein at room temperature, and then incubated overnight with a 1:2,000 dilution of the primary antibody, zebra finch specific anti-mesotocin receptor (Spring Valley Laboratories, Woodbine, MA), at 4ºC with gentle agitation. The membranes were washed thrice in 1X TBST for a period of 5 minutes for each wash before incubation with the secondary antibody a goat anti-rabbit IgG-HRP (Santa Cruz, Santa Cruz, CA) at a dilution of 1:6,000 for 3 hours at room temperature with gentle agitation. The membrane was then washed twice with 1X TBST for 5 minutes each wash and a final time with 1XTBS (50 mM Tris.HCl, pH 7.4, 150 mM NaCl) to ensure the removal of excess secondary antibody before bands were detected via chemiluminescence with the Amersham ECL Plus™ Western Blotting Detection Reagents A/B (GE Healthcare Biosciences, Pittsburgh, PA) and exposure to film. Films were developed using a Konica Minolta SRX-101A (Wayne, NJ) film processor.

Immunohistochemical Analysis

Tissue Preparation and Perfusion

A zebra finch was obtained from our aviary and was anesthetized with a 50mcl injection of Equithesin as previously described. The Cole Parmer Master Flex peristaltic pump (Vernon Hils, IL) was set up and ice-cold 1X PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4) was allowed to flow through the machine to remove air bubbles and a 30½g needle was attached. Once the proper level of anesthesia for the bird was obtained, the feathers on the neck and chest area were removed. Two cuts were made, one at the neck area and one at the shoulder of the descending vena cava. An incision was made at the bottom of the breast bone and the rib cage is opened to expose the heart. The bird was secured to a disposable pad. The needle attached to the pump was inserted into the right atria and the pump was turned on. Blood flow was observed to flow from the top incision which insured proper flow of the 1X PBS into the brain of the animal. Perfusion was continued until the fluid exuding from the top cut became clear. The pump was stopped and was switched to a 4% paraformaldehyde solution in 1X PBS which was allowed to flow until significant rigor was found in the animal's neck. The brain was carefully removed and placed into a solution of 4% paraformaldehyde overnight for post-fixation at 4ºC. Only white blood free brains were used for tissue analysis. Post-fixed brains were blocked parasagittally down the midline and stored in a 20% sucrose solution (1X PBS, 5% Sodium Azide) at 4ºC for at least 24 hours before use in experiments.

Brain Sectioning

Blocked brains will then be removed from storage and glued to a microtome chuck using super-glue and aligned to allow minimal movement upon sectioning. The microtome chuck was attached to the sample tray by the tightening of a screw and the sample tray was attached to the vibratome. A stainless steel injector blade was cleaned with acetone and secured onto the lateral knife holder which was then attached to the vibratome. Sections were made using a Leica

VT1000S vibratome (Bannockburn, IL) with the brain fully immersed in cold 1XPBS. 30 micron parasagittal sections were created and collected into ice-cold 1XPBS.

Immunohistochemistry

Preliminary experiments were performed to optimize conditions of the optimal primary and secondary antibody concentrations for the experiment and all experiments were conducted at room temperature. Brain sections were thrice washed in 1X PBS for 5 minutes per wash. Sections were then pretreated with a 1% H_2O_2 solution in 1X PBS to remove endogenous peroxidases for 30 minutes and then washed three times in 1X PBS (5 minutes per wash). A 5% solution of normal goat serum (NGS) in 0.3% Triton-X was used as a blocking buffer and tissues were allowed to block for 30 minutes before the addition of the primary antibody. The zebra finch specific anti-mesotocin receptor antibody (Spring Valley Laboratories, Woodbine, MA) was the primary antibody used at a dilution of 1:1000 in a 1X PBS solution (1X PBS, 1% NGS, 0.02% Sodium Azide) and tissues were incubated overnight. Excess primary antibody was removed with three 5 minute washes in 1X PBS before the addition of the secondary antibody, Biotinylated Goat Anti-Rabbit IgG Antibody (VECTASTAIN® ABC kit, Vector, Burlingame, CA) at a dilution of 1:3000 in a 0.3% Tri-X Solution (0.3% Tri-X, 1.5% NGS) for one hour. During this time the ABC reagent (VECTASTAIN® ABC kit) was prepared with equal amounts of A (Avidan DH) and B (Biotinylated Horseradish Peroxidase H) in 1X PBS and this solution was allowed to mix for one hour prior to use. Following incubation with the secondary antibody tissue sections were washed thrice in 1X PBS for 5 minutes per wash. Tissues were then incubated with the prepared ABC reagent for one hour and washed 3 times in 1X PBS for 5 minutes per wash. The DAB (3, 3'-diaminobenzidine) solution was prepared using the DAB substrate kit from Vector (Burlingame, CA) according to the manufactures' instructions and

tissues were reacted until a rusty color appeared. Tissues were then washed a final three times in 1XPBS for 5 minutes per wash before being mounted on Fisherbrand™ Superfrost Plus slides (Fairlawn, NJ) using cooled 0.5% gelatin solution (225 bloom, Type B Bovine gelatin). Mounted sections were allowed to dry overnight before dehydration using solutions containing increasing amounts of ethanol (70%, 95%, 100%) and xylene and coverslipping with Permount™ (Fisher Scientific, Fairlawn, NJ). Coverslipped slides were allowed to fully dry, cleaned, and examined in various CNS brain song regions (i.e. Area X, lMAN, HVC, etc) at 40, 100 and 1000 X magnification using Olympus BX51 microscope with Nomarski DIC optics (Center Valley, PA). Images of the slides were taken using a Spot Insight QE digital camera (Spot™ Imaging Solutions, Sterling Heights, MI) and Image-Pro Plus software (Media-Cybernetics, Silver Spring, MD) under identical calibrated exposure conditions and background-corrected.

Measurement of Optical Density of Anti-Zebra Finch Mesotocin Receptor Staining

There were five gray-scale images captured at 40X magnification per age and brain region examined. The borders of the regions of interest were traced using Image-Pro Plus analysis software, and the software computed the optical densities within each hand-traced region. The images for each of five age groups were captured in a single session under identical, calibrated exposure conditions. The optical density values for the five images of each brain region for each of the five ages were calculated and the maximal mean value was determined. This maximal mean was then used to transform the optical density data into a percentage maximal OD for ease in graphical analysis.

In-situ Hybridization

Tissue Preparation and Perfusion

Tissue preparation was as previously described except for precautions taken to ensure RNA integrity. All solutions used were created with DEPC-Tx water, and all equipment was cleaned with RNase AWAY (Molecular BioProducts, Inc., San Diego, CA) as per the manufacturers' instructions if feasible. All glassware was rinsed with DEPC-Tx water and autoclaved before use. Following perfusion with 4% paraformaldehyde the brain was carefully removed and placed into a solution of 4% paraformaldehyde overnight for post-fixation at 4ºC. Post-fixed brains were blocked parasagittally down the midline and were sectioned immediately to avoid RNA degradation.

Brain Sectioning

Brain sectioning was as previously described except for precautions taken to ensure RNA integrity. All solutions used were created with DEPC-Tx water, and all equipment was cleaned with RNase AWAY (Molecular BioProducts, Inc., San Diego, CA) as per the manufacturers' instructions. All glassware was rinsed with DEPC-Tx water and autoclaved before use. Sections were made using a Leica VT1000S vibratome (Bannockburn, IL) with the brain fully immersed in cold 1X PBS. 30 micron parasagittal sections were created and collected into treated and autoclaved glass scintillation vials containing ice-cold 2X SSC.

In-Situ Hybridization

The 2X SSC was removed from the scintillation vials and the sections were re-suspended in 1 mL of freshly prepared pre-hybridization buffer (50% formamide, 10% dextran sulfate, 2X SSC, 1X Denhart's solution (0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinyl-pyrrolidone), 50 mM DTT, 0.5 mg/mL of denatured salmon sperm DNA) warmed to 48ºC. Sections were allowed to incubate for one hour at 48ºC. A labeled cDNA probe was prepared as previously detailed in section V, part 5 and added to the vials containing the brain sections (1 x

10^7 CPM total radioactivity per vial) without allowing the vials to cool. Vials were incubated overnight with gentle rotation at 48ºC using a HybAid hybridization oven (Fisher Scientific, Fairlawn, NJ). Sections were then washed with the following concentrations of SSC for 15 minutes at 48ºC: 2X SSC, 2X SSC, 1X SSC, 0.5X SSC, 0.25X SSC, 0.125X SSC, 0.125X SSC. Washing progress was monitored with the Bicron PGN hand-held Geiger counter (Brooke Clarke, Ukiah, CA) and washing was stopped once the radioactivity decreased significantly. Tissues were then mounted on previously subbed Fisherbrand™ Superfrost Plus slides (Fairlawn, NJ) using cooled 0.5% gelatin solution (225 bloom, Type B Bovine gelatin). Mounted sections were allowed to dry overnight before being dipped into liquid emulsion.

Coating of Slides with Liquid Emulsion

The entire process was completed under darkroom conditions unless otherwise specified using only a KODAK Adjustable Safelight Lamp, Model B (Kodak, Rochester, NY) for illumination. Amersham Hypercoat™ LM-1 liquid emulsion (GE Healthcare Biosciences, Pittsburgh, PA) was placed into a water bath warmed to 43ºC and allowed to melt for 15 minutes. The melted emulsion was then poured into a glass dipping chamber. Each slide of interest was dipped vertically into the emulsion for 5 seconds, slowly withdrawn, and allowed to drain in a vertical position for 5 seconds. Slides were then placed on a paper towel in a near vertical position to set for 5 minutes before being placed into a slide box containing a small amount of anhydrous silica gel. The slide box was wrapped with layers of aluminum foil to ensure a light-tight environment for the slides and then the box was stored at 4ºC for 3 weeks to expose the emulsion.

Development of Coated Slides

The entire process was completed under darkroom conditions unless otherwise specified using only a KODAK Adjustable Safelight Lamp, Model B (Kodak, Rochester, NY) for illumination.

The slides were removed from storage and allowed to equilibrate to room temperature for 60 minutes. Working stock solutions of the Ilford Hypam fixer (1:4), Ilford Ilfostop Stop Bath (1:19), and Phenisol developer (1:4) were prepared (AEH Business Solutions, Houston, TX). Exposed slides were placed into a slide holder and into the developer for 5 minutes with gentle agitation every minute. The slides were then transferred to the stop solution for 30 seconds before being incubated in the fix solution for 10 minutes with gentle agitation. Slides were washed in water under a constant gentle flow for 15 minutes. Autoradiograms were allowed to dry overnight before dehydration using solutions containing increasing amounts of ethanol (70%, 95%, 100%) and xylene and coverslipping with Permount™ (Fisher Scientific, Fairlawn, NJ). Coverslipped slides were allowed to fully dry before being cleaned and examined in various CNS brain song regions (i.e. Area X, lMAN, HVC, etc) at 40 and 100 X magnification using an Olympus BX51 microscope with Nomarski DIC optics (Center Valley, PA). As with the immunohistochemistry slides images of the slides were taken using a Spot Insight QE digital camera (Spot™ Imaging Solutions, Sterling Heights, MI) and Image-Pro Plus software (Media-Cybernetics, Silver Spring, MD) under identical calibrated exposure conditions and background corrected.

Measurement of Optical Density of Silver Grains

Measurement of the optical density of silver grains present in the autoradiograms was similar to that detailed in immunohistochemistry experiments. There were five gray-scale images captured at 40X magnification per age and brain region examined. The borders of the regions of interest were traced using Image-Pro Plus analysis software, and the software computed the optical densities within each hand-traced region. The images for each of five age groups were

captured in a single session under identical, calibrated exposure conditions. The optical density values for the five images of each brain region for each of the five ages were calculated.

Auditory learning experiments

Drug Preparation

In order to obtain average weights for zebra finch fledglings, five young birds were weighed for each age of treatment (days 5-9 post hatch) and their weights were averaged. An OT stock solution of 1 mg/mL was created using sterile 1X PBS and all drug dilutions using sterile 1X PBS were made from this stock. Drug treatment solutions were created in a manner that assured the dosage of OT desired would be administered in a 50µL volume. Please see Table 2.1.

Drug Administration Schedules

Nests of zebra finch hatchlings were randomly assigned to treatment groups so that an entire nest received the same treatment. At day five post hatch zebra finches were injected with 50 mcl subcutaneously over the pectoralis muscle with either OT or vehicle prior to the start of the 14-hour light cycle for a total of five days (days 5-9). A preliminary behavioral experiment assayed the effects treatment with three doses of OT (300 µg/kg, 1000 µg/kg, and 3000 µg/kg) and a vehicle control. Doses were chosen based on a review of the literature. See Table 2.2. This initial experiment was refined due to the lack of statistical significant differences between doses on effects examined (song learning) and a high rate of death in the highest dose employed (3000 µg/kg). A larger study was conducted using the lowest dose shown effective in our preliminary experiment as well as a lower dose in an effort to evaluate the dose response relationship. Treatment groups were as follows: 300 µg/kg OT; 30 µg/kg OT; and vehicle.

Animal Tutoring

The zebra finch fledglings were banded for identification at 15 ± 2 days before they fledged the nests and were then allowed to remain with their parents until 25 ± 2 days when they were removed, group housed and raised by surrogate females. Male zebra finches were identified by secondary sex characteristics such as the appearance of the black bar feathers on the breast at 25 ± 2 days. After identification young male zebra finches were tutored by the same adult male in constant auditory and visual contact until 50 days of age. This period of tutor exposure exceeds that required for vocal learning (Funabiki and Konishi 2003). The animals were then moved to a cage with visual but not auditory isolation and allowed to mature. Visual isolation will prevent potential cross-tutoring from other juveniles (Morrison and Nottebohm 1993).

Song Recording

Digital recordings of songs produced were made at approximately 100 days of age from the treated male zebra finches. Birds were recorded simultaneously in randomly assigned groups of 8. Prior to recordings birds were allowed to habituate to recording chambers and handled daily for 1 week. Recordings were made using a custom, computerized monitoring system that the laboratory has had extensive experience with. The Avisoft SASLab Pro software (Berlin, Germany) compared continuous live recordings to programmed frequency criteria of song bouts (Johnson, Soderstrom et al. 2002; Soderstrom and Johnson 2003; Soderstrom and Tian 2004). Recordings that match frequencies and duration of song were saved by the computer as audio files in WAV format. This system allowed for real-time monitoring of the totality of a bird's vocal output.

Song Analysis

Ten-song bout recordings per animal were selected randomly for analysis. Song parameters were measured by an observer blind to treatment. Methods for measuring note duration and

internote intervals were based on previously established protocol used in the laboratory (Soderstrom and Tian 2004). Song stereotypy scores will be determined using the method described by (Scharff and Nottebohm 1991) which quantifies song stereotypy through the linearity of the syllables produced in phrases and the conisistency of the of the sequence of syllables contained within phrases. Phrase linearity is evaluated through a "linearity score": linearity = different song notes/transition types. In this equation a transition type is the order in which one syllable follows another. In a completely linear phrase sequence, each syllable has only one transition type, and the linearity score = 1. Syllable consistency is scored as: Consistency = typical transitions/total transitions. This equation expresses the frequency with which a particular ordering of syllables within a phrase occurs. The typical transition type is defined as the syllable sequence that occurs most frequently. The stereotypy score combines measures of linearity and consistency: Stereotypy = (linearity +consistency) / 2. Normal adult males stereotypy scores vary from 0.7 - 0.9 (Whitney, Soderstrom et al. 2000). Scores lower than this are generally associated with immature song (e.g. plastic song stereotypy scores range from 0.5 - 0.6).

Statistical Analysis

Immunohistochemistry and In-Situ Hybridization

Data analyses and statistical computations were performed in Systat 12 (Systat Software, Inc., Chicago, IL) and Microsoft® Office Excel 2007 PC software (Microsoft®, Mission Viejo, CA). The quantification of optical densities was determined from digital images of brain regions of interest captured under identical exposure conditions at 40x magnification. Tissues in experiments were reacted together to ensure identical staining conditions. Color images were obtained, background corrected, and converted to an eight-bit grey scale before being saved in

TIFF format from Image-Pro Plus. The TIFF images were then arranged into figures using Adobe Illustrator. No brightness, contrast or other image modifications were made. Brain regions were then manually traced and the optical densities within each traced region were determined by the Image Pro Plus Software. The resulting measurements were transformed to percentages of the maximal mean OD determined across all age groups and brain regions to control for variance in staining conditions. Maximal mean densities were then calculated and compared across age groups by brain regions using ANOVA and Tukey's HSD (Honestly Significant Difference) post hoc tests where appropriate. P values of less than or equal to 0.05 were considered as a statistically significant difference.

Song Analysis

Data analyses and statistical computations were performed in Systat 12 and Microsoft Excel PC software. The quantification of song parameters were measured as described above. Comparisons of the effect between treatments on parameters measured were made using analysis of variance (ANOVA) and Tukey's HSD post hoc tests where appropriate. P values of less than or equal to 0.05 were considered as a statistically significant difference.

Figure 2.1 Diagram of capillary transfer set-up for transfer of a RNA agarose gel onto a nitrocellulose membrane. Following set up, the whole apparatus is covered with a layer of plastic wrap to maintain humidity and a large weight is set on top (approximately 500 grams).

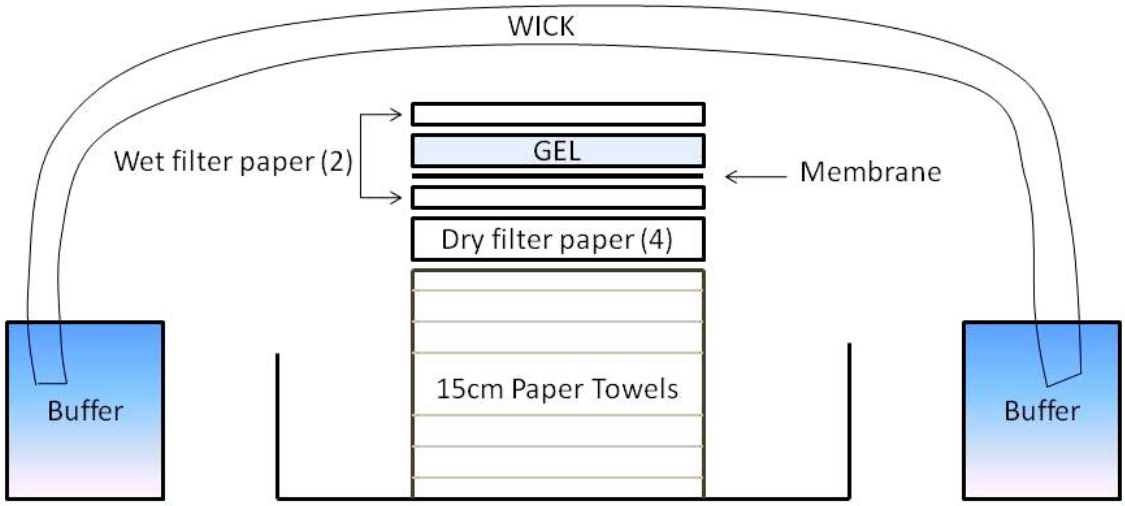

WICK
Wet filter paper (2)
GEL
Membrane
Dry filter paper (4)
15cm Paper Towels
Buffer
Buffer

Table 2.1 Oxytocin preparation. A stock solution of oxytocin (1 mg/mL) was created in sterile 1XPBS and this was used to prepare oxytocin for subcutaneous injections (50 μL per injection).

Oxytocin Stock = 1mg diluted with 1ml 1XPBS (1mg/1ml)

	30µg/kg	300µg/kg	1000µg/kg	3000µg/kg
Day 5	0.072mg	0.72mg	2.4mg	7.2mg
Day 6	0.102mg	1.02mg	3.44mg	10.2mg
Day 7	0.118mg	1.18mg	3.96mg	11.8mg
Day 8	0.151mg	1.512mg	5.04mg	15.12mg
Day 9	0.209mg	2.094mg	6.98mg	20.94mg

STOCK/1XPBS (µL)

	30µg/kg	300µg/kg	1000µg/kg	3000µg/kg
Day 5	1.5/998.5	14.4/985.6	48/952	144/856
Day 6	2.0/998	20.4/979.6	68.8/931.2	204/796
Day 7	2.5/997.5	23.6/976.4	79.2/920.8	236/764
Day 8	3.0/997	30.24/969.76	100.8/899.2	302.4/697.6
Day 9	4.2/995.8	41.88/958.12	139.2/860.4	418.8/581.2

Figure 2.2 Results of literature search of doses of peripheral oxytocin used to produce a variety of effects (Cushing and Carter 2000; Heinrichs, Meinlschmidt et al. 2004; Petersson, Diaz-Cabiale et al. 2005; Kramer, Choe et al. 2006; Bales, Plotsky et al. 2007; Bales, van Westerhuyzen et al. 2007; de Oliveira, Camboim et al. 2007; Domes, Heinrichs et al. 2007; Hollander, Bartz et al. 2007; Pournajafi-Nazarloo, Papademeteriou et al. 2007).

	Animal	Route	Dosages (mcg/kg)	Effect	Author
1	Prarie Voles	IP 1 day old pup	1000, 2000, 4000, 8000	maternal social	Bales, KL
2	Rat	IP 1 day old pup	1000	↓mRNA levels of OTR, ANP, eNOS in ♥	Pournajafi-Nazarloo, H
3	Human	Intranasal	171	↑ "mind reading" - getting social cues	Domes, G
4	Prarie Voles	IP 1 day old pup	1000	↓vasopressin receptors at 60 days	Bales, KL
5	Rat	IP	0.4	memory alteration , ↓ plasma G.C's	de Oliveira, LF
6	Human	IV	71	↑retention of social cognition	Hollander, E
7	Human	IV	171	memory alteration	Heinrichs, M
8	Rat	S.C.	1000	↓ nociepetion	Petersson, M
9	Prarie Voles	IP 1 day old pup	3000	CNS cFos expression	Kramer, KM
10	Prarie Voles	S.C.	85.71, 428.55, 1714.29	partner preferance	Cushing, BS

CHAPTER THREE: APPLICATION OF RESULTS OF THE ZEBRA FINCH GENOME SEQUENCING PROJECT TO DEVELOP PROBES AND ANTIBODIES TO STUDY MESOTOCIN NEUROCHEMISTRY; A SYSTEM RELEVANT TO VOCAL AND SOCIAL BEHAVIOR

Abstract

Oxytocin and OT-related peptides have an established role in the production of social behaviors in both humans and animals but little is known about the role of these peptides in communication (Lim and Young 2006; Carter, Grippo et al. 2008; Donaldson and Young 2008; Guastella, Mitchell et al. 2008). The zebra finch represents a promising animal model for the study of such peptides and their role in vocal learning and development. In order to study central mesotocin neurochemical signaling in the zebra finch both traditional cloning techniques and the identification of coding sequences in-silico were employed. Here we detail genome-derived zebra finch sequences important to mesotocin neurochemistry including proteins important for production of endogenous ligands, and expression of receptors and metabolic enzymes. This knowledge has allowed for the development of nucleic acid probes and an antibody to study expression of the zebra finch mesotocin receptor. Northern blotting analysis revealed a message of 4.6 kb for the mesotocin receptor within an adult (100 days) male brain. Western blotting results indicate that this receptor is expressed in the male brain and in the gastro intestinal (GI) tract with the appearance of two immunolabelled bands at approximately 44.1 kDa and 22.4 kDa in the brain and the appearance of one immunolabelled band at 44.1 kDa in the GI tract. These results indicate a potential role for meostocin signaling in zebra finch song production.

Introduction

Mesotocin is a neurohypophysial peptide that is the non-mammalian homolog of OT. It differs from OT by a single conserved amino acid at the eighth amino acid (Leucine to an Isoleucine) See Table 1.1 for an illustration.. All vertebrates are thought to express two orthologous nonapeptides functionally related to OT or vasopressin. Marsupials, avian species, and non-mammalian rhipidistians express mesotocin and vasotocin, which are hypothesized to be orthologs of mammalian OT and vasopressin (Searcy, Walthers et al. 2009). Each of these related nonapeptides bind to and activate genetically- and functionally-related G-protein coupled receptors, and each of these receptors plays important roles in the modulation of physiology and behavior. Well-established physiological consequences of OT receptor activation include the stimulation of uterine smooth muscle contractions during parturition and milk ejection during lactation (Gimpl and Fahrenholz 2001). Recent studies support a distinct role for central OT signaling in social behaviors (Carter, Grippo et al. 2008).

It has been well-established that OT signaling is involved in the mediation of complex social behaviors such as social memory, trust, pair-bond formation in both experimental animals and human beings (Donaldson and Young 2008). Oxytocin knockout mice (null mutation in the OT gene) are more aggressive, demonstrate a lesser degree of maternal behavior, are more prone to social isolation, and show a significant decrease in their ability to recognize other mice after repeated encounters (Winslow and Insel 2002). In prairie voles, OT administration has been shown to induce pair bonding in both male and female voles (Wang and Aragona 2004). Oxytocin signaling within human amygdala has been associated with modulation of social cognition and fear. When compared with placebo, intranasal application of OT reduces activation and coupling of the amydala to brainstem regions implicated in the manifestation of fear (Kirsch, Esslinger et al. 2005). Numerous studies demonstrate a link between human OT

levels and feelings of trust, an enhanced perception of faces, and the improvement of social cognition (Zak, Kurzban et al. 2005; Guastella, Mitchell et al. 2008; Guastella, Mitchell et al. 2008).

Deficits in OT signaling have been implicated in the etiology of Autism Spectrum Disorders. Autism is a heritable disorder and genotyping has revealed that OT receptor gene mutations are correlated with diagnosis of autism within Chinese Han, American Caucasian, and Finnish populations (Wu, Jia et al. 2005; Ylisaukko-oja, Alarcon et al. 2006; Jacob, Brune et al. 2007). Males are five times more likely to be diagnosed with autism than females are, and this sex difference suggests that OT (a possibly sexually dimorphic peptide) may be a critical factor in the development of the disease (Carter 2007). Gender differences in both the peptide OT and the OT receptor have been measured in some but not all species. Oxytocin levels tend to be higher in females and the synthesis of both OT and the OT receptor are to a certain extent regulated by estrogen (Witt, Carter et al. 1991; Insel and Shapiro 1992; Zingg, Rozen et al. 1995; Gimpl and Fahrenholz 2001; Nomura, McKenna et al. 2002; Zingg and Laporte 2003; Yamamoto, Cushing et al. 2004). No gender differences in the levels of the OT receptor within the CNS have been reported in the rat, but an increase in OT receptor binding was observed in the medial prefrontal cortex of the female prairie vole (Uhl-Bronner, Waltisperger et al. 2005; Smeltzer, Curtis et al. 2006) It has also been demonstrated that significant decreases in plasma OT levels and in circulating forms (a decrease in the bioactive nonapeptide form versus an increase in plasma levels of the inactive form with three amino acid C-terminal peptide extensions also known as OT-X) were found when age-matched autistic children were compared with non-autistic children (Modahl, Greene et al. 1998; Greene, Fein et al. 2001). The extended form of OT (OT-X) is a precursor of the active OT comprised of nine amino acids and its proteolysis is thought to be

accomplished by one or more of several pro-hormone convertases such as convertase 2 and convertase 5 (Gabreels, Swaab et al. 1998). Furthermore, intravenous OT treatment has been shown to improve symptoms of autism such as retention of social cognition and a reduction of repetitive behaviors in a randomized, placebo-controlled, double-blind crossover investigation in adults diagnosed with an autistic spectrum disorder (Hollander, Novotny et al. 2003; Hollander, Bartz et al. 2007).

Autism spectrum disorder is associated with alterations of both social and language skill, and although numerous studies have implicated OT signaling in the modulation of social behaviors, there are limited data on the extent to which OT signaling is directly involved in vocal development. It had been observed that OT-deficient mice emit fewer ultrasonic emissions than wild type pups upon separation from their mothers (Winslow and Insel 2002). A lack of information regarding potential involvement of OT signaling in vocal development may be due to a lack of appropriate animal models. Since song birds like the zebra finch learn a form of vocal communication, they represent a promising model for evaluating effects of OT-related peptides on vocal development. Neurobiology of both song learning and production has been extensively studied in the zebra finch, and thus the functional relationships are known between brain circuitry, neural features, and song behavior. One known problem with using the zebra finch as an animal model for the investigation of the molecular components of mesotocin signaling and its relation to vocal development was the lack of an established genome.

Genomic information of a species is particularly useful because it eliminates the need for time-consuming and difficult cloning techniques to generate sequences for nucleic acid probes and epitope identification for raising antibodies. With publication of the zebra finch genome, scientists can now rapidly identify and produce probes to study all components of a

neurochemical system (ligand, receptor, and metabolism). One issue with shotgun sequence assembly of the large genome of a eukaryotic species is the fact that the sequences produced are merely draft sequences. Sequencing error rates vary but average at about 1% of the sequence generated (Weber and Myers 1997). Further testing such as the actual cloning of the receptor using primers designed based on the sequence information generated by a shotgun sequence assembly from first strand cDNA is needed to verify the putative sequences published.

Materials and Methods

Except where otherwise noted, all materials and reagents were purchased from Fisher Scientific (Fair Lawn, NJ), Sigma (St. Louis, MO) or Invitrogen (Carslbad, CA). Immunochemicals were purchased from Vector (Burlingame, CA).

Traditional Cloning: 3'RACE (Rapid Amplification of cDNA Ends)

Total RNA was isolated from a freshly dissected male zebra finch brain using a phenol-based isolation reagent (Trizol). First strand cDNA was synthesized using random primers and reverse transcriptase obtained in a kit (Invitrogen Superscript 1º Strand Synthesis System for RT-PCR), using the manufacturer's protocol. A 3' RACE PCR strategy was employed to clone the zebra finch mesotocin receptor. First-strand cDNA was used as a template for PCR amplification using oligo-dT_{17} and a degenerate zebra finch mesotocin receptor ATG-GAATTC-TT(T/C)TT(T/C)ATGAA(A/G)C). This resulted in amplification of cDNA of approximately 500bp. This product was then cloned into a bacterial vector and sequenced. Insert size was verified by plasmid isolation and digestion with EcoR1. From this 3' RACE PCR method, 492 base pairs of the 3' end of the zebra finch mesotocin receptor sequence (including the stop codon) were determined. The sequence data obtained was subjected to homology searches using

NCBI's BLAST (Basic Local Alignment Search Tool) program for verification (see Fig 3.2 for multiple-species alignment).

In-Silico Cloning

Once the shotgun zebra finch sequence became available for BLAST, the 492 base pairs of the receptor cloned were used for a homology search. Results of this search included a predicted mesotocin receptor sequence coding sequence. In order to gather as much information as possible about the mesotocin signaling system in the zebra finch, we obtained the cDNA sequence information using BLAST searches of cDNA and translated protein sequences from other mammals of the mesotocin pre-propeptide including neurophysin, the mesotocin receptor, and zebra finch oxytocinase-like protein (Figure 3.1, 3.2, and 3.3). All alignments shown were produced using the ClustalW2 program found at http://www.ebi.ac.uk/Tools/clustalw2/index.html. Alignments included sequence information on the protein of interest from *gallus gallus*, *homo sapiens*, and *rattus norvegicus*.

Northern Blot Analysis

Standard northern blotting protocols were used (T. Brown 1999). Briefly, total RNA (5 µg) was resolved on a formaldehyde-containing agarose gel. Gels were stained with ethidium bromide and intensities of ribosomal RNA bands assessed to confirm that equal amounts of RNA had been loaded from each sample. The gels were then transferred to nitrocellulose membranes via overnight capillary transfer and immobilized by UV cross-linking. Blots were prehybridized in a solution containing 50% formamide, 0.8 M NaCl, 20 mM PIPES (pH 6.5), 0.5% SDS, 100 µg/ml sonicated herring sperm DNA and hybridized in the same solution containing 1×10^6 counts per minute (cpm)/ml of ^{32}P-labeled probe. ^{32}P-dCTP-labeled probes were synthesized with random hexamer primers and Klenow fragment using the zebra finch mesotocin receptor PCR fragment

described below as a template. The template used for cDNA probe synthesis was a 648 bp fragment of cDNA encoding the zebra finch mesotocin receptor. This mesotocin receptor cDNA (corresponding to nucleotides 385-1033), was PCR amplified with the following primers: forward primer = 5'-GGAATGTTCGCTTCCACCTA-3', reverse primer = 5'-CATCAGGTCGTGGAAGAGGT-3'. PCR product size was verified by visualization on a 1.2% agarose gel and staining with ethidium bromide. The cDNA probe was verified by restriction enzyme digestion (Figure 3.5) and by Southern blotting (Figure 3.6). Briefly a fragment of DNA from the mesotocin receptor (648 base pair fragment used as the template for probe synthesis) and one fragment of zebra finch cannabinoid receptor (~1 kb) were amplified by PCR and ran on a 1.5% agarose gel along with a plasmid harboring the 492 basepair fragment cloned by 3'RACE which was transferred via capillary transfer to a nitrocellulose membrane. The DNA on the resulting membrane was denatured, cross-linked, and dried. The dried blot was then treated as described above for Northern Blot analysis. Wei Xi Qin assisted with both the northern blotting analysis and the southern blotting.

Mesotocin Receptor Antibody Production

Following repeated attempts to identify a commercially-available antibody that would selectively label the zebra finch mesotocin receptor protein it was concluded that preparation of a zebra finch-specific anti–mesotocin receptor antibody was necessary. This was successfully done using deduced amino acid sequence and identification of a suitable epitope with the help of Spring Valley Laboratories, Inc (Woodbine, MA). The resulting antisera, raised in rabbit, targets a 16-amino-acid region within the intracellular loop portion of the zebra finch mesotocin receptor (LLSSSTRYLKSRPAC) which encompassed residues Lys 347 – Cys 361 (See Figure 3.6). The predicted epitope is TRYLKS.

Immunoblotting

The immunochemical specificity of the affinity purified antiserum was demonstrated by Western blot staining of bands with molecular weight of approximately 44 kDa and 21 kDa, and reversal of staining following preabsorption of antibody with the immunizing peptide. Crude membrane protein from adult male zebra finch brain (100 days of age) was isolated by homogenization in ice-cold buffer (0.32 M sucrose in 5 mM Tris, pH 7.4) followed by two centrifugations to remove cellular debris. The resulting protein pellet was resuspended in 50 mM Tris, 5mM $MgCl_2$ at a pH of 7.4. The protein sample was quantified using the BCA method. The BCA Protein Assay combines the reduction of copper by protein in an alkaline solution with the colorimetric detection of the cuprous cation by bicinchoninic acid. This BCA/copper complex has a linear absorbance at 562 nm with increasing protein concentrations and this linear absorbance is used to create a linear regression curve using standard known protein concentrations to which the absorbance of the samples of unknown concentration are compared (Noble and Bailey 2009). A total of 20 ug of protein was separated by electrophoresis on a 12% polyacrylamide gel and transferred to a PVDF membrane. The blot was blocked in a 10% casein solution in 1X TBST (50 mM Tris.HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20), then probed with the anti- zebra finch mesotocin receptor antibody (1:2,000) and a goat anti-rabbit HRP-conjugated secondary antibody (1:6,000). One blot from each experiment was incubated with the primary antibody at a 1:2,000 dilution and 26 μM of the immunizing peptide in 10% Casein solution in 1X TBST at 4º C overnight to serve as a negative control (Figure 3.9). Detection was with ECL Plus and film exposure of 10 minutes (GE Healthcare Biosciences, Pittsburgh, PA). The ECL Plus kit is based on the enzymatic generation from the conjugated horseradish peroxidase on the secondary antibody of an acridium ester which produces an intense light that

can expose film (Osborn 2000). Similar experiments were conducted using protein isolated from adult male zebra finch brain, liver, and gastrointestinal tissue as illustrated in Figure 3.9.

Results

Zebra Finch Mesotocin Receptor Identification

A multiple-species alignment of the deduced amino acid sequence encoding mesotocin and OT receptor orthologs is presented in Figure 3.2. The zebra finch mesotocin receptor nucleotide sequence shares a 73% identity with that of the human OT receptor, a 69% identity with that of the rat OT receptor, and an 88% identity with that of the chicken OT-like receptor. The translated protein is 93.1% homologous to the chicken OT-like receptor, 76.4% homologous to the human OT receptor, and 77.4% homologous to the rat OT receptor. A 2 dimensional graphical representation of the receptor as it is located within the cellular membrane is illustrated in Figure 3.6. The calculated molecular mass of the zebra finch mesotocin receptor comprised of 390 residues is 44.23 kDa. This is comparable with the predicted molecular mass of the chicken mesotocin receptor (44.05 kDa), the human OT receptor (42.78 kDa), and the OT receptor found in the rat (42.87 kDa).

Zebra finch Mesotocin Pre-propeptide Identification

The pre-propeptide of mesotocin contains the hormone, a processing signal, and the carrier protein neurophsyin. A multi-species alignment of the zebra finch pre-propeptide with the chicken, human and rat pre-propeptides is illustrated in Figure 3.1. As noted previously, avian species such as the zebra finch utilize mesotocin rather than OT and these two peptides differ by one amino acid. It is noted that the zebra finch mesotocin cDNA sequence shares an 88% identity with both the rat and human OT nucleotide sequence and a 100% identity with the chicken. The neurophysin nucleotide sequence found in *homo sapiens* shares a 68% identity

with the amino acid sequence determined to be that of neurophysin in the zebra finch while the rat and zebra finch neurophysin nucleotide sequences were found to have 72% homology. The deduced amino acid sequence for the pre-propeptide was determined to share 59.5% homology with the chicken, a 62.4% with the human sequence, and a 63.9% homology to that of the sequence found in the rat.

Identification of Sequence Encoding an Oxytocinase-related Enzyme: a Protein Involved in Mesotocin Metabolism

A cDNA sequence apparently encoding a zebra finch ortholog of oxytocinase, the enzyme responsible for the cleavage and inactivation of OT, is presented in Figure 3.3. It was determined that the zebra finch nucleotide sequence of oxytocinase has a 69% homology with the human gene and a 68% homology with the rat oxytocinase also known as leucyl/cystinyl aminopeptidase. The alignment of the protein demonstrated that the zebra finch oxytocinase-related enzyme shares a 67% identity with that of the rat and human variants of oxytocinase and a 77% homology with that of an oxytocinase like protein found in the chicken. The predicted molecular masses of each of the variants of oxytocinase are comparable (zebra finch – 117.4 kDa, chicken – 116.3 kDa, human – 117.35 kDa, rat – 117.2 kDa).

Northern Blotting

Northern blots of 5 μg total zebra finch RNA isolated from brain, liver, and gastrointestinal tract tissue, demonstrate the presence of a mesotocin receptor mRNA of approximately 4.6 kb (based on a standard curve generated from migration of ribosomal RNAs, Figure 3.8). This mRNA size is in reasonable agreement with message sizes of the toad (3.9 kb) and the tammar wallaby (4.1kb) (Akhudnova A 1996; Siebel AL 2005). Predominant expression was observed in brain, with little detectable message observed mRNA isolated from liver and gastrointestinal tract.

Lower-level mesotocin receptor mRNA expression in liver and gastrointestinal tract corresponds to similarly lower density expression of receptor protein observed in western blotting experiments with expression being non-detectable in the liver (see Fig 3.9).

Immunoblotting

Western blotting resulted in staining of two bands with apparent molecular weight of 44.1 kDa and 21.4 kDa as calculated by linear regression analysis located within the male zebra finch brain as illustrated in Figure 3.9. The predicted mass of the protein was 44.2 kDa based on the deduced cDNA sequence which is consistent with values reported in the literature for the chicken (44 kDa), the mouse (42.8 kDa), and the human (42.7 kDa) OT receptors.

Discussion

We were able to clone a portion of the mesotocin receptor using degenerate primer PCR, and through in-silico cloning via BLAST based on the shotgun sequence of *Taeniopygia guttata* we were able to obtain predicted cDNAs encoding the mesotocin receptor, the mesotocin ligand, neurophysin, and the zebra finch like oxytocinase. Neurophysin, along with OT, is a cleavage product of preprooxyphysin. It is transported, stored, and released simultaneously with OT however its biological function remains unclear (Gimpl and Fahrenholz 2001). Most of the knowledge of oxytocinase is based on its function during pregnancy since it has been shown that the serum level of oxytocinase or placental leucine aminopeptidase (P-LAP) increases during gestation and it appears to function in maintaining homeostasis of several peptide hormones such as OT and vasopressin during pregnancy (Rogi, Tsujimoto et al. 1996). The enzyme cleaves the peptide bond between N-terminal cysteine and adjacent tyrosine residues of OT and thereby inactivates the hormone. It also degrades vasopressin and angiotensin III effectively. Oxytocinase has also been found in human spermatozoa and it is hypothesized that it may play a

role either directly or indirectly during fertilization (Roy and Ratnan 1990). Oxytocinase has been demonstrated to be present in multiple tissues of the body in the absence of pregnancy such as vascular endothelial cells, gastrointestinal mucosal cells, epithelial cells of hepato-biliary, pancreato-biliary, bronchial-alveolar and renal tubular systems as well as islet cells of pancreas and neurons in the central nervous systems (Nagasaka, Nomura et al. 1997).

The advantage of having coding sequences is that it allows for the design of antibodies and nucleic acid probes to study protein and mRNA distribution within a particular model system. For our study, we used the coding sequence of the mesotocin receptor to design a nucleic acid probe and an antibody to determine the distribution of the receptor within the zebra finch. Northern blotting allowed for the determination of the size of the mRNA message corresponding to the mesotocin receptor and confirmed its presence within the brain of the adult male zebra finch. This mRNA size is in reasonable agreement with message sizes of the toad (3.9 kb) and the tammar wallaby (4.1 kb) (Akhudnova A 1996; Siebel AL 2005). Predominant expression was observed in brain, with little detectable message observed with in RNA isolated from liver and gastrointestinal tract. Lower-level mesotocin receptor mRNA expression in liver and gastrointestinal tract corresponds to similarly lower density expression of receptor protein observed in western blotting experiments with expression being non-detectable in the liver (see Figure 3.7).

The antibody was used to determine the molecular mass and distribution of the mesotocin receptor protein within selected tissues of the male zebra finch. Western blots indicated distinct immunolabelled bands within the brain of 44.2kDa and 21.4 kDa. Other western blot data published for the OT receptor typically show an immunolabelled band of higher molecular mass (55-65 kDa) likely due to the gylcosylation of the protein. It is feasible that our methods of

protein isolation removed gylcosylations or they exist in modified form within the zebra finch. The appearance of a smaller 21.4 kDa labeled band may indicate a splice variant of the mesotocin receptor within the CNS or it may be truncated or otherwise metabolized forms of the protein. These labeled bands were not present in blots incubated with the blocking peptide prior to incubation with the primary antibody. Western blotting of protein isolated from multiple tissues from an adult male zebra finch (brain, gastrointestinal tract, liver) demonstrates the two distinct bands within the brain (44.2 kDa and 24.1 kDa), the 44.2 kDa labeled band in the GI, and no labeling in the liver. The lack of the lower 24.1 kDa band in the GI suggests that a truncticated form of mesotocin receptor may be expressed in the brain or that processing may vary for the receptor within the CNS.

Previous studies have determined that OT distribution varies widely within the CNS of mammals and it is thought that these differences in expression may be related to the differences in complex social behaviors displayed in these animals (Young, Huot et al. 1996). There is also a wealth of data that implicates alterations in OT signaling with many behavioral disorders including autism spectrum disorders (Hollander, Novotny et al. 2003; Hollander, Bartz et al. 2007; Rojas Walh 2007; Guastella, Einfeld et al. 2010). Autistic individuals display dysfunctions in three main behavioral areas: repetitive behaviors, social deficits, and language abnormalities. While evidence exists to demonstrate the function of OT signaling in social behaviors, there is little evidence of the importance of this nonapeptide in vocal development. By using the sequence data obtained during the zebra finch genome sequencing project and traditional cloning techniques, we have been able to identify sequences that represent proteins integral to the mesotocin signaling system within the zebra finch. This allowed us to determine if the mesotocin receptor is expressed within the zebra finch CNS. Future experiments will detail the

mesotocin receptor distribution within in the brain of the male zebra finch in order to determine if they are present within brain nuclei associated with song learning and vocal motor control. The presence of mesotocin receptors in these areas would indicate a potential role for mesotocin signaling in either the production of or learning of song.

Figure 3.1 Multi-species alignment of the zebra finch pre-propeptide for mesotocin. The areas encoding the signal peptide, the hormone, and neurophysin are demonstrated. Black shading of an amino acid represents conservation with all 3 species chosen for the alignment (chicken, human, rat). Gray shading of an amino acid indicates homology with the chicken sequence. This figure was prepared with the assistance of Robin Yang.

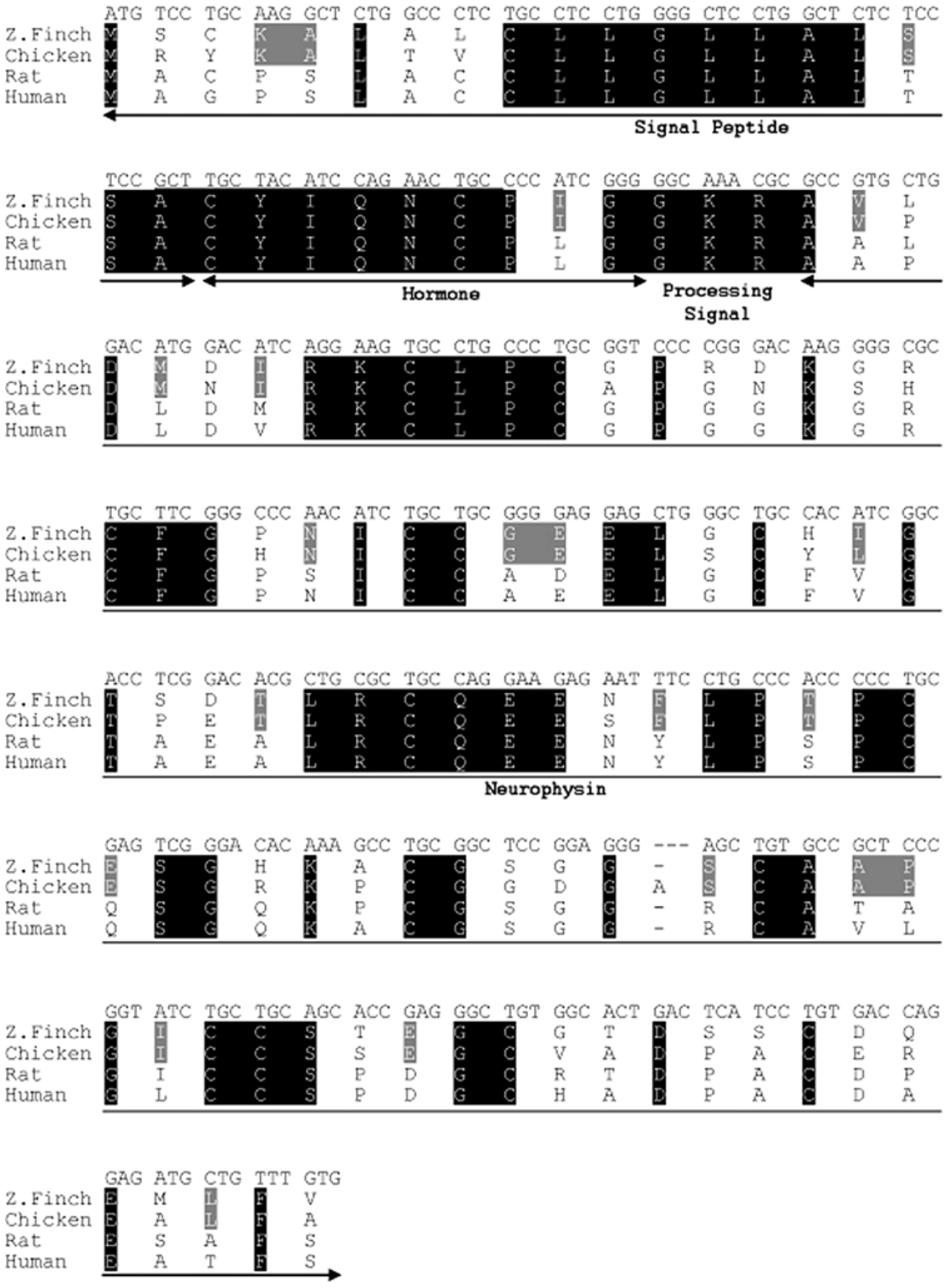

ATG TCC TGC AAG GCT CTG GCC CTC TGC CTC CTG GGG CTC CTG GCT CTC TCC
Z.Finch M S C K A L A L C L L G L L A L S
Chicken M R Y K A L T V C L L G L L A L S
Rat M A C P S L A C C L L G L L A L T
Human M A G P S L A C C L L G L L A L T
Signal Peptide
TCC GCT TGC TAC ATC CAG AAC TGC CCC ATC GGG GGC AAA CGC GCC GTG CTG
Z.Finch S A C Y I Q N C P I G G K R A V L
Chicken S A C Y I Q N C P I G G K R A V P
Rat S A C Y I Q N C P L G G K R A A L
Human S A C Y I Q N C P L G G K R A A P
Hormone
Processing Signal
GAC ATG GAC ATC AGG AAG TGC CTG CCC TGC GGT CCC CGG GAC AAG GGG CGC
Z.Finch D M D I R K C L P C G P R D K G R
Chicken D M N I R K C L P C A P G N K S H
Rat D L D M R K C L P C G P G G K G R
Human D L D V R K C L P C G P G G K G R
TGC TTC GGG CCC AAC ATC TGC TGC GGG GAG GAG CTG GGC TGC CAC ATC GGC
Z.Finch C F G P N I C C G E E L G C H I G
Chicken C F G H N I C C G E E L S C Y L G
Rat C F G P S I C C A D E L G C F V G
Human C F G P N I C C A E E L G C F V G
ACC TCG GAC ACG CTG CGC TGC CAG GAA GAG AAT TTC CTG CCC ACC CCC TGC
Z.Finch T S D T L R C Q E E N F L P T P C
Chicken T P E T L R C Q E E S F L P T P C
Rat T A E A L R C Q E E N Y L P S P C
Human T A E A L R C Q E E N Y L P S P C
Neurophysin
GAG TCG GGA CAC AAA GCC TGC GGC TCC GGA GGG --- AGC TGT GCC GCT CCC
Z.Finch E S G H K A C G S G G - S C A A P
Chicken D S G R K P C G G D G A S C A A P
Rat Q S G Q K P C G S G G - R C A T A
Human Q S G Q K A C G S G G - R C A V L
GGT ATC TGC TGC AGC ACC GAG GGC TGT GGC ACT GAC TCA TCC TGT GAC CAG
Z.Finch G I C C S T E G C G T D S S C D Q
Chicken G I C C S S E G C V A D P A C E R
Rat G I C C S P D G C R T D P A C D P
Human G L C C S P D G C H A D P A C D A
GAG ATG CTG TTT GTG
Z.Finch E M L F V
Chicken D A L F A
Rat E S A F S
Human E A T F S

Figure 3.2 Multi-species alignment of the zebra finch mesotocin receptor. Black shading of an amino acid represents conservation with all 3 species chosen for the alignment (chicken, human, rat). Gray shading of an amino acid indicates homology with the chicken sequence. This figure was prepared with the assistance of Robin Yang.

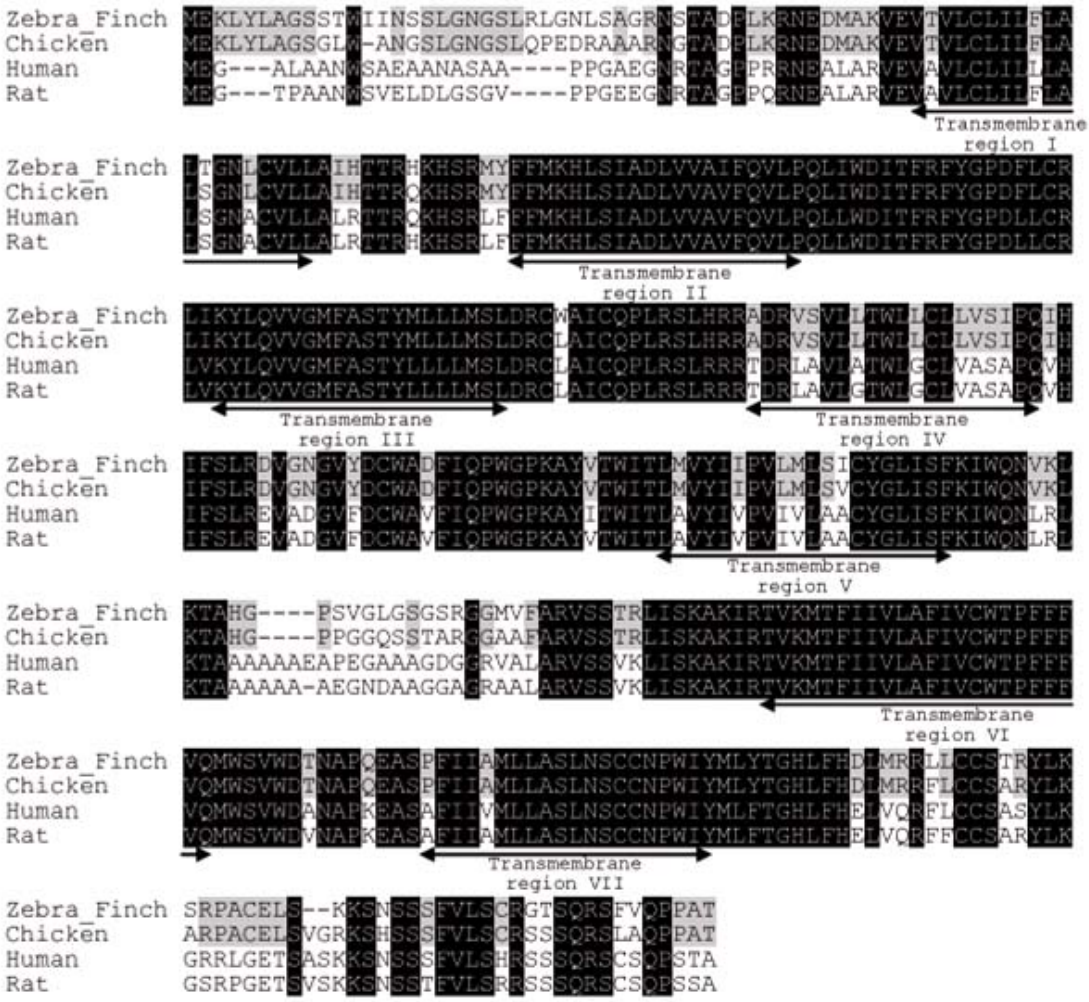

Zebra_Finch
Chicken
Human
Rat
Transmembrane region I
Transmembrane region II
Transmembrane region III
Transmembrane region IV
Transmembrane region V
Transmembrane region VI
Transmembrane region VII

Figure 3.3 Multi-species alignment of the zebra finch oxytocinase like enzyme. Black shading of an amino acid represents conservation with the 3 species chosen for the alignment (chicken, human, and rat). Gray shading of an amino acid indicates homology with the chicken sequence. This figure was prepared with the assistance of Robin Yang.

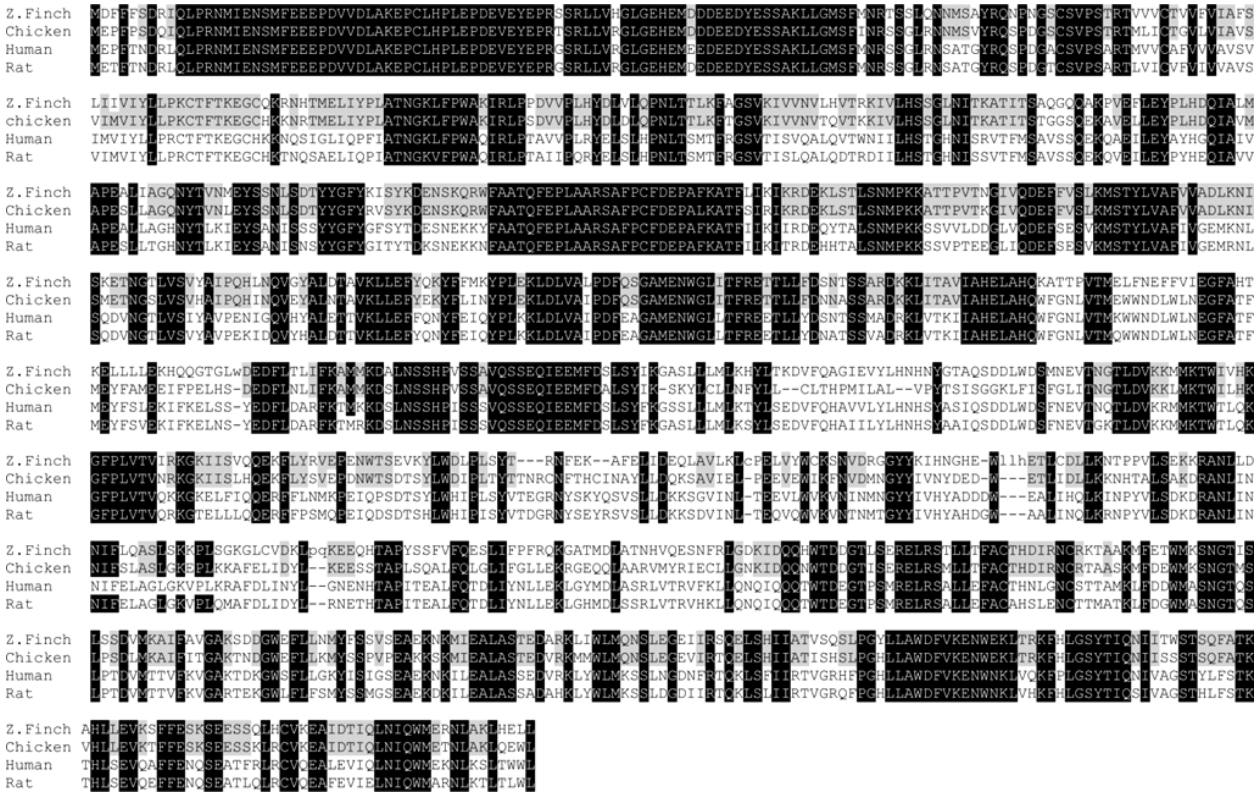


Figure 3.4 Graphical representation of the zebra finch mesotocin receptor as it lies within the cell membrane. The target of the mesotocin antisera is shaded in grey.

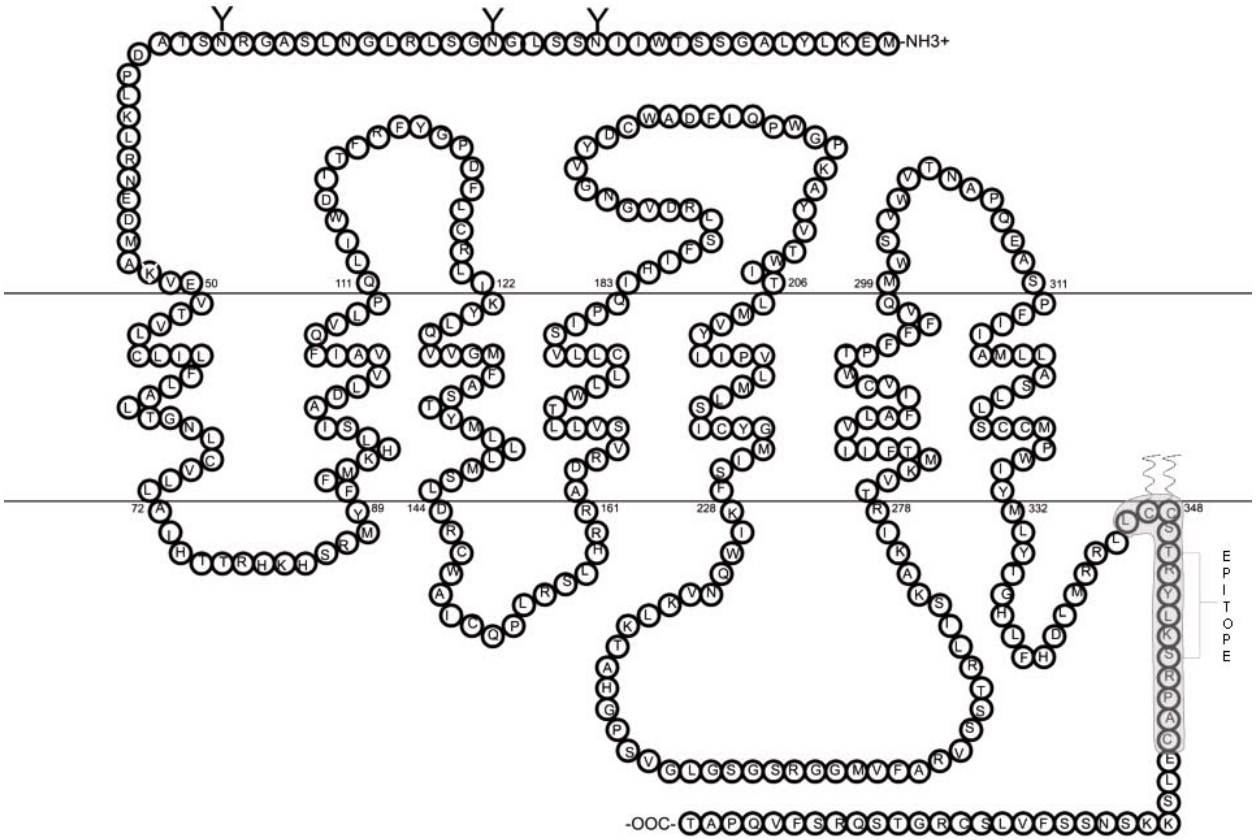

-NH3+
-OOC-
EPITOPE

Figure 3.5 ApaI digestion of 648 base pair cDNA probe template created PCR amplification with the following primers: forward primer = 5'-GGAATGTTCGCTTCCACCTA-3', reverse primer = 5'- CATCAGGTCGTGGAAGAGGT-3 to verify sequence fidelity. Gel products of 350bp and 298bp were expected based on restriction digest mapping of 648bp DNA fragment.

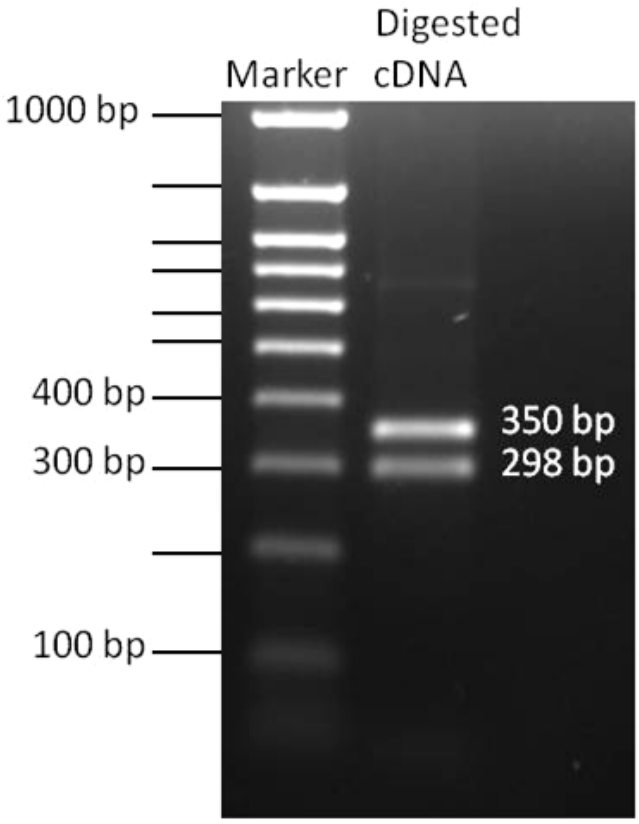

Digested
Marker cDNA
1000 bp
400 bp
300 bp
100 bp
350 bp
298 bp

Figure 3.6 Southern Blot analysis of binding of cDNA probe. (Panel A) A 1.5% Agarose gel was loaded with a1kb fragment of the zebra cannabinoid receptor (negative control, labeled as CB1), the 581 bp fragment of the zebra finch mesotocin receptor (positive control, labeled as 581bp), and a plasmid known to harbor approximately 500 bases of the 3'end of the zebra finch mesotocin receptor (labeled as 3ZfMstR). (Panel B) The DNA was then trasnfered to a nitrocellulose membrane and probed with the cDNA probe created with the Klenow enzyme. The hybridized blot was then washed to remove excess radioactivity and exposed to film for 30 minutes at -80ºC.

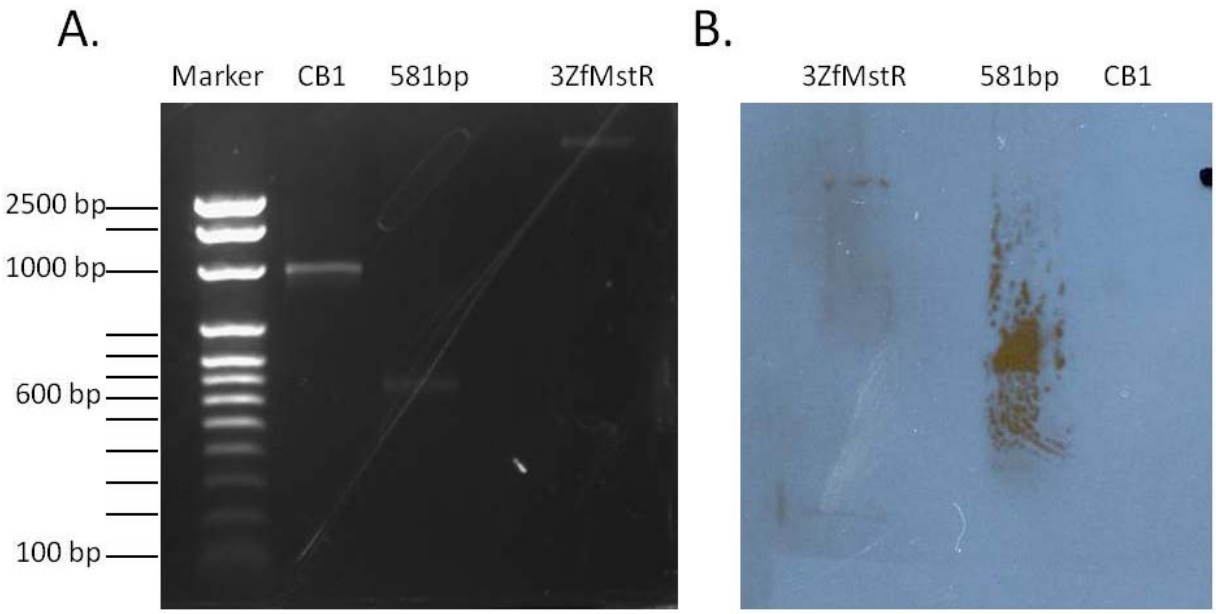

A.
Marker
CB1
581bp
3ZfMstR
2500 bp
1000 bp
600 bp
100 bp
B.
3ZfMstR
581bp
CB1

Figure 3.7 Northern Blot. Mesotocin receptor cDNA probe and total RNA (5 μg) from an adult male brain, liver, and gastrointestinal tract. The approximate sizes of the 28S, 18S, and 5S RNA, and the zebra finch mesotocin receptor transcript (4.6 kb) are indicated. Calculation of the message size was determined by linear regression analysis using the distance migrated by the 28S, 18S, and 5S ribosomal bands and their known size.

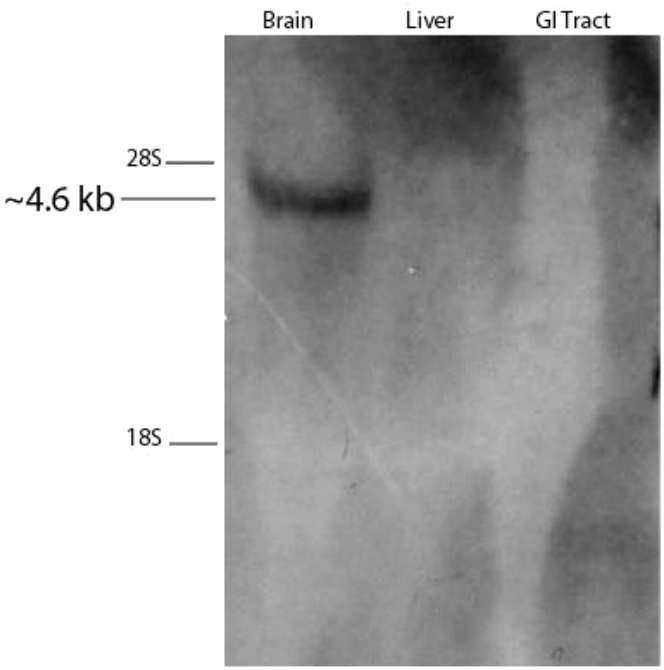

Brain
Liver
GI Tract
28S
~4.6 kb
18S

Figure 3.8 Linear regression analysis of the distance migrated of the ribosomal bands (28S, 18S, 5S) to determine the mesotocin receptor message size in northern blot.

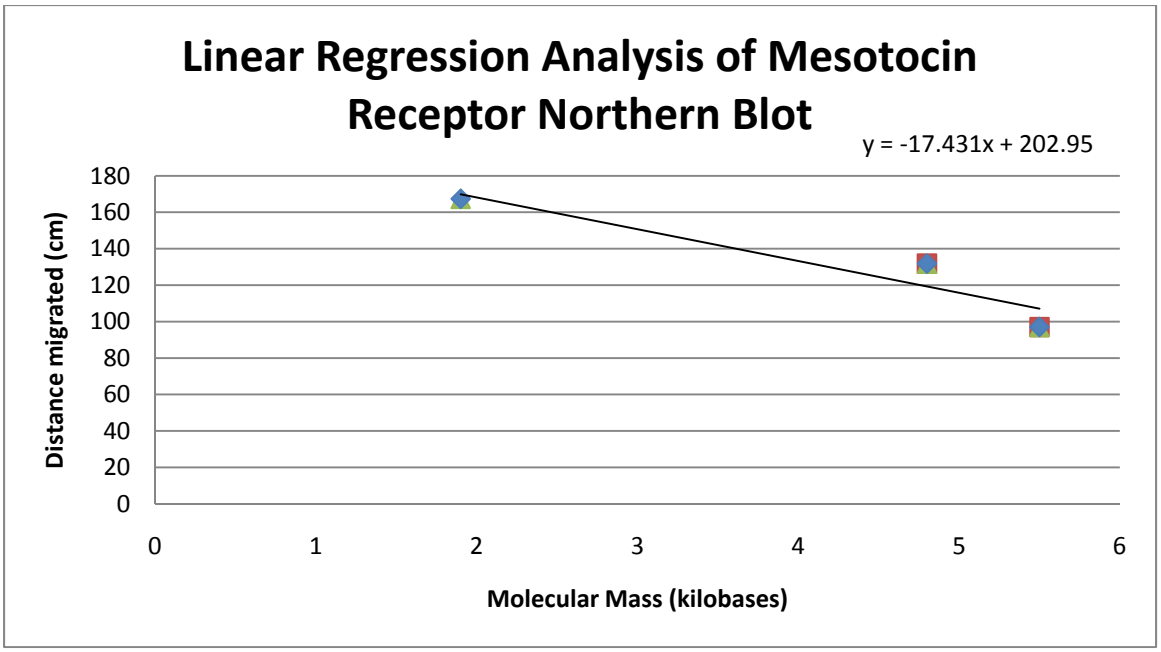
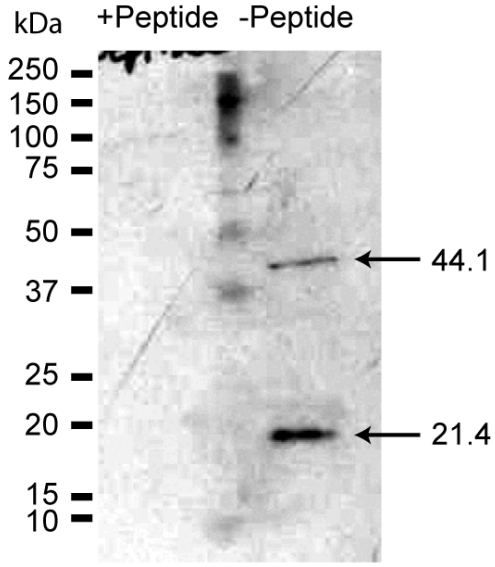

Linear Regression Analysis of Mesotocin Receptor Northern Blot
y = -17.431x + 202.95
Distance migrated (cm)
180
160
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
Molecular Mass (kilobases)

Figure 3.9 Western Blot Analysis of Zebra Finch Tissues using an anti-zebra finch mesotocin receptor antibody. 20 ug of membrane protein from each organ were separated on a 12% acrylamide gel and transferred to a PVDF membrane. The blots were then probed with the zebra finch specific anti-mesotocin receptor antibody (1:2000) and a goat anti-rabbit HRP secondary antibody (1:6,000). Detection was with ECL and film exposure of 10 mins. A) Blot containing 20 μgs of adult male zebra finch brain protein that was incubated with or without the zebra finch mesotocin receptor antibody blocking peptide for 1 hour prior probing with the anti-mesotocin receptor antibody as described above. Molecular mass was determined by linear regression analysis. B) Blot containing 20 μgs of protein from adult male liver, brain, and gastrointestinal tissues. The figure in Panel A was provided by Wei Xi Qin and prepared by Dr. Ken Soderstrom.

A – Western Blot with or without blocking peptide



B – Multiple tissues Western Blot

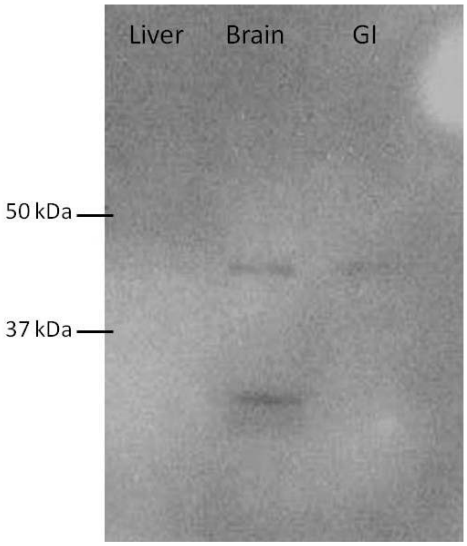
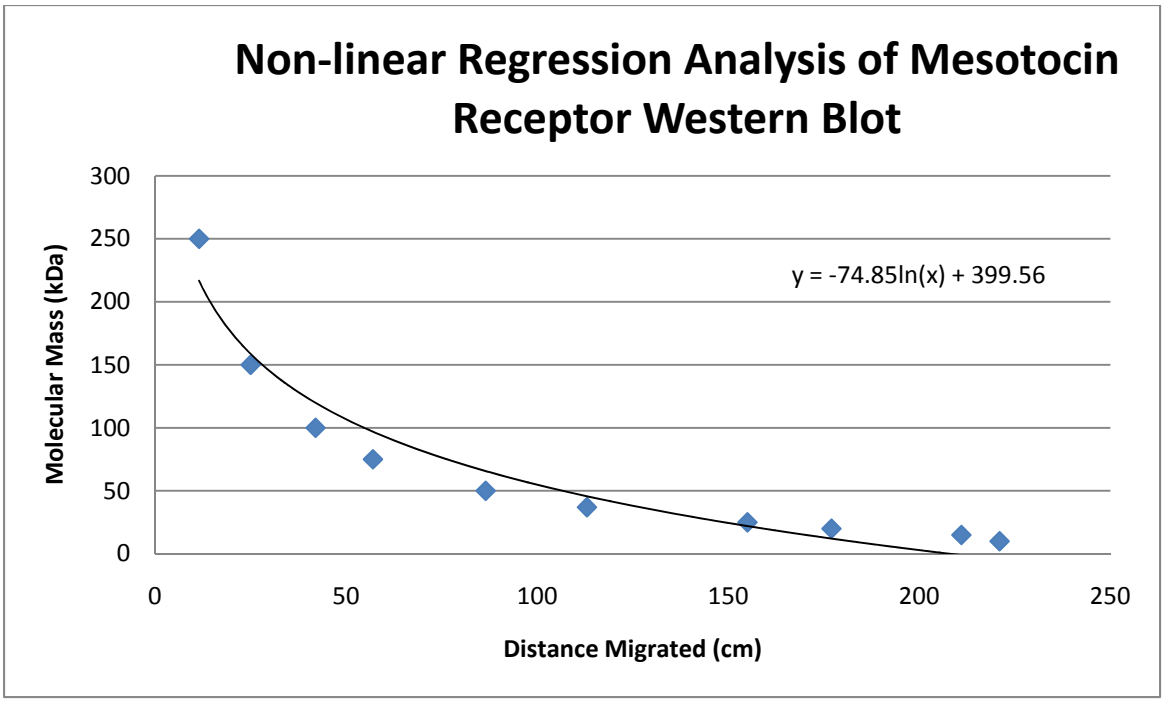


Figure 3.10 Non-linear regression analysis of the molecular weight of the labeled bands of the mesotocin receptor using the molecular weight and distance migrated of the bands of the molecular weight ladder.


Non-linear Regression Analysis of Mesotocin Receptor Western Blot
Molecular Mass (kDa)
300
250
200
150
100
50
0
y = -74.85ln(x) + 399.56
0
50
100
150
200
250
Distance Migrated (cm)

CHAPTER FOUR: DEVELOPMENTAL PATTERN OF MESOTOCIN RECEPTOR DISTRIBUTION IN BRAIN REGIONS IMPORTANT TO ZEBRA FINCH (TAENIOPYGIA GUTTATA) SONG LEARNING AND CONTROL

Abstract

Oxytocin receptors are distinctly expressed within regions of the mammalian CNS including the nucleus accumbens, the prelimbic cortex, and the caudate-putamen. Interestingly, expression patterns vary across species that display various degrees of social behavior (c.f. monogamous- vs. promiscuous-pair bonding voles, (Lim and Young 2006)). The pattern of distribution of oxytocin-related receptors has not been established in animals that undergo vocal development. Since song birds learn a form of vocal communication, they represent a promising model for evaluating the central distribution of OT-related peptide receptors in animals capable of vocal development. As an avian species, zebra finches utilize mesotocin rather than OT signaling (these nonapeptides differ by a single amino acid). To study mesotocin receptor distribution in zebra finch brain, a polyclonal antibody was raised for use in immunohistochemical experiments. In sections of fixed zebra finch brain tissue, mesotocin receptor expression pattern was determined at various stages of vocal development: 10 days, 25- [auditory learning], 50- [subsong], 75- [plastic song], and 100-days of age [crystallized song]. Expression of the mesotocin receptor was detected within areas associated with either song learning (Area X, lMAN), song production (HVC, RA), or auditory inputs (L2) at 25, 50, 75, and 100 days of age with the peak of receptor expression occurring at 50 days of age. Mesotocin receptor expression was apparent in HVC and Area X at 10 days of age in male zebra finches, but other song nuclei remained unstained. In-situ hybridization experiments were also done to assess mesotocin

receptor mRNA expression at 25, 50, 75, and 100 days and the results indicate significant expression of mesotocin mRNA in the vocal motor song region HVC at 50, 75, and 100 days. These results indicate a potential role for meostocin signaling in zebra finch song production.

Introduction

Song birds such as the zebra finch have been established as a uniquely useful animal model for studying vocal development. Neurobiology of both song learning and production has been extensively studied, and thus functional relationships are known between brain circuitry, neural features, and song behavior (Nottebohm, Stokes et al. 1976; Bottjer, Miesner et al. 1984; Sohrabji, Nordeen et al. 1990; Scharff and Nottebohm 1991) . The neuronal circuitry underlying song learning and production is contained within several discrete interconnected brain regions that include prominent striatal (Area X), thalamic (DLM), and telencephalic (lMAN, HVC, RA) components. Each of these regions performs different functions in both song learning and production. For example, lesions of RA and HVC interfere with the production of memorized song, while lesions of rostral forebrain song regions (Area X, lMAN) prevent vocal learning but not production of learned song (Nottebohm, Stokes et al. 1976; Bottjer, Miesner et al. 1984; Sohrabji, Nordeen et al. 1990; Scharff and Nottebohm 1991). It is of note that CNS regions associated with song control undergo substantial changes in neuronal number and connectivity and it has been proposed that these changes coincide with vocal learning (Bottjer and Johnson 1997). Since the zebra finch learns a form of vocal communication during distinct and well defined phases and because their neurobiology of song learning and production is well characterized, these animals can be used to investigate whether specific receptor populations may be involved in altered vocal learning.

Autistic individuals display dysfunctions in three main behavioral areas: repetitive behaviors,

social deficits, and language abnormalities. Various studies have demonstrated a role for OT in social behaviors such as fear, social recognition, and monogamous pair bonding initiation (Wang and Aragona 2004; Kirsch, Esslinger et al. 2005). Animal and human clinical data implicates a causal relationship between altered OT levels and repetitive behaviors (Drago, Pederson et al. 1986; Hollander, Novotny et al. 2003). The involvement of OT in vocal development has not yet been well investigated. It has been observed however that mouse pups with the null mutation in the OT gene emit fewer ultrasonic emissions than wild type pups upon separation from their mothers (Winslow and Insel 2002). Therefore we are interested in studying the distribution of the mesotocin receptor (the avian analog of the OT receptor) in an animal capable of vocal learning such as the zebra finch.

If mesotocin signaling was involved with song learning and production, then it would be expected that the receptors would be distributed in song control regions. In order to test this hypothesis, we utilized a combination of immunohistochemical analysis and in-situ hybridization to determine the localization of this receptor.

Materials and Methods

Except where otherwise noted, all materials and reagents were purchased from Fisher Scientific (Fair Lawn, NJ) or Sigma (St. Louis, MO). Immunochemicals were purchased from Vector (Burlingame, CA).

Animals

Male zebra finches used in these experiments were bred in the aviary and sexed at approximately 25 days of age by a PCR method previously established by our laboratory (Soderstrom, 2007). Birds were housed in flight aviaries maintained at the ambient temperature of 78ºF and given free access to mixed seeds (SunSeed VitaFinch), grit, water, and cuttlebone. The light-dark

cycle was maintained at LD 14:10 hours. Animals were cared for and experimental procedures were conducted according to protocols approved by East Carolina University's Animal Care and Use Committee.

Immunohistochemistry

Brain tissue was prepared for immunohistochemistry experiments by overdosing birds (n of 4 per age group) with Equithesin and transcardially-perfusing birds with cold phosphate buffered saline (PBS; pH 7.4) followed by phosphate-buffered 4% paraformaldehyde, pH 7.0. The perfusion-fixed brains were removed and post-fixed by immersion overnight in phosphate-buffered 4% paraformaldehyde, pH 7.0 at 4° C. Once fixed, brains were blocked parasagittally down the midline and stored at 4° C in 20 % sucrose until tissue from an entire set of age conditions were collected to be processed together so as to ensure comparable reaction conditions. Male zebra finch brains were collected in groups of 4 per age group (10, 25, 50, 75, 100 days). Blocked brains were then removed from storage and glued to a microtome chuck so they could be sectioned using a vibrating microtome parasagittally (lateral to medial) to produce 30-μm sections of zebra finch brain. Tissue sections were collected and thrice washed in PBS. Sections were incubated in 0.3 % hydrogen peroxide solution for 30 minutes to remove endogenous peroxidases, blocked with 5 % goat serum for 30 minutes, and incubated overnight in solution containing a primary antibody at previously optimized dilution (1:1,000). The next day sections were washed thrice in PBS (pH 7.4) and incubated in a previously optimized dilution of peroxidase conjugated secondary antibody solution (1:3,000) for one hour. Samples were washed three times with PBS and secondary labeling was visualized with a DAB solution. Each experiment included negative control sections not exposed to primary antibody that were shown to be not immunreactive. Mounted tissues were examined in various CNS brain song

regions (i.e. Area X, lMAN, HVC, etc) at 40,100 and 1000 X using Olympus BX51 microscope with Nomarski DIC optics. Images of the slides were taken using a Spot Insight QE digital camera and Image-Pro Plus software (Media-Cybernetics, Silver Spring, MD) under identical calibrated exposure conditions and background-corrected.

Measurement of Optical Density of Anti-Zebra Finch Mesotocin Receptor Staining

There were five x 40 gray-scale images captured per subject and brain region. The borders of the regions of interest were traced using Image-Pro Plus analysis software, and the software computed the optical densities within each hand-traced region. The images for each of five age groups were captured in a single session under identical, calibrated exposure conditions. The optical density values for the five images of each brain region for each of the five ages were calculated and the maximal mean value was determined. This maximal mean was then used to transform the optical density data into a percentage maximal OD for ANOVA comparisons across age groups.

In-Situ Hybridization

Levels of messenger RNA of the mesotocin receptor in various brain regions were examined using a free-floating, fixed tissue method for *in situ* hybridization (J.W. Jahng 1997). Brain tissue was prepared for *In-situ* hybridization experiments in a method similar to the one used in the immunohistochemistry experiments. Male birds of various ages (25, 50, 75, 100 days) were overdosed with Equithesin and transcardially perfused birds with cold phosphate buffered saline (PBS; pH 7.4) followed by phosphate-buffered 4% paraformaldehyde, pH 7.0. The perfusion-fixed brains were removed and post-fixed by immersion overnight in phosphate-buffered 4% paraformaldehyde, pH 7.0 at 4° C. Once fixed, brains were blocked parasagittally down the midline and sectioned immediately to preserve mRNA integrity. Blocked brains were then glued

to a microtome chuck so they could be sectioned using a vibrating microtome parasaggitally (lateral to medial) to produce 30-μm sections of zebra finch brain. The sections were collected into ice cold 2XSSC solution (0.3 M NaCl, 0.03 M NaCitrate) prepared with DEPC-treated water. Sections were re-suspended in pre-hybidization buffer (50% formamide, 10% Dextran Sulfate, 2X SSC, 1X Denharts, 0.5M DTT, 0.5 mg/mL denatured salmon sperm DNA) and incubated for one hour at 48ºC. ^{32}P -labeled cDNA probes (1 x 10^7 CPM) were synthesized as described previously and then added to hybridization reactions. The reactions were incubated overnight at 48Cº. Sections were then washed in decreasing concentrations of SSC. RNase A (100 μg, Qiagen) was added to one set of alternate sections as a negative control. Detection of the signal was accomplished by coating slide-mounted sections with photographic emulsion (Hypercoat LM-1 from Amersham BioSciences). The slides were incubated at 4°C for 3 weeks. After developing with Ilford Hypam fixer and Phenisol developer as per the manufacturer's instructions (AEH Business Solutions), the autoradiograms were coverslipped with Permount (Fisher). Mounted tissues were then examined by microscopy as detailed above for immunohistochemical experiments.

Data Analysis and Statistics

Data analyses and statistical computations were performed in Systat 12 and Microsoft Excel PC software. The quantification of optical densities was determined from digital images of brain regions of interest captured under identical exposure conditions at 40x magnification. Once these images were background corrected and converted to grey scale, brain regions were manually traced and the optical densities within each traced region were determined by the Image Pro Plus Software. The resulting measurements were transformed to percentages of the maximal mean OD determined across all age groups and brain regions to control for variance in

staining conditions. Maximal mean densities were then calculated and compared across age groups by brain regions using ANOVA and Tukey's HSD post hoc tests where appropriate. Probabilities of $p \leq 0.05$ were taken to be statistically significant.

Digital Photography

Images shown in Figures were taken at magnifications of 40x, 100x, and 1000x with an Olympus BX51 microscope equipped with Normarski optics and a Spot Insight QE digital camera which was controlled by Image-Pro plus software. Images in Figures 4.1-4.7 were captured by Dr. Ken Soderstrom. The images in Figures demonstrate different brain regions from the same five animals whose tissues were reacted together to ensure identical staining conditions. Color images were obtained, background corrected, and converted to an eight-bit grey scale before being saved in JPEG format from Image-Pro Plus. The JPEG images were then arranged into figures using Microsoft Office Word 2007 and Adobe Photoshop. No brightness, contrast or other image modifications were made.

Results

Immunohistochemistry

General staining features

The general staining patterns of the zebra finch mesotocin antibody are summarized in low power micrographs provided in Figure 4.1. These images demonstrate the distinct staining of several brain regions essential for vocal development and communication in the telencephalon that both wax and wane over the course of the animal's vocal development. Staining is also present in a distinct pattern in the striatum and hippocampus. The staining appeared to be a diffuse pericellular stain of the large somata throughout the telencephalon that was more intense in areas associated with either the learning or production of song. The staining in the caudal

striatum appeared to be a pericellular stain that included some neuronal fiber projections that was at its most intense in the early stages of development (10 and 25 days).

Anti-mesotocin Receptor Staining of the Zebra Finch Telencephalic Song Regions

i. Robust Nucleus of the Arcopallium (RA)

RA is a nucleus located in the caudal-ventral telencephalic portion of the zebra finch brain that is essential for vocal motor output. Input to this nucleus is from HVC (High Vocal Center) and it outputs to the tracheosyringeal portion of the hypoglossal nucleus (nXIIts) which projects to the muscles of the syrinx vocal organ (for a more complete review, please see (Bottjer and Johnson 1997). The pattern of anti-mesotocin receptor staining located within RA throughout the zebra finch's development is detailed in Figure 4.2. At ten days of age, the staining of this nucleus was not discernable above that of background staining (mean OD = 0.089 ± 0.0019) Fig. 4.2, Panel A,B. The level of staining became more distinct in animals at 25 days of age and continues to be maintained throughout the remainder of the bird's vocal development Fig. 4.2, Panel C-J. This staining is characterized by both neuropil and puncta staining of the smaller cell bodies located within RA. There are the occasional patches of unstained cells evident in 25, 50, and 75 day old animals. Receptor density counts determined that a peak level of staining was present in sections from 50 day old animals as indicated in Figure 4.8 (mean OD = 0.201 ± 0.010, p = 0.000 Tukey's HSD following ANOVA). The staining intensity varied significantly among the age groups, but a significant difference in staining intensity was not demonstrated between two groups, 100 (mean OD = 0.113 ± 0.010) and 75 days of age (mean OD = 0.128 ± 0.010, p = 0.279 Tukey's HSD following ANOVA) and 75 and 25 days of age (mean OD = 0.136 ± 0.018, p = 0.825 Tukey's HSD following ANOVA).

ii. HVC

The High Vocal Center or HVC is vocal motor nucleus located caudal-dorsal area of the telencephalon that is required for vocalization in the zebra finch. As detailed above, this nucleus projects to RA and ultimately influences motor output to the muscles of the syrinx vocal organ. Staining within HVC was visible in all age groups. At 10 days, a small but distinct HVC nucleus is visible (Figure 4.3, panel A,B). The staining pattern is a diffuse staining of neuropil that is consistent throughout the course of the bird's vocal development. The intensity of staining was demonstrated to be at its highest at 50 days of age (mean OD = 0.177 ± 0.011, $p = 0.000$ Tukey's HSD following ANOVA) (Fig 10). The density of mesotocin receptor expression was significantly different at 10 days of age (mean OD = 0.139 ± 0.005, $p = 0.000$ Tukey's HSD following ANOVA), 25 days of age (mean OD = 0.125 ± 0.009, $p < 0.000$ Tukey's HSD following ANOVA), and 50 days of age with 10 days of age having more expression than that of 25 days of age but less than that of 50 days of age. There was no significant difference determined between the expression at 10 days of age and 25 days of age ($p = 0.179$ Tukey's HSD following ANOVA). While the receptor expression was found to significantly decrease at 75 (mean OD = 0.092 ± 0.011) and 100 (mean OD = 0.087 ± 0.010) days of age when compared to that of earlier ages ($p = 0.000$ Tukey's HSD following ANOVA), the intensity between the two ages was not significantly different ($p = 0.945$ Tukey's HSD following ANOVA).

iii. Auditory field L2 (L2)

This area is the primary auditory field in the zebra finch telencephalon and it receives input from the thalamic nucleus ovoidalis. Anti-mesotocin receptor antibody staining is evident in L2 starting at 10 days of age (mean OD = 0.130 ± 0.018) and its presence continues until adulthood as demonstrated in Figure 4.4. It is consistently characterized by a light and diffuse but distinct staining of L2 neuropil. The staining intensity is consistent from day 10 to day 25 (mean OD =

0.123 ± 0.008) (See Figure 4.8), but it is significantly increased to its peak expression level at 50 days of age (mean OD = 0.205 ± 0.029, p = 0.000 Tukey's HSD following ANOVA). As the bird ages to adulthood (75 and 100 days), the intensity of staining of the mesotocin receptor returns to that of its previous levels (75 days mean OD = 0.109 ± 0.018; 100 days mean OD = 0.137 ± 0.024).

iv. Area X within medial striatum (Area X)

The Area X nucleus within the striatum is critical to song learning in the zebra finch and it has projections to DLM of the thalamus which in turn projects to lMAN. Each of these regions play a vital role in the process of song learning since a lesion in any one of these areas impairs a bird's ability to learn its song. The staining pattern of the anti-mesotocin antibody is a distinct pericellular staining around unstained cell bodies (Figure 4.5). The staining pattern varies in throughout development. The appearance of this pattern starts at day 10 of age where there are discretely labeled pericellular processes around cell bodies with some evidence of puncta staining. There are unlabelled cell bodies of neurons present as well (Fig. 4.5, Panel A,B). This pattern of pericellular staining around unlabeled cell bodies continues throughout development, but changes dramatically at adulthood. At 100 days, Area X appears to have a dense background of neuropil staining with an occasional cell body surrounded by pericellular staining, and a distinct increase in the staining of puncta (mean OD = 0.135 ± 0.015). Optical densities of anti-mesotocin receptor staining revealed that peak mesotocin receptor expression occurs at 50 days of age, and is significantly higher in density than at other ages (mean OD = 0.228 ± 0.013, p = 0.000, Tukey's HSD following ANOVA) (Figure 4.8). The intensity of staining is lowest at 10 days of age (mean OD = 0.091 ± 0.009), and increases significantly at 25 days of age (mean OD = 0.139 ± 0.014, p = 0.001 Tukey's HSD following ANOVA). There was no significant

difference found between ages 25, 75, and 100 days (25 and 75 days $p = 0.312$; 25 and 100 days $p = 0.995$; 75 and 100 days $p = 0.515$ Tukey's HSD following ANOVA).

v. Lateral Magnocelluar Nucleus of the Anterior Nidopallium (lMAN)

This nucleus, that receives input from DLM, is known to be critical for normal vocal development. At 10 days of age there is little anti-mesotocin receptor staining in this region as demonstrated in Figure 4.6. At day 25 the region appears distinctly stained (mean OD = 0.141 ± 0.016). The pattern of this lMAN staining is characterized by diffuse neuropil staining along with some stained puncta surrounding cell bodies. This staining pattern is maintained and intensifies by day 50 (mean OD = 0.186 ± 0.034). Following peak densities at day 50, staining density decreases, and the appearance of stained puncta are reduced ($p = 0.012$ Tukey's HSD following ANOVA). As summarized in Figure 10, the intensity of the mesotocin receptor antibody stain is very low at 10 days, increases significantly at day 25 ($p = 0.000$ Tukey's HSD following ANOVA), and continues to rise to its peak at day 50 ($p = 0.012$ Tukey's HSD following ANOVA). From there the level of expression decreases with significance at days 75 and 100 (50 to 75 days $p = 0.000$; 50 to 100 days $p = 0.000$; 75 to 100 days $p = 0.794$ Tukey's HSD following ANOVA).

vi. Caudal striatum

The caudal striatum is an area that contains dopaminergic projections similar to that of mammalian striatum. We have demonstrated that there is a distinct population of anti-mesotocin receptor reactive neurons in this brain region but no quantitative measurements were taken due to difficulty in defining the region. At day 10 there is the appearance of a distinct staining of soma, puncta, and fiber tracts (Figure 4.7). This staining pattern continues to day 25 where the staining becomes more pericellular, but the staining of the puncta and fiber tracts remain. At 50 days, the

mesotocin receptor antibody staining appears to decrease and is limited to that of a pericellular nature with some puncta. This qualitative trend of a decrease in labeling continues to day 75. By adulthood the intensity of mesotocin receptor staining in the striatum appears to increase dramatically to a level similar to that in the earlier stages of development from a visual inspection of the staining. The pattern of staining is pericellular with distinctly stained puncta and fiber tracts.

In-Situ Hybridization

The spatial telencephalic distribution of expression of mRNA encoding mesotocin receptors is summarized in Figure 4.9. Sections containing both RA and HVC were hybridized with a ^{32}P-labeled zebra finch mesotocin receptor cDNA probe. Results indicate the song region HVC distinctly expresses mesotocin receptor mRNA (Figure 4.9). At 25 days of age the mesotocin receptor expression was not distinct as compared to the archopallium (mean OD = 0.066 ± 0.010, p = 0.067 following paired t-test). The expression of mesotocin receptor mRNA in HVC increased dramatically at 50 days as there was a high density of labeled cells in the song nucleus as compared to the surrounding areas (mean OD = 0.146 ± 0.009, p = 0.029 following paired t-test). This pattern of high density of cells expressing mesotocin mRNA continued to be evident in HVC at ages 75 (mean OD = 0.114 ± 0.020, p = 0.022 following paired t-test) and 100 (mean OD = 0.142 ± 0.015, p = 0.000 following paired t-test) days of age.

Discussion

Mesotocin receptors were expressed in areas of the zebra finch telencephalon that are vital for vocal learning and production of song such as RA, HVC, lMAN, L2, and Area X and in other areas not involved in song learning such as the caudal striatum and hippocampus. The OT receptor, equivalent to the avian mesotocin receptor, has a distinct pattern of expression in

mammalian CNS including nucleus accumbens, the prelimbic cortex, and the caudate-putamen. Interestingly, expression patterns vary across species that display various degrees of social behavior (c.f. monogamous- vs. promiscuous-pair bonding voles) and are highly variable amongst all mammalian species (Lim and Young 2006). Other receptors implicated in vocal development such as the CB1 cannabinoid receptor are also localized in CNS song regions lMAN and Area X (integral for vocal learning) and regions HVC and RA (required for song production)(Soderstrom and Tian 2006).

Our results demonstrate a distinct pattern of mesotocin receptor distribution located within areas previously demonstrated to be critical in song learning (area X, lMAN), auditory perception of song (L2), and vocal motor production of song (HVC, RA) of the zebra finch. This pattern of staining changes as a function of time and corresponds to noted periods of vocal learning. The early presence of labeled cells within HVC, L2, and Area X at 10 days of age is interesting because these animals have yet to start the process of learning their song but they do use vocalizations in a characterized begging behavior to stimulate parental feeding. In the earliest days of their life this begging behavior has both vocal and visual components, but as the fledglings age it becomes entirely a vocal behavior to initiate feeding (Muller and Smith 1978). Mesotocin receptors remain labeled in vocal motor regions such as RA and HVC as the bird ages and this would suggest a role in sensory-motor learning and vocal production. The increase in mestocin receptor labeling in areas integral for vocal learning (Area X and lMAN) which peaks at 50 days and declines with time highlights a possible role for the receptor in learning-related signaling in the zebra finch. This waning of mesotocin receptor expression as the animal ages and completes vocal learning lends further credence to the idea that mesotocin signaling is an important component of the normal processes of vocal development (vocal learning, perception,

and production) in these animals. It is known that in mammals the pre-adolescent stage corresponds to an increase in myelenation and refinement of synaptic connections within the CNS as it matures to adulthood (Bourgeois and Rakic 1993). Our in-situ hybridization data confirmed the presence of mesotocin receptor mRNA in the HVC at all ages examined further supporting a role for mesotocin signaling in the vocal production of sound and song. The presence of mesotocin receptors within the auditory field L2 at all ages examined indicates a potential role of mesotocin signaling in the auditory perception of song. Birds must be able to hear themselves practice to produce a normal song both as a juvenile and as an adult as it was determined that adult birds only retained 36% of their songs following bilateral cochlear removal compared to the 90% of the song retained by the control birds 16 weeks after surgery (Nordeen and Nordeen 1992). This distinct labeling of neuropil during periods of active auditory processing such as the auditory learning phase where the young bird is listening to and memorizing the song of an adult suggests a feasible role for mesotocin in synapse maturation.

Mesotocin mRNA has been determined to be present in each of the four song regions (Area X, lMAN, RA and HVC) and in other areas not associated with song learning such as the preoptic area, hippocampus, septal nuclei, nucleus taeniae, and the paraventricular nucleus (Xie, London et al. 2010). It was determined through the use of two radioligands, [(^{125})I]ornithine vasotocin analog ([(^{125})I]OVTA) and [(^{125})I]linear VP antagonist ([(^{125})I]HO-LVA) that vasotocin receptors (avian ortholog of vasopressin) were present throughout the telencephalon, diencephalon, midbrain, and brainstem with RA showing a population of receptors labeled. However since competitive binding assays demonstrated that both ligands used were displaced by vasotocin and mesotocin (though mesotocin had a lower binding affinity), it could not be conclusively determined that only vasotocin receptors were labeled (Leung, Goode et al. 2009). Oxytocin–

like receptors have been found in other species who demonstrate forms of vocal communication. Oxytocin immunoreactive neurons have been found in auditory cortices of the mustached bat and densely distributed in the ventral telencephalon and areas within the hypothalamus and brainstem encompassing all sites involved vocal-acoustic integration of the plain midshipman fish (a species of fish who have adapted their airbladder for sound production) (Goodson, Evans et al. 2003; Prasada Rao and Kanwal 2004). The distribution of the OT receptor was recently investigated in two species of singing mice using autoradiography and moderate expression was found in the medial preoptic area, an area linked with vocalization.

Other regions of distinct mesotocin receptor expression include avian hippocampus and caudal striatum where there are increased levels of labeled mesotocin receptors in the striatum of young birds that decreased throughout development until adulthood where they again increased. Reward-related learning has been associated with both ventral and dorsal striatum, and these areas are known to contain OT and dopamine receptors in mammals (Skuse and Gallagher 2008). In human the interplay between these two receptors are thought to be responsible for the cognition of trust an important component of the formation of social bonds by activating areas such as the substantia nigra, the globus pallidus, the bed nucleus of the stria terminalis, and the VTA (Skuse and Gallagher 2008). While mestocin receptors were not visualized within the substantia nigra or the VTA in this study, the globus pallidus and the bed nucleus of the stria terminalis are located in this area of striatum that is distinctly labeled by the anti-mesotocin receptor anti-body. The existence of mesotocin receptors within striatal tissue could provide a potential role for mesotocin signaling in behaviors associated with learning and reward. Area X in zebra finches was shown to activate dopaminergic neurons by a projection to the ventral pallidum which then projects to other dopaminergic regions upon response to hearing the birds

own song which alludes to a component of reward in the song learning process in these animals (Gale and Perkel 2010).

The mammalian ortholog of mesotocin, OT, has previously been established as an important modulator of social behaviors (Lim and Young 2006; Carter, Grippo et al. 2008; Stein 2009). Oxytocin knockout mice (null mutation in the OT gene) are more aggressive and prone to failing to recognize other mice after repeated social encounters than are wild type mice. These behavioral differences were eliminated following OT administration (Winslow and Insel 2002). Recently it has been discovered that targeted delivery of antisense DNA directed against specific sequences of the mRNA of the mouse OT receptor in the medial amygdala blocks normal social recognition in female mice (Choleris, Little et al. 2007). Oxytocin also functions in the neurochemical regulation of prairie vole pair bonding. In these animals OT administration induces pair bonding in both male and female voles (Wang and Aragona 2004). In male zebra finches it was determined that a central infusion of mesotocin had no effect on aggressive behaviors, intersexual affiliation, partner preference, or male courtship singing suggesting that pair bonding is not influenced by mesotocin in this species (Goodson, Lindberg et al. 2004). Mesotocin has, however, been demonstrated to have an effect on the social behavior of zebra finches as peripheral administration of an OT antagonist (OTA - desGly–NH^2,d(CH^2)5[Tyr(Me)2, Thr^4]OVT) significantly reduced the time zebra finches spent with large groups and with familiar birds. The central infusion of mesotocin caused an opposite effect significantly increasing the amount of time spent in a social group in these animals (Goodson, Schrock et al. 2009). While these effects appeared to be mainly restricted to that of the female they do highlight a role for mesotocin signaling in the social behavior of the zebra finch.

Our results illustrate a distinct pattern of expression of the mesotocin receptor in brain regions previously established as vital for zebra finch vocal learning and production. The receptors are also present in the caudal striatum and the hippocampus important for social memory and reward. These findings correlate with central OT receptor distribution patterns reported in other animals. The in-situ hybridization experimental results confirmed that the mRNA of mesotocin receptors is also present in areas of vocal development. The intense pericellular staining within song regions during the sensor-motor states of vocal development (25, 50, 75 day old groups) suggest a relevant role for mesotocin signaling during normal vocal learning. The peak of mesotocin receptor expression correlates to a period of peak vocal learning in these animals (age 50 days).

Oxytocin signaling has been implicated in the etiology of autism in the literature. Accumulating evidence demonstrates that peripheral administration of OT (widely used in the induction of contractions at parturition) distributes to the central nervous system (CNS), particularly in newborns (McEwen 2004). It has been previously determined that OT administration is capable of altering social behaviors such as pair bond formation, trust, and facial memory (Carter, Williams et al. 1992; Zak, Kurzban et al. 2005; Donaldson and Young 2008; Rimmele, Hediger et al. 2009). It is our hypothesis that early exogenous exposure to OT may alter normal zebra finch vocal development, and this is the subject of future experiments.

Figure 4.1 Images (40X) of immunohistochemical staining with anti-mesotocin receptor antibody of the male zebra finch brain at various ages in vocal development. All tissues sections were reacted together. Figure was prepared by Dr. Ken Soderstrom.

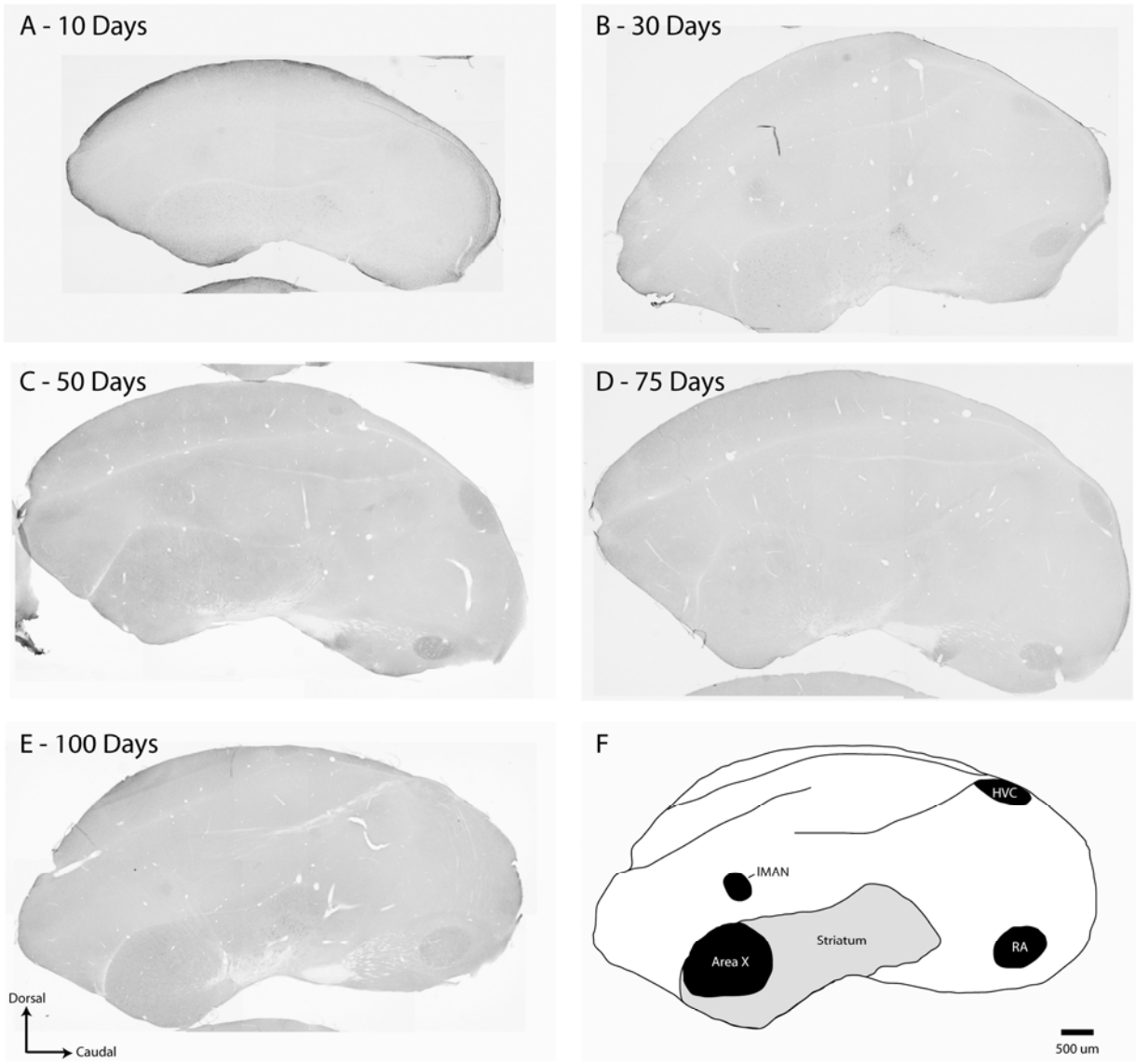
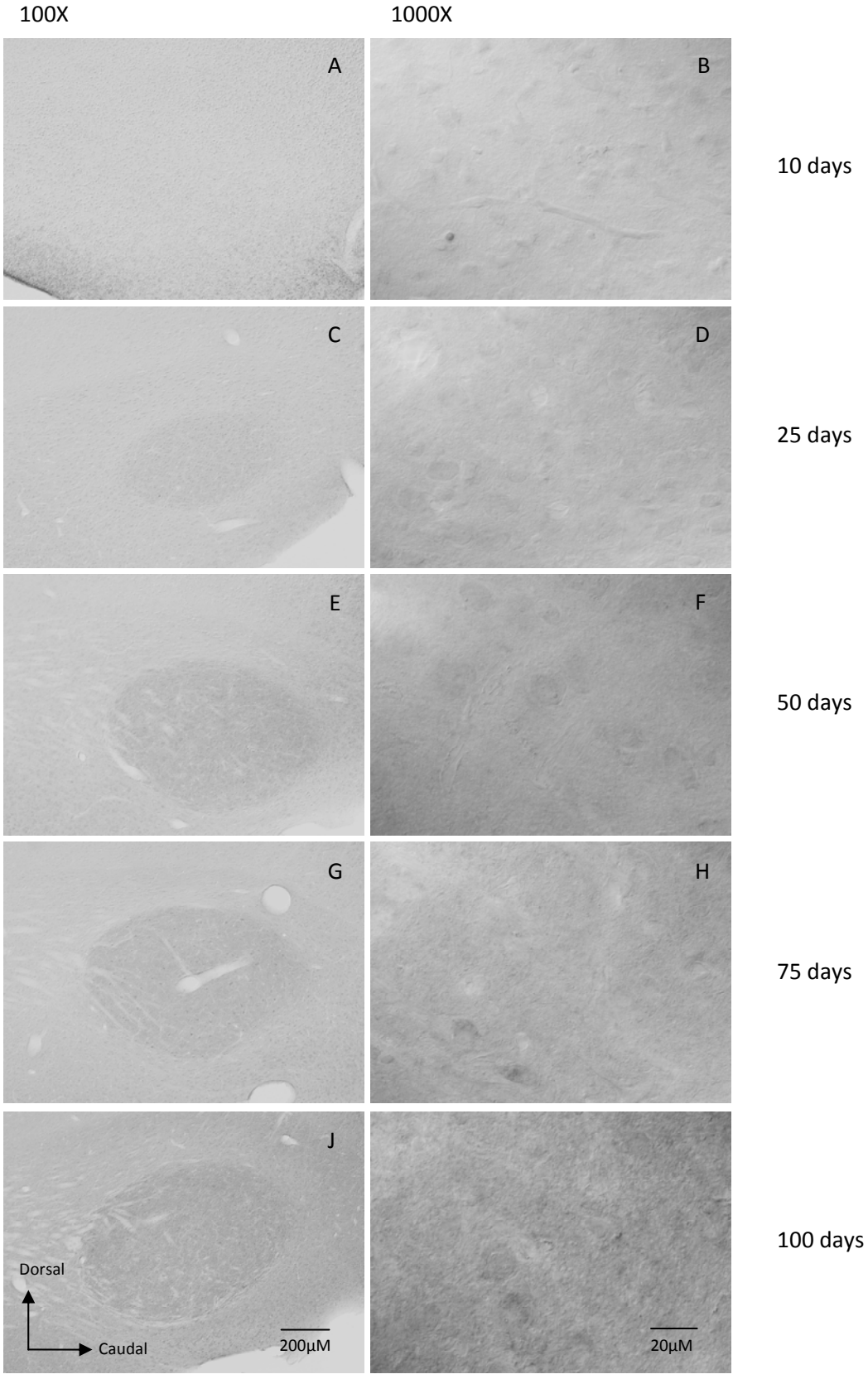

A - 10 Days
B - 30 Days
C - 50 Days
D - 75 Days
E - 100 Days
Dorsal
Caudal
F
HVC
IMAN
Striatum
Area X
RA
500 um

Figure 4.2 Anti-mesotocin receptor antibody staining of Robust Nucleus of the Arcopaollium (RA) at different stages of vocal development. See Figure 4.1F for the location of the RA relative to other brain regions. A, B: Staining at ten days is not discernable above background. C,D: Staining within RA becomes distinct by 30 days and is characterized by neuropil and puncta staining of smaller cell bodies. E-J: The pattern of staining of neuropil and puncta of smaller cell bodies is maintained at 50, 75, and 100 days of age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1000X
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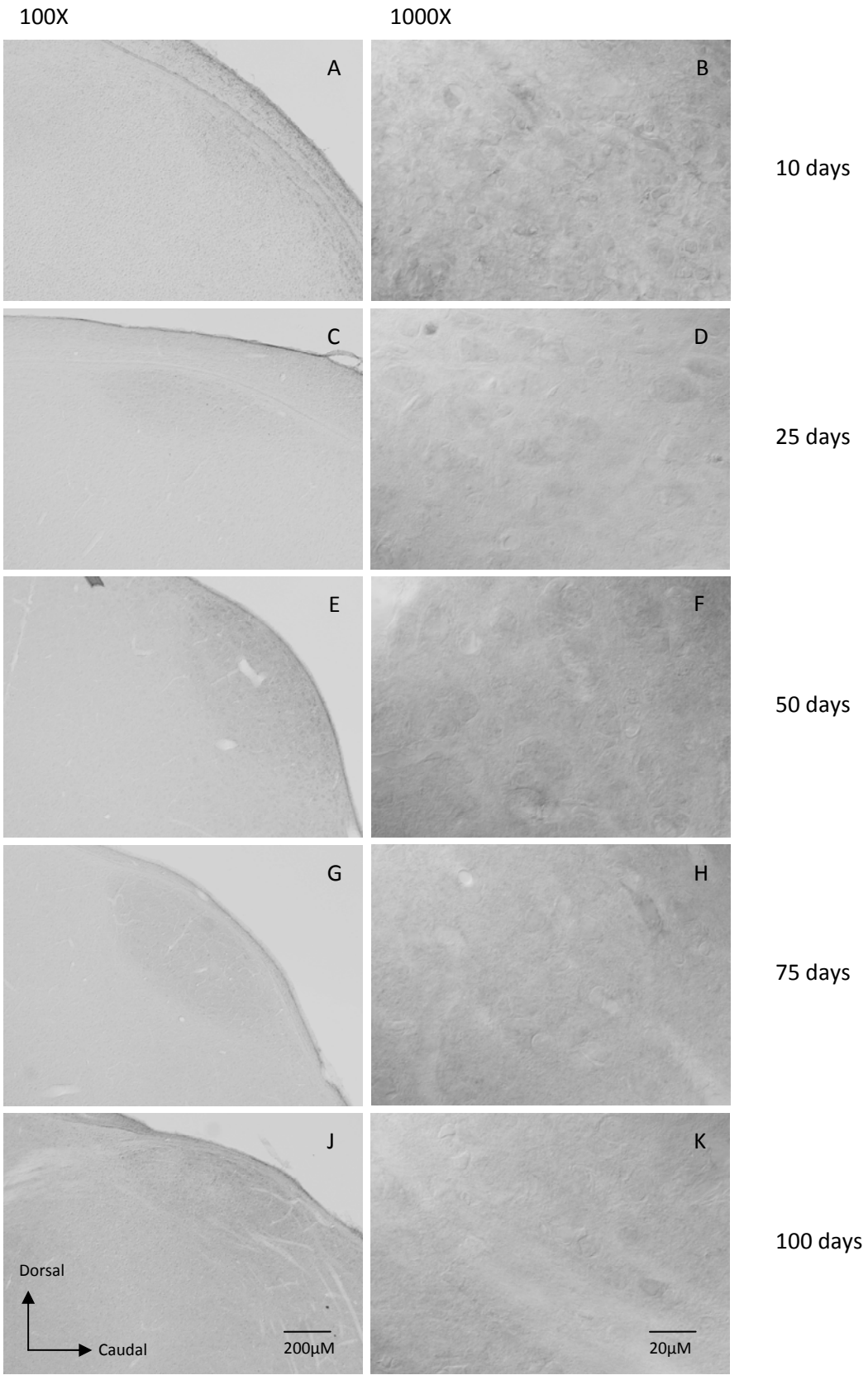
10 days
C
D
25 days
E
F
50 days
G
H
75 days
J
100 days
Dorsal
Caudal
200μM
20μM

Figure 4.3 Anti-mesotocin receptor antibody staining of HVC at different stages of vocal development. See Figure 4.1F for the location of the HVC relative to other brain regions. A, B: Staining at ten days of a small HVC nucleus is visible. C,D: Staining within HVC becomes more pronounced by 30 days and is characterized by diffuse staining of neuropil. E-J: The pattern of staining of neuropil is maintained at 50, 75, and 100 days of age


100X
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50 days
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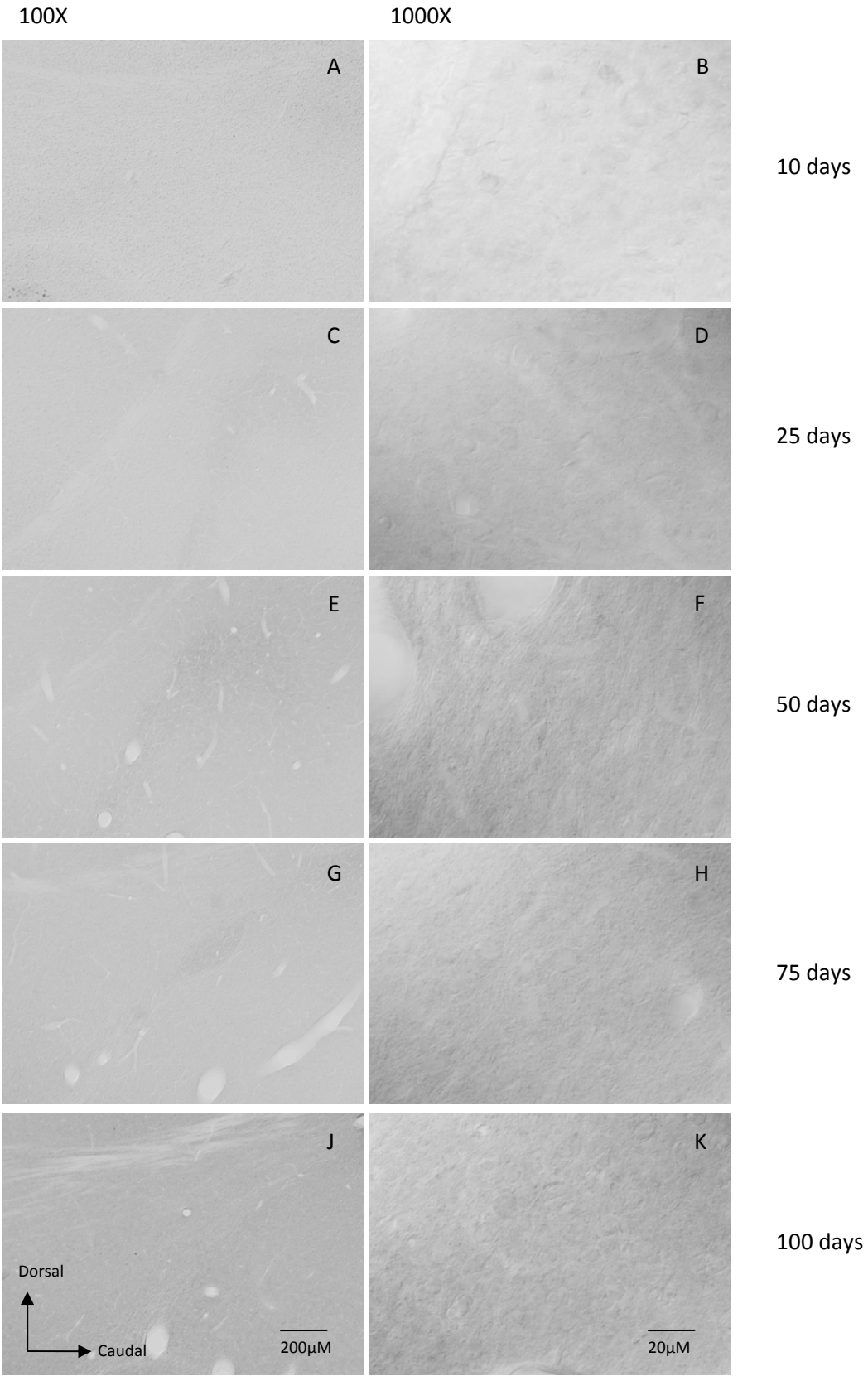
Dorsal
Caudal
200μM
20μM

Figure 4.4 Anti-mesotocin receptor antibody staining of Auditory Field L2 at different stages of vocal development. A, B: Labeling of the mesotocin receptor in L2 starts at ten days and is characterized by a diffuse staining of neuropil. C-J: The pattern of staining of neuropil maintained at 50, 75, and 100 days of age.


100X
1000X
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75 days
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100 days
Dorsal
Caudal
200µM
20µM

Figure 4.5 Anti-mesotocin receptor antibody staining of Area X of the medial striatum at different stages of vocal development. See Figure 4.1F for the location of the Area X relative to other brain regions. A, B: At ten days of age the staining appears to label pericellular processes with some evidence of puncta staining. C,D: The pericellular staining of cell bodies becomes more distinct . E-H: The pattern of staining of cell bodies in a pericellular fashion continues at 50 and 75 days of age. I, J: The staining pattern changes at 100 days to a denser background of neuropil staining with the occasional cell body that is stained pericellularly. The staining of puncta increases dramatically at adulthood.

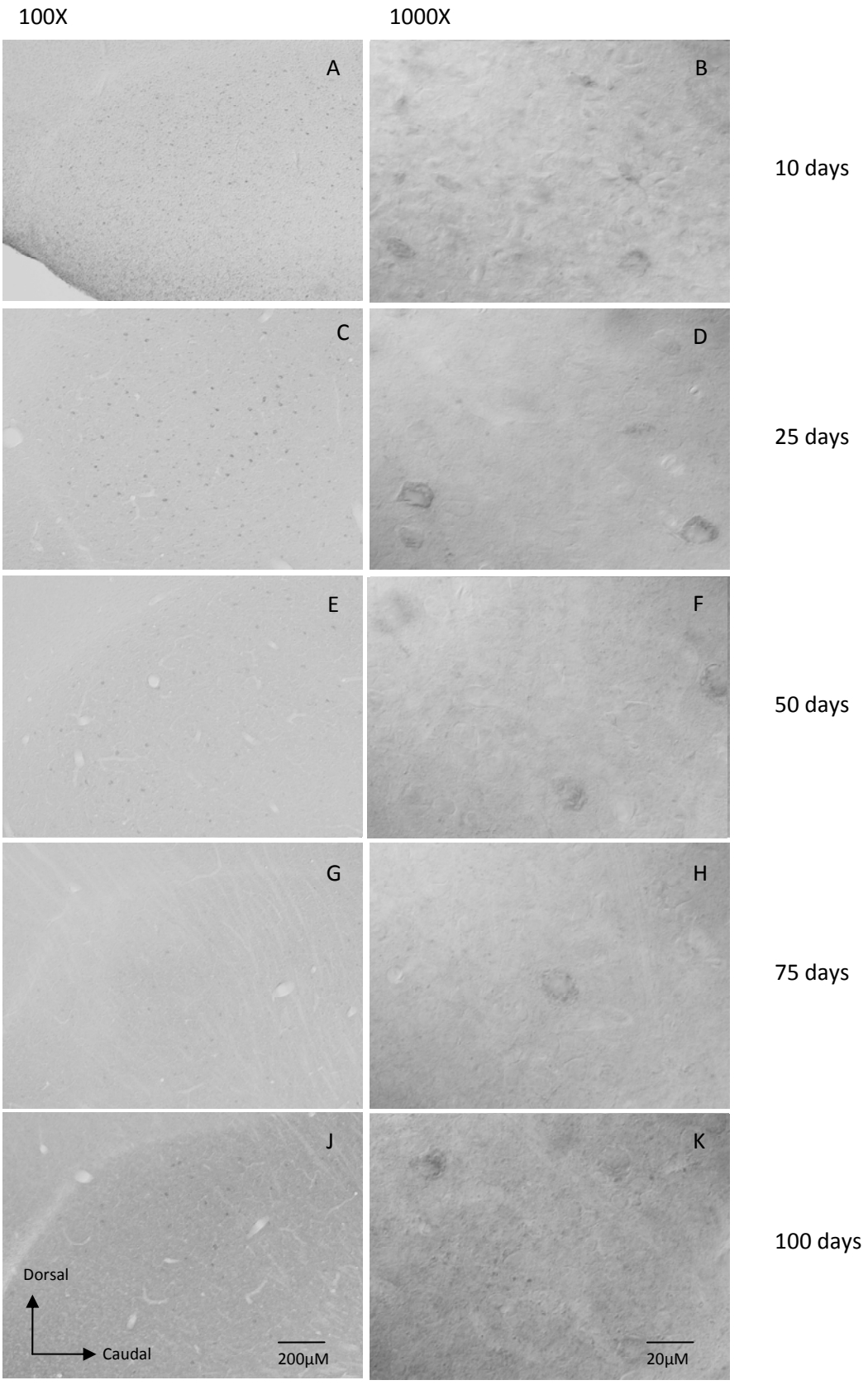

100X
1000X
A
B
10 days
C
D
25 days
E
F
50 days
G
H
75 days
J
K
100 days
Dorsal
Caudal
200µM
20µM

Figure 4.6 Anti-mesotocin receptor antibody staining of the Lateral Magnocelluar Nucleus of the Anterior Nidopallium (lMAN) at different stages of vocal development. See Figure 4.1F for the location of the lMAN relative to other brain regions. A, B: Staining of the mesotocin receptor is not evident at this age. C,D: A distinct stain of diffuse neuropil along with some puncta appears at 30 days. E-F: This pattern of staining continues and intensifies at day 50. G-J: The staining decreases as the bird ages (75, 100 days) to adulthood to that of background staining.

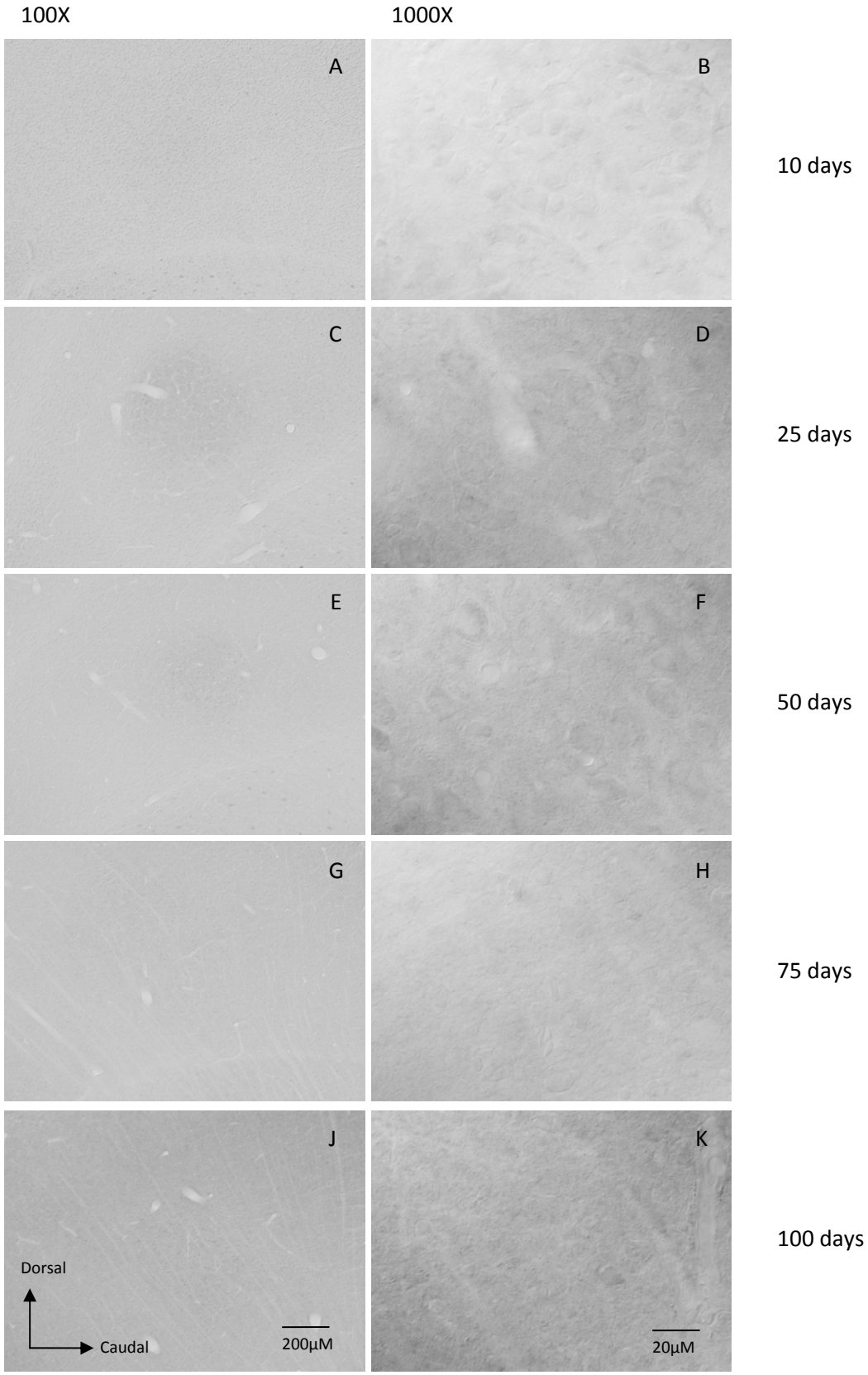

100X
1000X
A
B
10 days
C
D
25 days
E
F
50 days
G
H
75 days
J
K
100 days
Dorsal
Caudal
200µM
20µM

Figure 4.7 Anti-mesotocin receptor antibody staining of the caudal striatum at different stages of vocal development. A, B: The pattern of stained cells includes soma, their puncta, and their projecting fiber tracts at 10 days. C,D: This pattern continues at 30 days but the stain becomes more pericellular in nature. E-H: This pattern of staining decreases and is now characterized by a pericellular stain that includes some puncta. I-J: The staining dramatically returns to the pattern and intensity seen in the juvenile birds at 100 days of age with stained soma, their puncta, and their projecting fiber tracts.

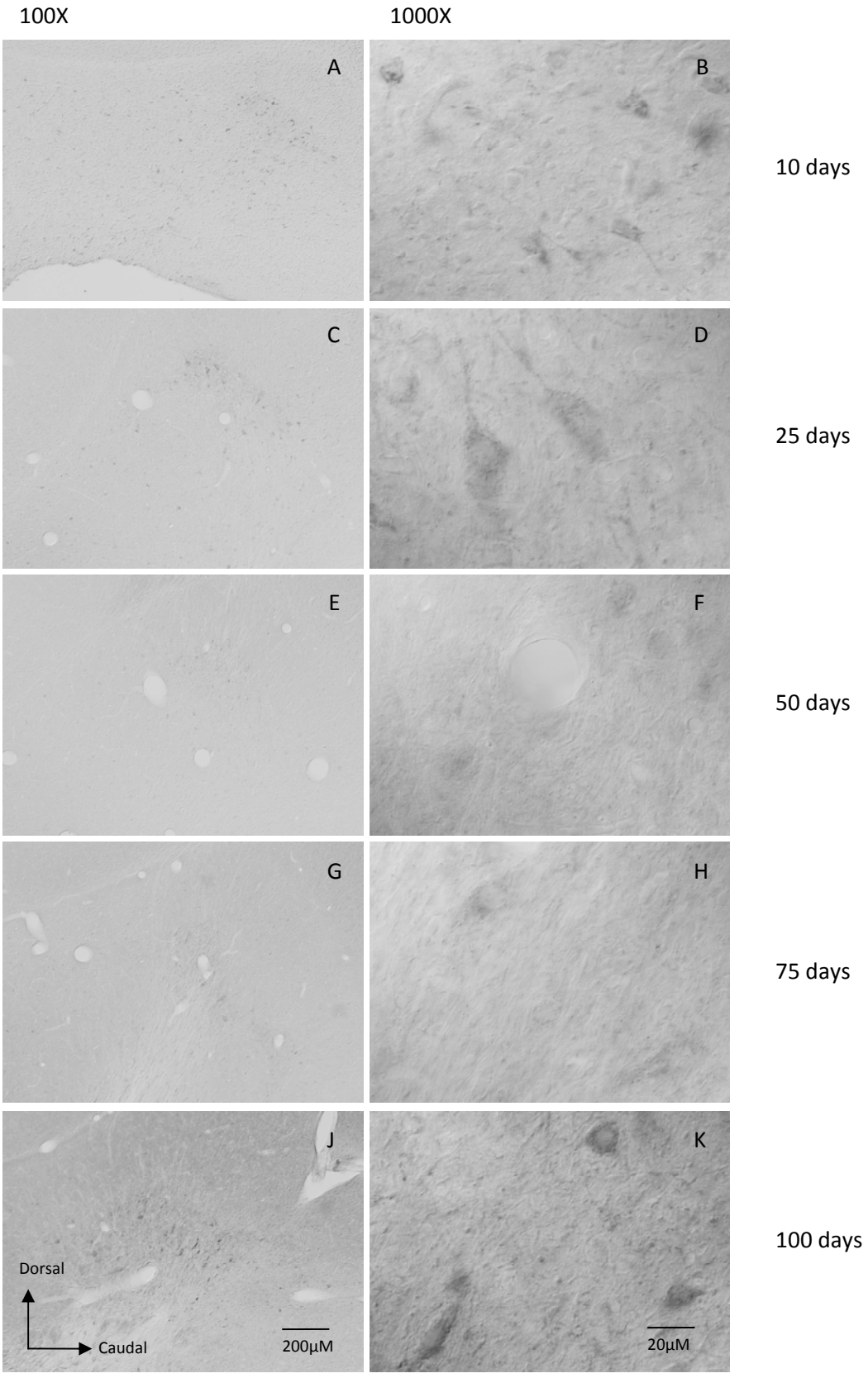

100X
1000X
A
B
10 days
C
D
25 days
E
F
50 days
G
H
75 days
J
K
100 days
Dorsal
Caudal
200µM
20µM

Figure 4.8 Comparison of optical density of mesotocin receptor labeling in telencephalic song regions across different age groups. In all areas selected for comparison, the peak of labeling density occurred at 50 days of age. This is then followed by a general decrease in labeling density at adulthood. RA, robust nucleus of the acropallium; HVC is used as a proper name; L2, primary auditory field; area X, area X of the medial striatum; lMAN, lateral magnocellular nucleus of the anterior nidopallium.

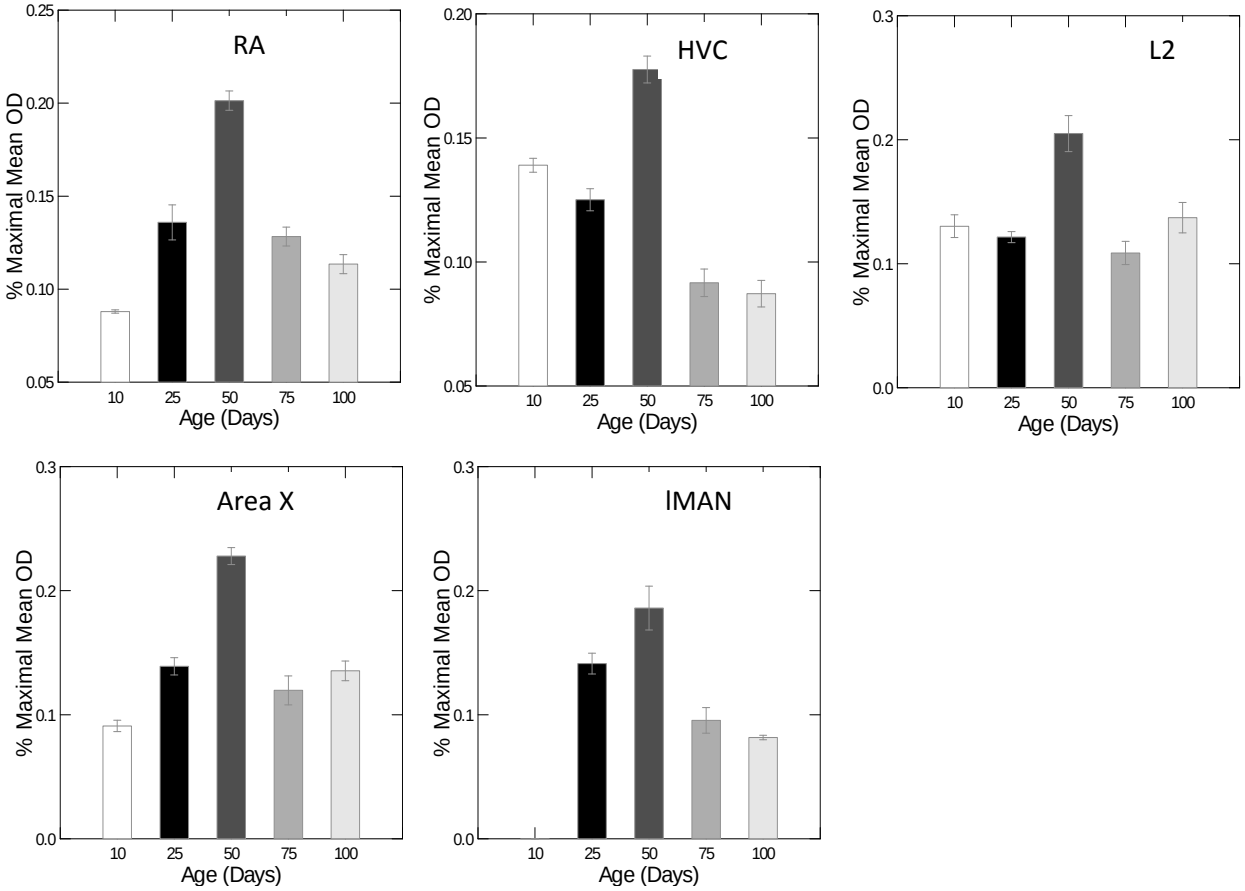

RA
HVC
L2
Area X
lMAN
% Maximal Mean OD
Age (Days)
10
25
50
75
100

Figure 4.9 Expression of the mesotocin receptor mRNA in HVC (a zebra finch song region) as determined by *in situ* hybridization. All micrographs are at 100X magnification. (A) HVC in a 25 day old male. The level of message is barely discernable above background. (B) HVC in a 50 day old male demonstrates the increased level of mesotocin receptor mRNA. (C) HVC in a 75 day old male where the density of labeled message is still increased above that of the surrounding archopallium. (D) HVC of a 100 day old male illustrating labeled mesotocin receptor mRNA.

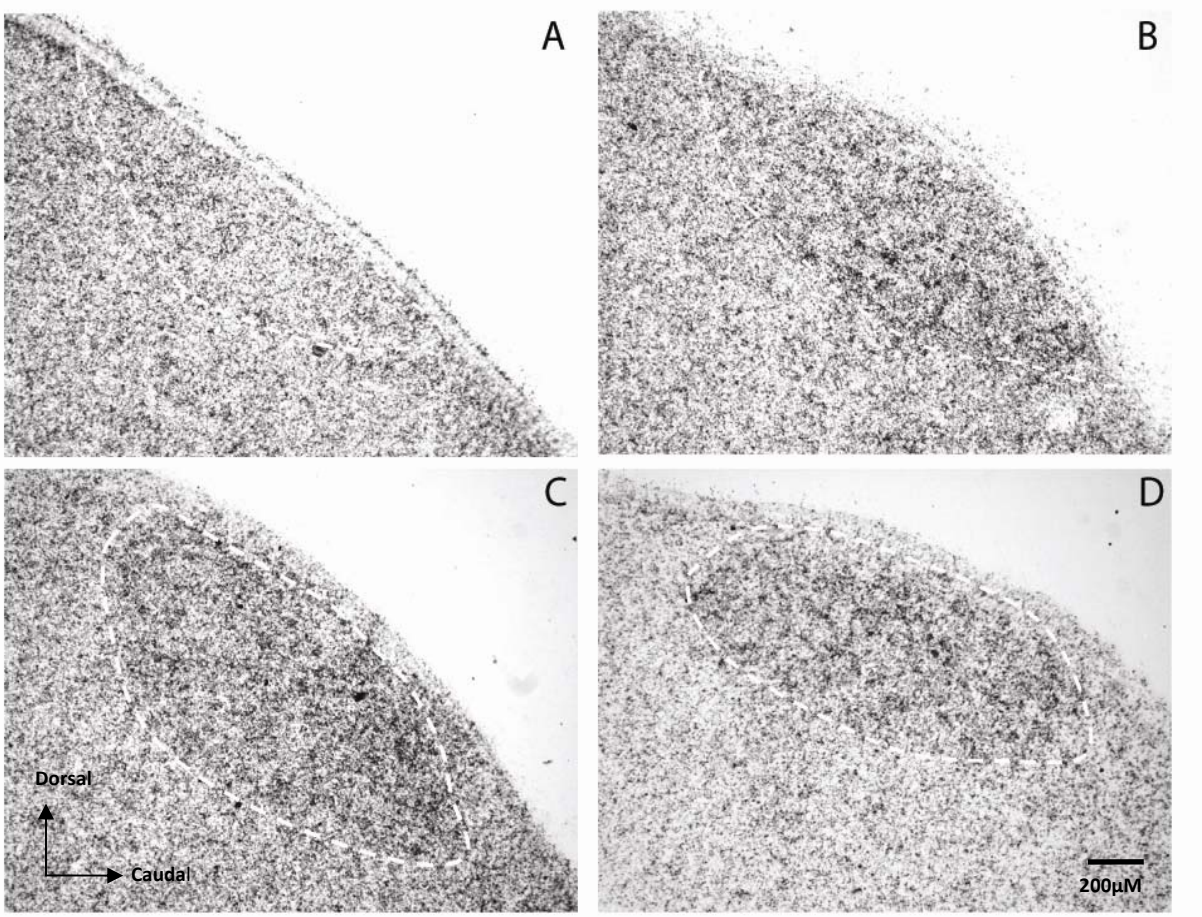
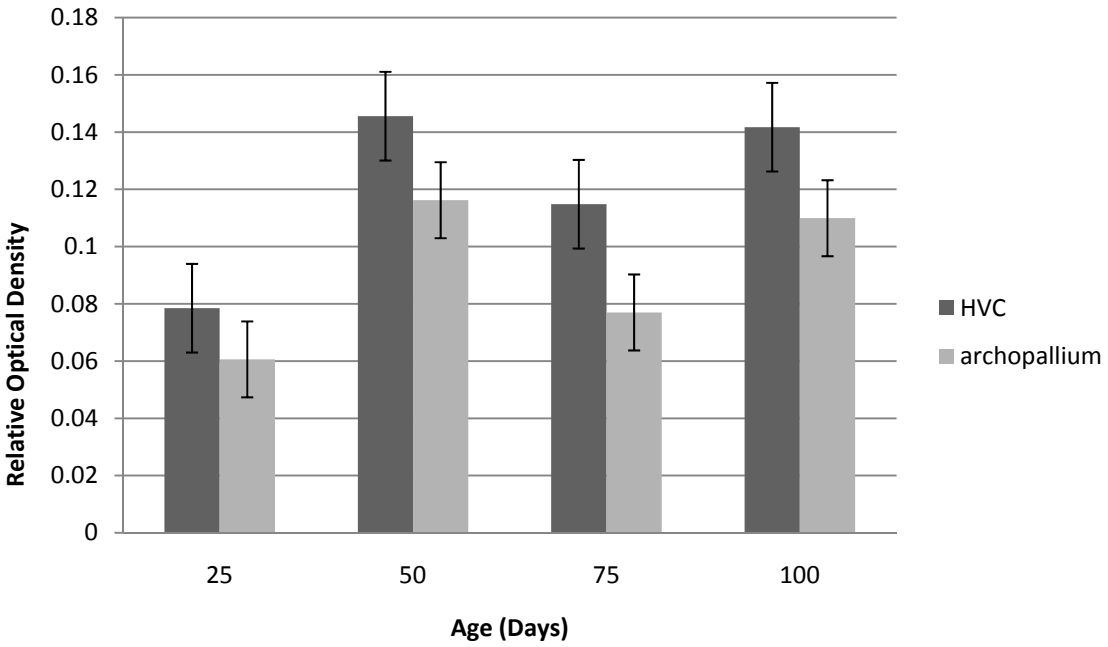

A
B
C
D
Dorsal
Caudal
200µM

Figure 4.10 Comparison of optical density of labeled cells with a mesotocin receptor probe in the telencephalic song region HVC across different age groups versus the surrounding archopallium. Significant expression of labeled cells was detected at 50 days of age (mean OD = 0.146 ± 0.009, $p = 0.029$ following paired t-test) and continued at 75 days of age (mean OD = 0.114 ± 0.020, $p = 0.022$ following paired t-test) and 100 days of age (mean OD = 0.142 ± 0.015, $p = 0.000$ following paired t-test). Shown are mean percentages ± S.E.M from counts of five sections from four birds. .


0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Relative Optical Density
25
50
75
100
Age (Days)
HVC
archopallium

CHAPTER FIVE: EARLY EXPOSURE TO OXYTOCIN ALTERS ZEBRA FINCH VOCAL PATTERNS

Abstract

Oxytocin has been found to alter the neural circuitry involved in controlling social behaviors in humans and is a proposed treatment for individuals suffering from Autism Spectrum Disorder, which is a disorder characterized by both social and language deficits (Hollander, Novotny et al. 2003; Kirsch, Esslinger et al. 2005; Domes, Heinrichs et al. 2007; Hollander, Bartz et al. 2007). Humans are exposed to synthetic OT in the uterus when their mother's labor is augmented with Pitocin (Rojas Wahl 2004; Alfirevic, Kelly et al. 2009). In order to investigate the possibility that exposure to exogenous OT can alter vocal development, zebra finch hatchlings were be injected with varying doses of OT. Young male birds were sexed according to the appearance of secondary sex characteristics at age 25 ± 2 days and then were allowed to mature and learn a song from an established adult male tutor. The resulting songs were recorded, analyzed, and compared to those of the control treated group. Song stereotypy and mean note duration were significantly decreased as a function of treatment in both OT dose groups (30 μg/kg, 300 μg/k) when compared to that of the vehicle treated group. No significant difference was found to exist in the mean optical density of anti-mesotocin receptor immunohistochemical staining in the four song regions examined (vocal motor regions HVC and RA and song learning regions lMAN and Area X) between the vehicle treated control group and the OT treated birds. Our results suggest that OT is capable of altering vocal development but its actions are not through a persistent alteration in mesotocin receptor densities.

Introduction

The zebra finch is an established animal model of vocal development (Bottjer, Glaessner et al. 1985). Male zebra finches learn a song pattern in a manner similar to human language acquisition, and the neurobiology of both song learning and production has been extensively studied (Scharff and Nottebohm 1991; Doupe and Kuhl 1999). Since the zebra finch learns a form of vocal communication during distinct and well defined phases, these animals can be used to investigate the effects of exogenous compounds on vocal learning (Soderstrom and Johnson 2003; Soderstrom and Tian 2004).

Male zebra finches learn a song that consists of a series of notes of a specific frequency and order during juvenile development through early adulthood and once a specific song pattern is learned, it does not change (Immelmann 1969; Arnold 1975; Bohner, Chaiken et al. 1990). There are at least two distinct developmental stages for song acquisition: auditory phase and sensory-motor phase (See Figure 1.5). In the auditory phase young male zebra finches first listen and memorize the song of an adult male. In the second stage of vocal learning the bird begins to "practice" this memorized song by learning to control their syrinx in order to produce vocalizations of a particular frequency and duration. The first songs produced by young zebra finches are highly variable and these vocalizations are referred to as sub-songs. Once distinct notes are produced by the bird the songs are slowly refined to match the song memorized during the auditory phase and the vocalizations produced during this period of practice and refinement are termed plastic songs. This refinement period requires sensorimotor learning or vocal practice where the bird must actively sing and compare its song to the memorized template and birds need be able to hear themselves practice to produce a normal song both as a juvenile and as an adult (Nordeen and Nordeen 1992). A final stereotyped song is consistently produced once the birds reach adulthood or approximately 90 days of age and it continues to be produced for the

rest of the bird's life.

Autistic individuals display dysfunctions in three main behavioral areas: social deficits, repetitive behaviors, and language abnormalities. A role for OT in complex social behaviors such as fear, social recognition, and monogamous pair bonding initiation has been established by recent studies (Wang and Aragona 2004; Kirsch, Esslinger et al. 2005; Choleris, Little et al. 2007). There is evidence of a causal relationship between altered OT levels and repetitive behaviors in both human and animal data (Drago, Pederson et al. 1986; Hollander, Novotny et al. 2003). However limited research exists to document the role of OT in vocal development in humans and in animal models. Oxytocin immunoreactive neurons have been found in auditory cortices of the mustached bat and densely distributed in the ventral telencephalon and areas within the hypothalamus and brainstem encompassing all sites involved vocal-acoustic integration of the plainfin midshipman fish (a species of fish who have adapted their airbladder for sound production) (Goodson, Evans et al. 2003; Prasada Rao and Kanwal 2004). Furthermore the production of vocalizations has been determined to increase plasma OT levels in humans (Grape, Sandgren et al. 2003; Seltzer, Ziegler et al. 2010).

The etiology of ASD is not well understood and many hypotheses as to the causes of this developmental disorder exist. One such hypothesis is that excessive amounts of OT particularly the large amounts used in the induction of labor could contribute to the development of ASD by the down-regulation of the OT receptor (Rojas Walh 2007). The OT receptor does internalize as a result of agonist stimulation and an alteration of receptor densities, even a transient one, may possibly result in a dysfunction at a critical period of newborn development (Gimpl and Fahrenholz 2001). Studies demonstrate a dose-dependent effect of OT on social behaviors where lower doses facilitate a particular behavior such as social recognition but higher doses

cause the disruption of social memory (Rojas Walh 2007). Here we use zebra finches to investigate the possibility early exogenous OT exposure will alter vocal development.

Materials and Methods

Except where otherwise noted, all materials and reagents were purchased from Fisher Scientific (Fair Lawn, NJ) or Sigma (St. Louis, MO). Oxytocin (Bachem, Torrance, CA) was dissolved in vehicle consisting of 1X phosphate buffered saline.

Animals

Male zebra finches used in these experiments were bred in our aviary, housed in flight aviaries maintained at the ambient temperature of 78ºF, and given free access to mixed seeds (SunSeed VitaFinch), grit, water, and cuttlebone. The light-dark cycle was maintained at LD 14:10 hours. At 25±2 days of age the birds were identified by secondary sex characteristics such as the appearance of the black bar feathers on the breast. The male birds were then removed from the care of their parents, raised in groups by surrogate females, and tutored by the same adult male until 50 days of age. Following tutor exposure, the animals were placed in visual but not auditory isolation for the remainder of the experiment. Animals were cared for and experimental procedures were conducted according to protocols approved by East Carolina University's Animal Care and Use Committee.

Auditory Learning Experiments

A preliminary behavioral experiment assayed the effects treatment three doses of OT (300 µg/kg, 1000 µg/kg, and 3000 µg/kg) and a vehicle control. Doses were chosen based on a review of the literature. See Table 2.2. This initial experiment was refined due to no statistical difference between doses on effects examined (mean number of notes, average note duration, and song stereotypy – $p = 0.94$, $p = 0.76$, and $p = 0.85$ respectively, two sample t-test) and an apparent

high rate of death in the high dose (3000 µg/kg). See Figures 5.1 and 5.2. A larger study was conducted using the lowest dose shown effective in our preliminary experiment and included a lower dose. Nests of zebra finch hatchlings were randomly assigned to the following treatment groups: 300 µg/kg OT; 30 µg/kg OT; and vehicle. At post-hatch day five, zebra finches were injected with 50 µl subcutaneously over the pectoralis muscle with either OT or vehicle prior to the start of the 14-hour light cycle for a total of five days (days 5-9). The animals were then allowed to remain with their parents until 25 ± 2 days when they were removed, group housed and raised by surrogate females. During this period the young male zebra finches were tutored by the same adult male in constant auditory and visual contact until 50 days of age where they were then moved to a cage with visual but not auditory isolation and allowed to mature. Visual isolation prevents potential cross-tutoring from other juveniles (Morrison and Nottebohm 1993). Digital recordings of songs produced were made at approximately 100 days of age from the treated male zebra finches (300 µg/kg, n = 11; 30 µg/kg, n = 5; vehicle, n = 7). See Figure 5.3 for a summary of treatment paradigm. Ten-song bout recordings were selected randomly for analysis. Song parameters were measured by an observer blind to treatment. Methods for measuring note duration and internote intervals were based on previously established protocol for our laboratory (Soderstrom and Tian 2004). Song stereotypy was determined using the method described by (Scharff and Nottebohm 1991) which quantifies song stereotypy through the linearity of the syllables produced in phrases and the consistency of the of the sequence of syllables contained within phrases. Phrase linearity is determined by a "linearity score" where linearity = different song notes/transition types. In this equation a transition type is the order in which one syllable follows another similar to the way one word follows another in a sentence. In a completely linear phrase sequence, each syllable has only one transition type, and therefore the

linearity score equals 1. Syllable consistency is scored as: Consistency = typical transitions/total transitions. This equation expresses the frequency with which a particular ordering of notes within a song occurs and determines if the bird consistently repeats the same pattern of notes every time they sing. The stereotypy score combines measures of linearity and consistency: Stereotypy = (linearity +consistency) / 2. Normal adult males stereotypy scores vary from 0.7 - 0.9 while lower scores are generally associated with an immature or plastic song (0.5 – 0.6, see Whitney et al., 2000). Data analyses and statistical computations were performed in Systat 12 and Microsoft Excel PC software. The quantification of song parameters were measured as described above. Comparisons of the effect between treatments on parameters measured were made using analysis of variance (ANOVA) and Tukey's HSD post hoc tests where appropriate. P values of less than or equal to 0.05 were considered as a statistically significant difference.

Immunohistochemistry

After song recordings were captured for each animal, the brain tissue was prepared for immunohistochemistry experiments to investigate possible changes in the mesotocin receptor densities in song regions. The animals were overdosed with Equithesin and transcardially perfused with cold phosphate buffered saline (PBS; pH 7.4) followed by phosphate-buffered 4% paraformaldehyde, pH 7.0. If the perfusion failed to produce a white blood-free brain suitable for immunohistochemistry, then the brain was removed from the experiment. The perfusion-fixed brains were removed and post-fixed by immersion overnight in phosphate-buffered 4% paraformaldehyde, pH 7.0 at 4° C. Once fixed, brains were weighed and then blocked parasagittally down the midline and stored at 4° C in 20 % sucrose until tissue from the entire set of animals from treatment conditions were collected to be processed together so as to ensure

comparable reaction conditions. Male zebra finch brains were collected from each of the treatment groups (300 μg/kg, n = 10; 30 μg/kg, n = 4; or vehicle, n = 6) and weighed to determine if any difference in body mass was produced as a result of treatment. Blocked brains were then removed from storage and glued to a microtome chuck so they could be sectioned using a vibrating microtome parasaggitally (lateral to medial) to produce 30 μm sections of zebra finch brain. Tissue sections were collected and thrice washed in PBS. Sections were incubated in 0.3 % hydrogen peroxide solution for 30 minutes to remove endogenous peroxidases, blocked with 5 % goat serum for 30 minutes, and incubated overnight in solution containing a primary antibody at previously optimized dilution (1:1,000). The next day sections were washed thrice in PBS (pH = 7.4) and incubated in a previously optimized dilution of peroxidase conjugated secondary antibody solution (1:3,000) for one hour. Samples were washed three times with PBS and secondary labeling was visualized with a DAB solution. Each experiment included negative control sections not exposed to primary antibody that were shown to be not immunreactive. Mounted tissues were examined in four CNS brain song regions (i.e. Area X (Area X within medial striatum), lMAN (Lateral Magnocelluar Nucleus of the Anterior Nidopallium), HVC, RA (Robust Nucleus of the Arcopallium)) at 100 and 1000 X using an Olympus BX51 microscope with Nomarski DIC optics. Images of the slides were taken using a Spot Insight QE digital camera and Image-Pro Plus software (Media-Cybernetics, Silver Spring, MD) under identical calibrated exposure conditions and background-corrected.

Measurement of Optical Density of Anti-Zebra Finch Mesotocin Receptor Staining

There were five x 40 gray-scale images captured per treatment and brain region by an observer blind to treatment conditions. The borders of the regions of interest were traced using Image-Pro Plus analysis software, and the software computed the optical densities within each hand-traced

region. The images for each of the three treatment groups were captured in a single session under identical, calibrated exposure conditions. The optical density values for the five images of each brain region for each of the treatment groups were calculated and the mean value was determined. Optical density values were then compared across age groups by brain regions using ANOVA and Tukey's HSD (Honestly Significant Difference) post hoc tests where appropriate. P values of less than or equal to 0.05 were considered as a statistically significant difference.

Results

Auditory Learning Experiments

There was no significant difference observed in the mass of the brains due to treatment with OT (PBS and 30 µg/kg $p = 0.87$, PBS and 300 µg/kg $p = 0.89$, 30 µg/kg and 300 µg/kg $p = 0.99$ Tukey's HSD following ANOVA). See Figure 5.4. No significant relationship was found to exist between treatment groups and the mean number of notes produced per song (PBS: 5.71 ± 0.95; 30 µg/kg: 5.2 ± 0.45; 300µg/kg: 6.2 ± 1.56) as analyzed by Tukey's HSD following ANOVA post hoc tests (PBS and 30 µg/kg $p = 0.75$, PBS and 300 µg/kg $p = 0.62$, 30 µg/kg and 300 µg/kg $p = 0.26$). A significant decrease in the mean note duration of songs was found when the high dose OT group (300 µg/kg) was compared to the vehicle treated control birds ($p = 0.00$ Tukey's HSD following ANOVA) but no significant difference was found between the high and low dose (30 µg/kg) groups ($p = 0.43$ Tukey's HSD following ANOVA) and the low dose and control times ($p = 0.089$ Tukey's HSD following ANOVA). The high dose group was found to have mean note durations of 0.152 ± 0.02 seconds; the low dose group had mean note durations of 0.176 ± 0.036 seconds; and the PBS group had mean note durations of 0.223 ± 0.052 seconds. OT treatment produced significant alterations in both consistency and linearity scores in both the

high and the low dose groups when compared to that of control birds (for consistency PBS and 30 µg/kg $p = 0.03$, PBS and 300 µg/kg $p = 0.01$, 30 µg/kg and 300 µg/kg $p = 1.00$, for linearity PBS and 30 µg/kg $p = 0.01$, PBS and 300 µg/kg $p = 0.02$, 30 µg/kg and 300 µg/kg $p = 0.82$ Tukey's HSD following ANOVA for both) and this difference resulted in a significant decrease in stereotypy scores (PBS and 30 µg/kg $p = 0.02$, PBS and 300 µg/kg $p = 0.01$, 30 µg/kg and 300µg/kg $p = 0.97$ Tukey's HSD following ANOVA). Vehicle treated animals had linearity, consistency, and stereotypy scores of 0.799 ± 0.066, 0.89 ± 0.11, and 0.843 ±0.085 respectively. The songs produced by the lower dose of OT (30 µg/kg) were characterized by an average linearity score of 0.648 ± 0.059, an average consistency score of 0.716 ± 0.077, and an average stereotypy score of 0.684 ± 0.065 while the songs produced by the higher dose of OT (300µg/kg) were characterized by an average linearity score of 0.675 ± 0.103, an average consistency score of 0.717 ± 0.123, and an average stereotypy score of 0.695 ± 0.110. Song analysis results are depicted in Figure 5.5. Songs produced by OT treated birds were typified by a disorganization of notes learned and a tendency toward repetition of notes within a song as demonstrated in Figure 5.6.

Immunohistochemistry

Mesotocin receptor densities within four song control nuclei (Area X, lMAN, HVC and RA) were examined in the treated birds used in the previously detailed behavioral experiment to determine if any persistent alterations in the density of the receptor existed as a function of early exogenous OT exposure. No significant relationship existed as a result of treatment on the mesotocin receptor densities for any of the areas examined. See Figure 5.7. Mesotocin receptor staining within the vocal learning song nuclei of Area X is typified by a dense background of neuropil staining with an occasional cell body surrounded by pericellular staining, and a distinct

increase in the staining of puncta. Mean optical densities of anti-mesotocin receptor staining in this song nuclei were as follows: 300 µg/kg – 0.099 ±0.013, 30 µg/kg – 0.099 ± 0.019, and vehicle – 0.104 ± 0.038 (PBS and 30 µg/kg $p = 0.95$, PBS and 300 µg/kg $p = 0.93$, 30 µg/kg and 300 µg/kg $p = 0.99$ Tukey's HSD following ANOVA). The pattern of staining of the mesotocin receptor in another song learning region of lMAN is characterized by diffuse neuropil staining along with some stained puncta surrounding cell bodies. High dose treated birds were found to have a mean OD of 0.075 ± 0.017, low dose birds had a mean OD of 0.078 ± 0.014, and vehicle treated birds had a mean OD of 0.084 ± 0.035 in this area (PBS and 30 µg/kg $p = 0.91$, PBS and 300 µg/kg $p = 0.77$, 30 µg/kg and 300 µg/kg $p = 0.98$ Tukey's HSD following ANOVA). The staining density of the zebra finch anti-mesotocin receptor antibody was examined in the vocal motor control regions RA and HVC. Within RA the staining is characterized by both neuropil and puncta staining of the smaller cell bodies and there is a diffuse staining of neuropil within HVC of the mesotocin receptor. Mean optical densities of the staining of RA were as follows: 300 µg/kg – 0.094 ± 0.023, 30 µg/kg – 0.097 ± 0.032, and vehicle – 0.111 ± 0.057 (PBS and 30 µg/kg $p = 0.99$, PBS and 300 µg/kg $p = 0.76$, 30 µg/kg and 300 µg/kg $p = 0.87$ Tukey's HSD following ANOVA). Typical staining patterns of each of the areas examined for the three treatment groups (300 µg/kg, 30 µg/kg, and PBS) are illustrated in Figures 5.8, 5.9, and 5.10.

Discussion

Oxytocin Effects on Song Learning

The altered song learning seen in OT treated zebra finches may be the result of OT effects on song learning, the production of song as a form of social communication or both. Other investigators have examined the effects of OT on other behavioral paradigms in the zebra finch. Goodson et al. (2004), determined that a central infusion of mesotocin had no effect on

aggressive behaviors, intersexual affiliation, partner preference, or male courtship singing in adult male zebra finches (Goodson, Lindberg et al. 2004). However mesotocin has been shown to be a potential facilitator of social behaviors within the zebra finch similar to that seen in other animals including humans given that the peripheral administration of an OT antagonist (OTA - desGly–NH2,d(CH2)5[Tyr(Me)2, Thr4]OVT) significantly reduced the time zebra finches spent with large groups and with familiar birds while the central infusion of mesotocin significantly increased the amount of time spent in a social group in these animals (Goodson, Schrock et al. 2009). Therefore a potential role may exist in the zebra finch for mesotocin in the production of social behaviors such as communication.

Oxytocin-induced reductions on the quality of song learned as examined by stereotypy scores when compare to that of the vehicle control treated birds could be an effect on memory which OT is known to influence (Gimpl and Fahrenholz 2001). Oxytocin is thought to have an amnestic function in labor and delivery in order to allow a woman to forget the pain of giving birth and a reduction of the activity of NMDA receptors whose substrates are highly implicated in learning and memory was found in cultured neurons following the introduction of OT to the media (Caruso, Agnello et al. 1993; Evans 1997). Intranasal OT administration was also found to impair memory performance in a cued recall test in adult males (Heinrichs, Meinlschmidt et al. 2004). Octopressin (OT related peptide; see Table 1.1) at low doses (3 μg/kg) was found to increase long term memory formation of a passive avoidance task, but a high dose of octopressin (60 μg/kg) was found to attenuate memory formation in the cuttlefish (Bardou, Leprince et al. 2010). It is possible that the mechanisms underlying the possible alteration in memory formation in the zebra finch could include an erroneous perception of song or an error in the encoding of song memories. Previous work has demonstrated mesotocin receptors in song control regions

associated with the processing of auditory stimuli such as the Auditory Field L2 in the zebra finch telencephalon. See Figure 4.4.

The decrease in mean stereotypy scores in the OT treated birds was a function of altered note timing and repetition of notes in songs as evidenced in Figure 5.6. Oxytocin treated birds displayed stereotypy scores (0.684 ± 0.0654 for the lower dose and 0.695 ± 0.110 for the higher dose) similar to those associated with plastic song production (0.5-0.6). Furthermore songs produced by the birds treated with the higher dose of OT share common features with immature songs such as subsongs and plastic songs. The first highly variable songs produced by the young zebra finch are termed subsongs and are analogous to vocal babbling. They are the young birds attempt at producing the notes initially memorized during the auditory phase from the adult male tutor. These vocalizations are slowly refined to that of the plastic song to match the memorized song. This refinement period requires sensorimotor learning or vocal practice where the bird must actively sing and compare its song to the memorized template (Nordeen and Nordeen 1992). Refer to Figure 1.5 for more information.

The subsong of zebra finches and is an early motor behavior that relies on the proper function of HVC. The electrolytic elimination of HVC in juvenile zebra finches produced unaffected subsongs, but the same procedure in older zebra finches (those in the plastic song stage and adults) caused the loss of all distinct song syllables and the production of songs similar to that of subsong (Aronov, Andalman et al. 2008). Evidence also exists linking the vocal motor region of HVC with proper note sequencing which is also altered in the OT treated birds (Vu, Mazurek et al. 1994). Mesotocin receptors have been demonstrated to be expressed in HVC from day 10 to adulthood (day 100) and the mRNA of the mesotocin receptor was also expressed within this area. See Figure 4.3 and Figure 4.9. The song analysis data and the presence of mesotocin

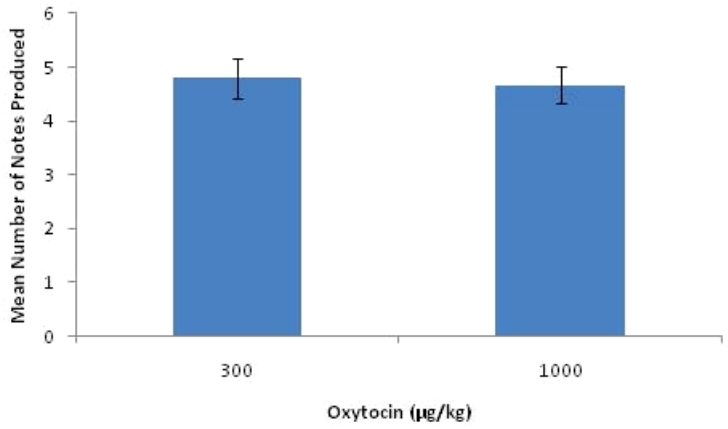
receptors in HVC suggests that early OT treatment may cause the persistently alter the transition from subsong production to that of the plastic song and eventually to the final adult stereotyped song.

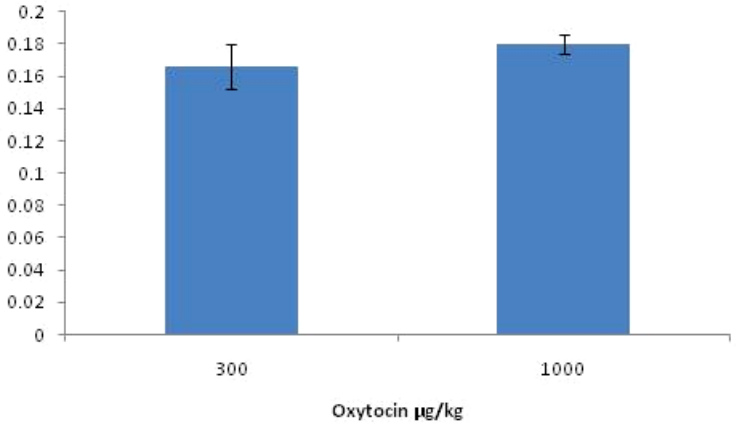
Oxytocin Effects on Adult Mesotocin Receptor Densities

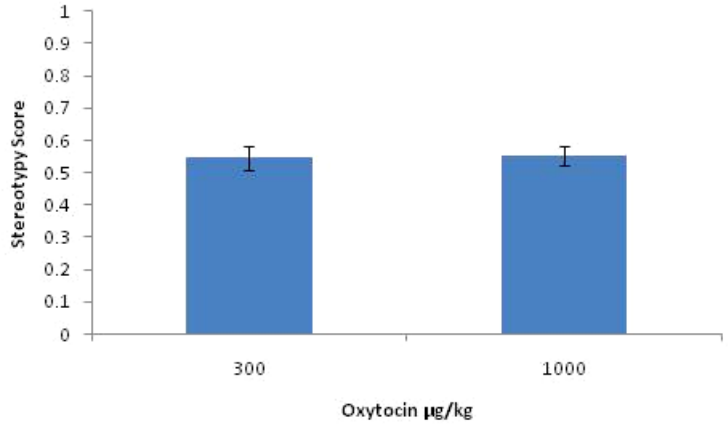
Early OT treatment had no significant persistent effect on the expression of the mesotocin receptors in any of the song control regions examined. While our results illustrate no long term effects on the population of mesotocin receptors, we cannot eliminate the possibility that there was a transient alteration in the receptor population following treatment. As stated previously the mesotocin receptor is a member of the OT G-coupled protein receptor superfamily and there is evidence of OT receptor internalization following agonist stimulation (Gimpl and Fahrenholz 2001). A treatment-induced effect on OT levels within the CNS of the zebra finch is another hypothesis that cannot be rejected at this time. There is evidence that early-life environmental effects such as handling and maternal seperation can persistently reduce OT neurons within the parvocellular region of the paraventricular (PVN) nuclei of the hypothalamus and this reduction can alter affiliative social behaviors in adult male rats (Todeschin, Winkelmann-Duarte et al. 2009). Therefore future experiments should investigate the possibility that either a change in mesotocin receptors could exist following the conclusion of treatment at day 10 or if the number of mesotocin neurons were modified within the PVN or supraoptic nuclei (SON) of the hypothalamus which might influence the development of song.

Figure 5.1 Song analyses. Individuals were either treated with OT (300 µg/kg [n = 5], 1000 µg/kg [n = 3], or 3000 µg/kg [n = 1]) or vehicle [n = 3] for five days (days 5-9) and then raised by the same adult tutor. Songs were recorded at adulthood and ten song files were randomly selected for analysis. Only one male treated with the high dosage of OT survived to adulthood and therefore he was excluded from analysis. Furthermore it was noted that there was no statistical difference on note stereotypy by OT treated animals due to dosage and thus the animals were pooled into one group (Panel A). Oxytocin treated birds displayed significantly less notes per song bout and decreased linearity, consistency, and stereotypy scores ($p = 0.039$ for notes per song and $p = 0.000$ for comparisons of linearity, consistency, and stereotypy scores as analyzed by two sample t-test). No significant difference was determined for average note duration ($p = 0.511$, two sample t-test) (Panel B). $*p \leq 0.05$.

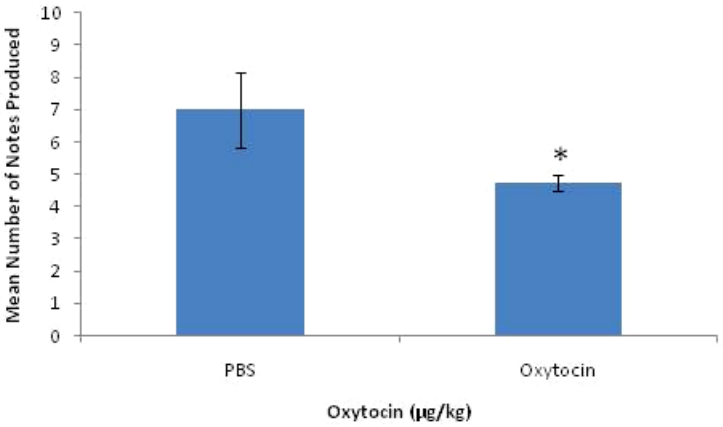
A.

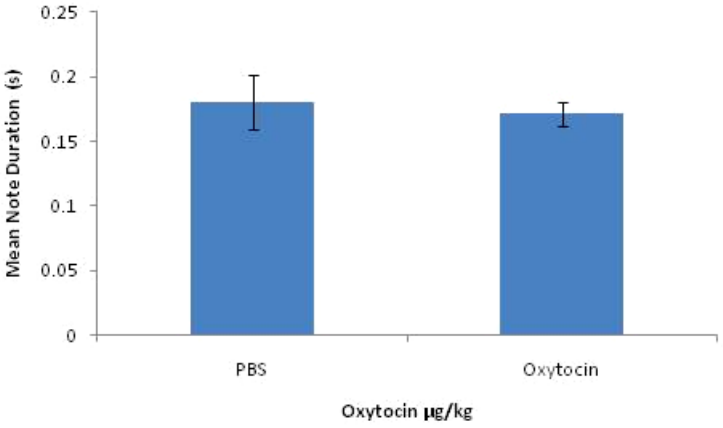

Mean Number of Notes Produced
6
5
4
3
2
1
0
300
1000
Oxytocin (µg/kg)


0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
300
1000
Oxytocin µg/kg


Stereotypy Score
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
300
1000
Oxytocin µg/kg

B.


Mean Number of Notes Produced
10
9
8
7
6
5
4
3
2
1
0
*
PBS
Oxytocin
Oxytocin (µg/kg)


Mean Note Duration (s)
0.25
0.2
0.15
0.1
0.05
0
PBS
Oxytocin
Oxytocin µg/kg

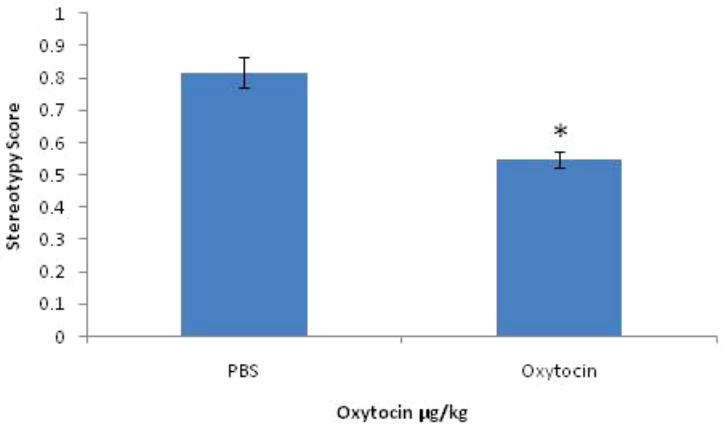

Stereotypy Score
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
PBS
Oxytocin
Oxytocin µg/kg

Figure 5.2 Death rate of birds that were either treated with OT (300 µg/kg, 1000 µg/kg, or 3000 µg/kg) or vehicle for five days (days 5-9).

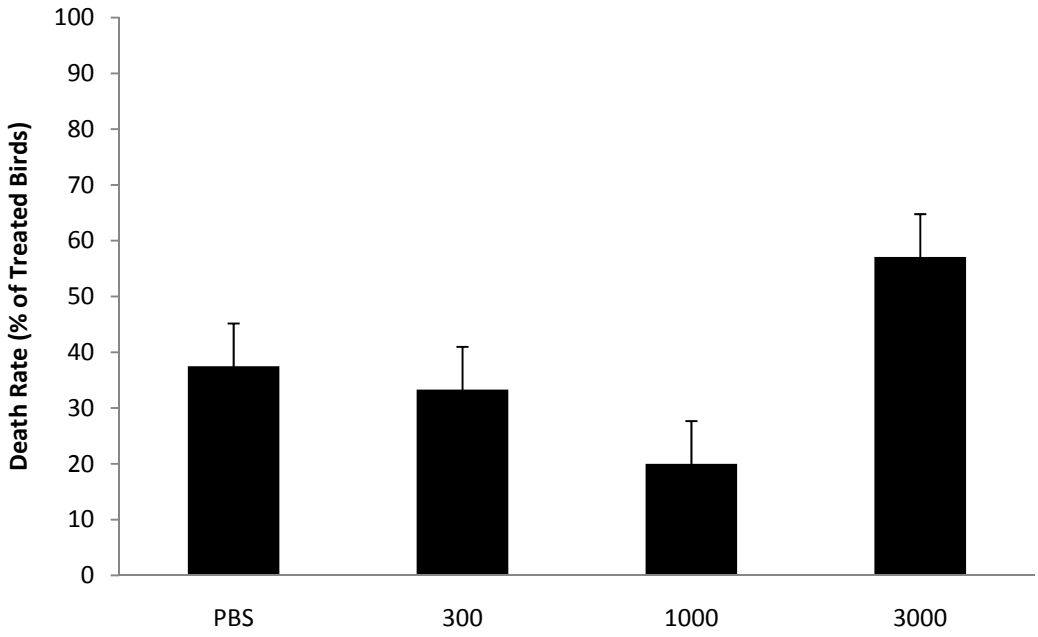

Death Rate (% of Treated Birds)
100
90
80
70
60
50
40
30
20
10
0
PBS
300
1000
3000

Figure 5.3 Treatment paradigm for the behavioral experiment.

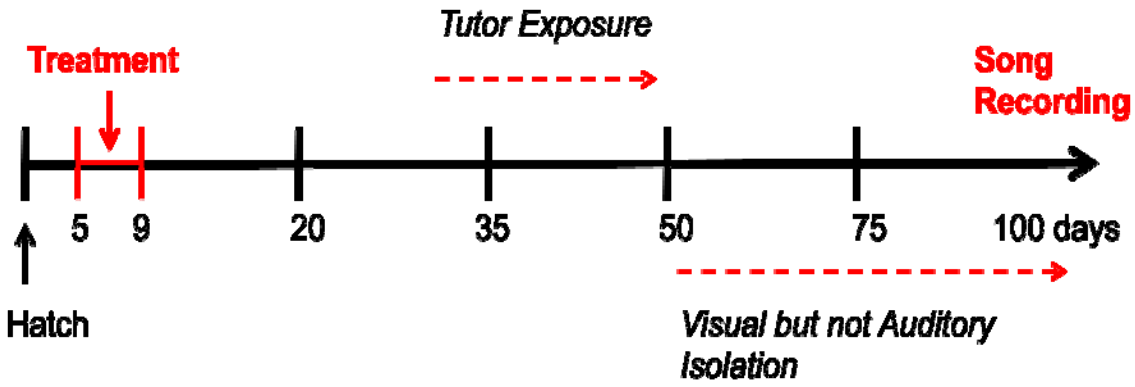

Tutor Exposure
Treatment
Song
Recording
5
9
20
35
50
75
100 days
Hatch
Visual but not Auditory
Isolation

Figure 5.4 Effect of OT treatment on brain weights. Individuals were either treated with OT (30 μg/kg or 300 μg/kg) or vehicle (PBS) for five days (days 5-9) and then allowed to grow till maturity (100 days). Animals (300mcg/kg – n=11, 30mcg/kg – n=5, vehicle – n=7) were then euthanized, their brains perfused with 4% paraformaldehyde, and removed for immunohistochemical analysis. Perfused brains were dissected parasagittally down the midline and weighed. PBS is labeled as 0 on the graph. No significant difference was observed due to treatment with OT (PBS and 30 μg/kg $p = 0.868$, PBS and 300 μg/kg $p = 0.890$, 30 μg/kg and 300 μg/kg $p = 0.987$ Tukey's HSD following ANOVA).

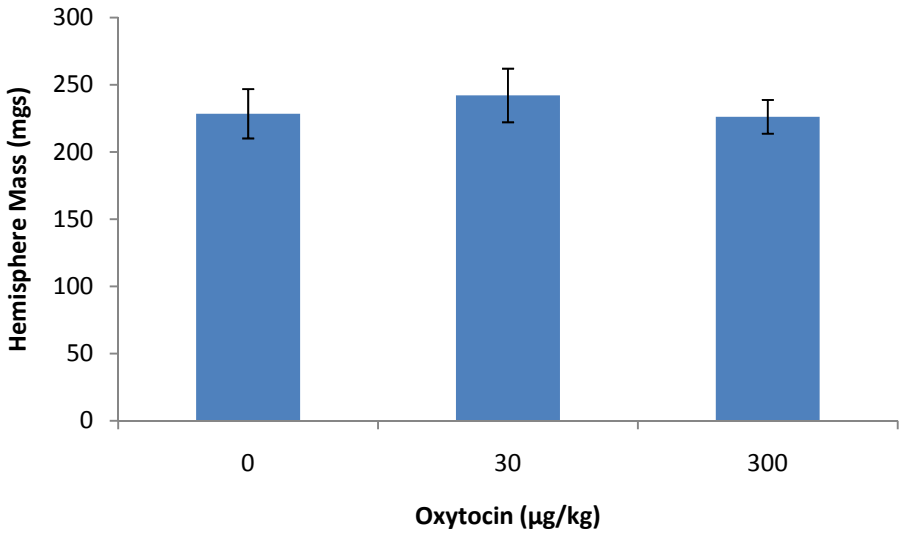
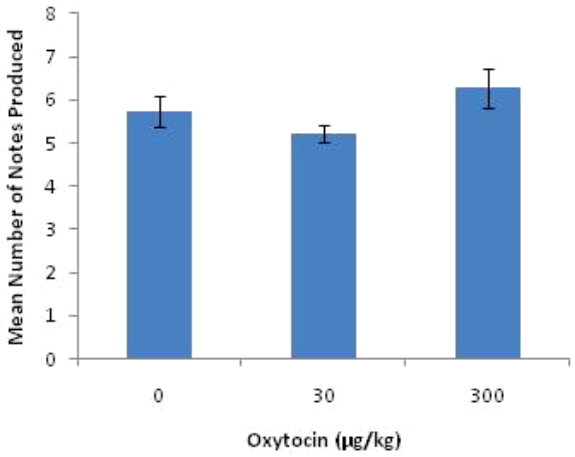
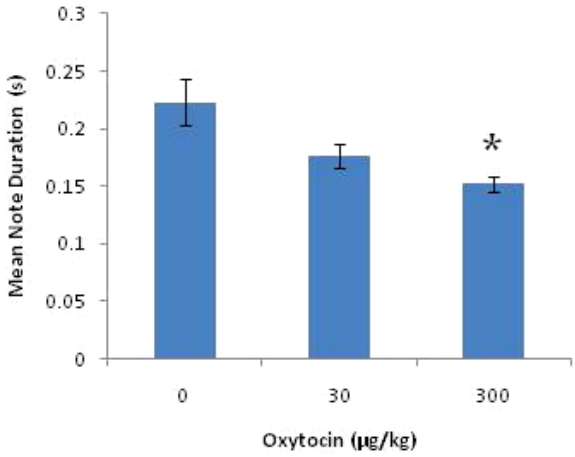
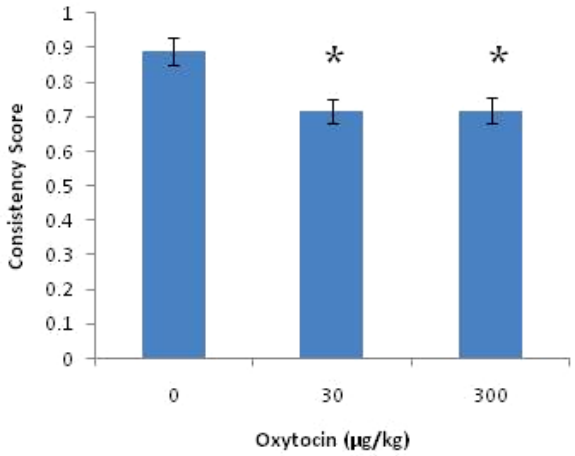
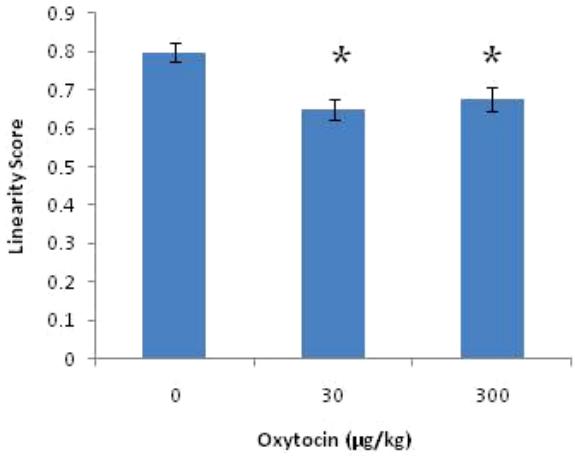

300
250
200
150
100
50
0
Hemisphere Mass (mgs)
0
30
300
Oxytocin (µg/kg)

Figure 5.5 Song analyses from behavioral experiment. Individuals were either treated with OT (30 μg/kg or 300 μg/kg) or vehicle for five days (days 5-9) and then raised by the same adult tutor (300mcg/kg – n=12, 30mcg/kg – n=6, vehicle – n=7). Songs were recorded at adulthood and ten song files were randomly selected for analysis. Oxytocin treated birds displayed significantly shorter average note durations and decreased linearity, consistency, and stereotypy scores (Tukey's HSD following ANOVA). No significant difference was determined for number of notes per song bout (Tukey's HSD following ANOVA). Significant statistical probabilities of $p \leq 0.05$ are designated on the graphs by an *, and PBS is labeled as 0.


Mean Number of Notes Produced
8
7
6
5
4
3
2
1
0
0
30
300
Oxytocin (µg/kg)


Mean Note Duration (s)
0.3
0.25
0.2
0.15
0.1
0.05
0
*
0
30
300
Oxytocin (µg/kg)


Consistency Score
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
0
30
300
Oxytocin (µg/kg)


Linearity Score
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
0
30
300
Oxytocin (µg/kg)

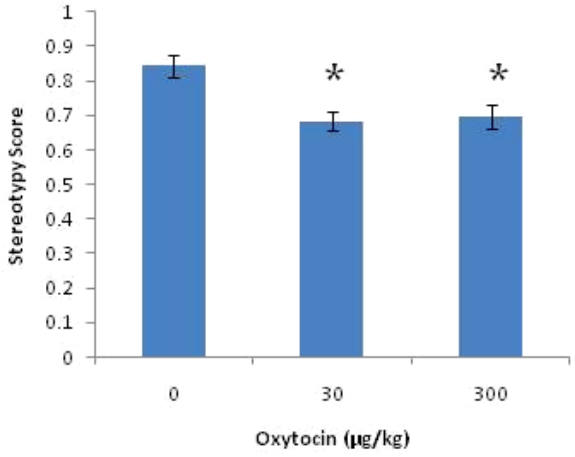
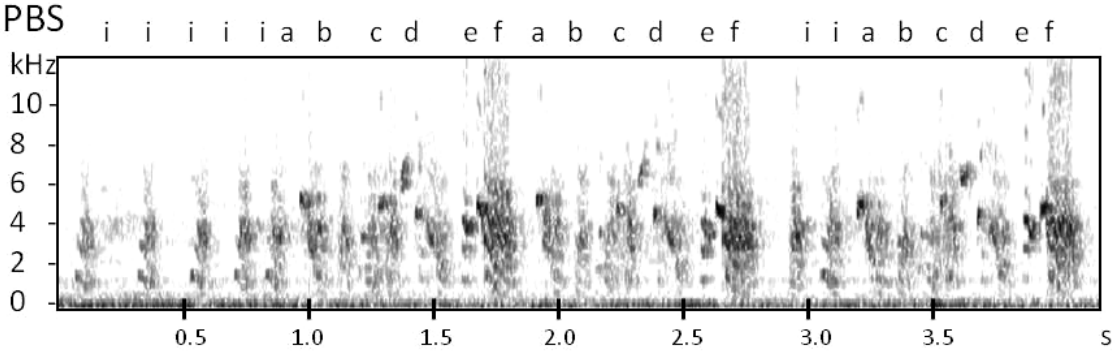
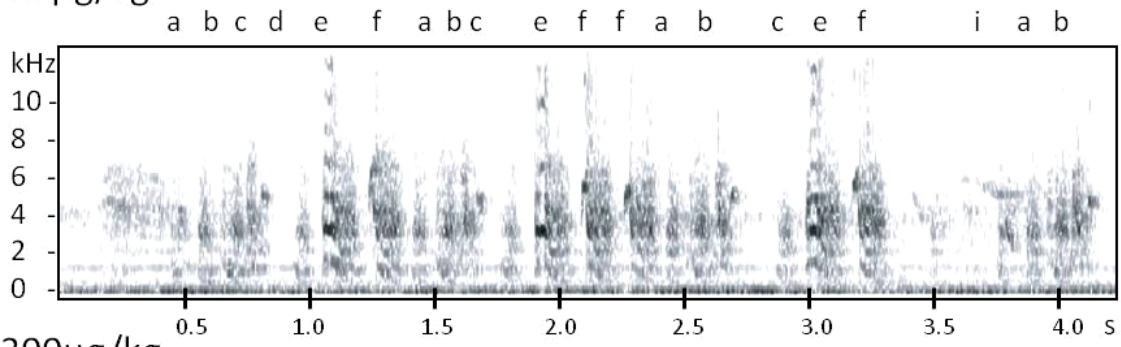

Stereotypy Score
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
0
30
300
Oxytocin (µg/kg)

Figure 5.6 Representative sonograms produced by treated birds. A. Vehicle treated animal G425 B. 30 μg/kg treated animal Y368 and C. 300 μg/kg treated animal G440. Introductory notes are labeled with an "i" and song notes are indicated alphabetically according to the typical order of production in the songs analyzed.

A - PBS



B – 30µg/kg



C - 300µg/kg

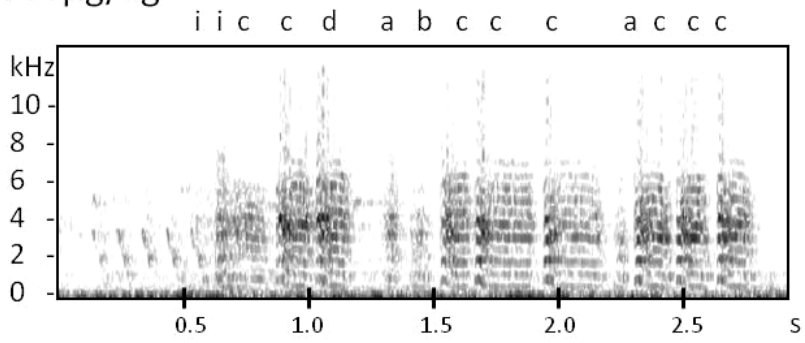
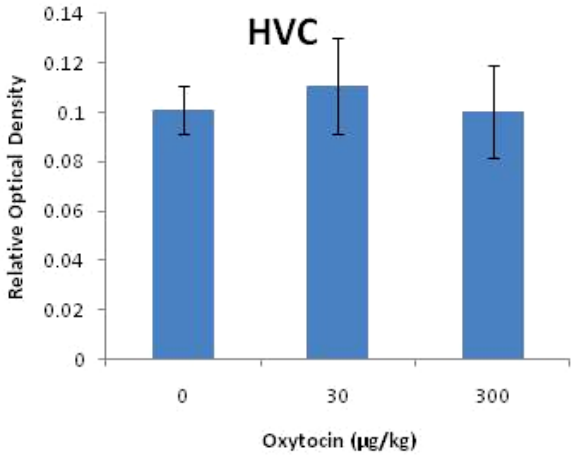
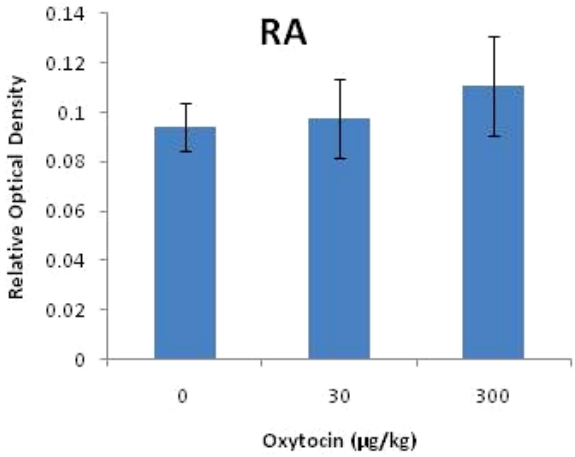
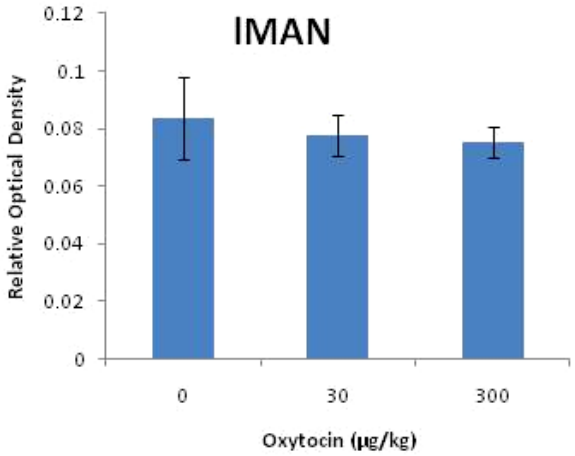


Figure 5.7 Comparison of optical density of mesotocin receptor labeling in telencephalic song regions across treatment groups. No significant difference in OD's was found as a result of treatment (Tukey's HSD following ANOVA). HVC is used as a proper name; RA, robust nucleus of the acropallium; lMAN, lateral magnocellular nucleus of the anterior nidopallium; Area X, Area X of the medial striatum.


HVC
Relative Optical Density
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
30
300
Oxytocin (µg/kg)


RA
Relative Optical Density
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
30
300
Oxytocin (µg/kg)


lMAN
Relative Optical Density
0.12
0.1
0.08
0.06
0.04
0.02
0
0
30
300
Oxytocin (µg/kg)

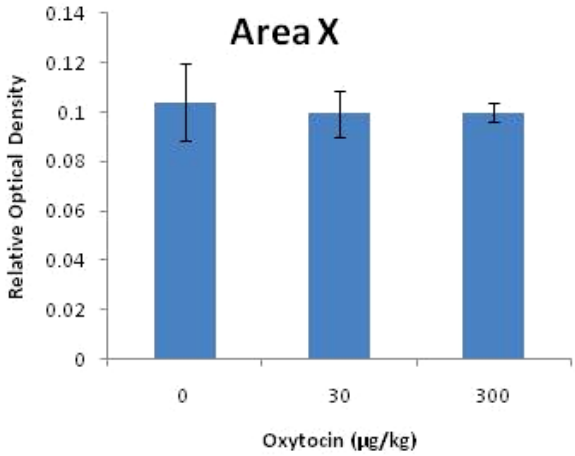

Area X
Relative Optical Density
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
30
300
Oxytocin (µg/kg)

Figure 5.8 Anti-mesotocin receptor antibody staining of song regions of a vehicle treated bird. A, B. HVC. C,D. RA (Robust Nucleus of the Acropallium). E,F. lMAN (Lateral Magnocellular Nucleus of the Anterior Nidopallium). G, H. Area X (Area X of the medial striatum).

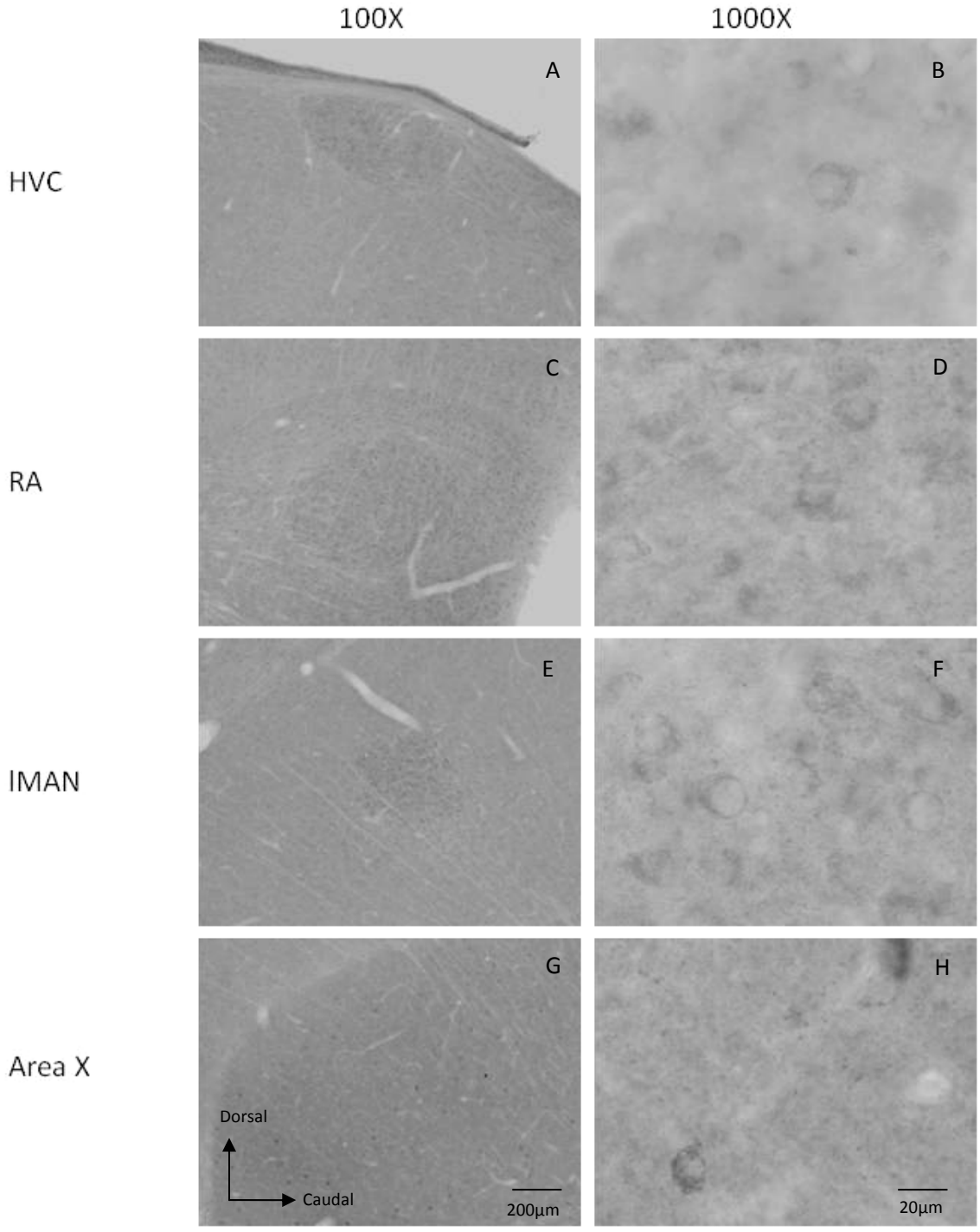

100X
1000X
HVC
RA
IMAN
Area X
A
B
C
D
E
F
G
H
Dorsal
Caudal
200µm
20µm

Figure 5.9 Anti-mesotocin receptor antibody staining of song regions of a bird treated with 30 µg/kg of oxytocin. A, B. HVC. C,D. RA (Robust Nucleus of the Acropallium). E,F. lMAN (Lateral Magnocellular Nucleus of the Anterior Nidopallium). G, H. Area X (Area X of the medial striatum).

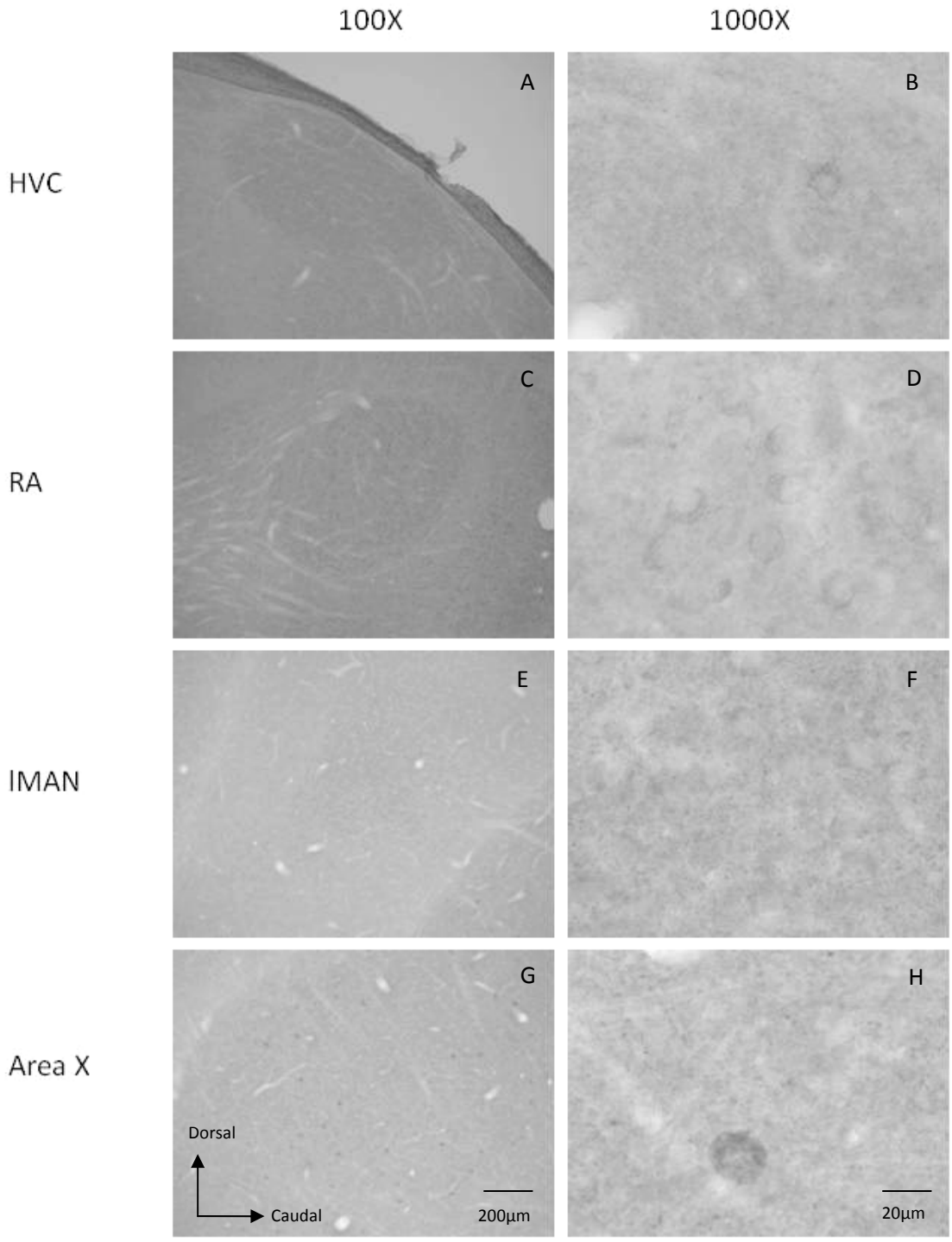
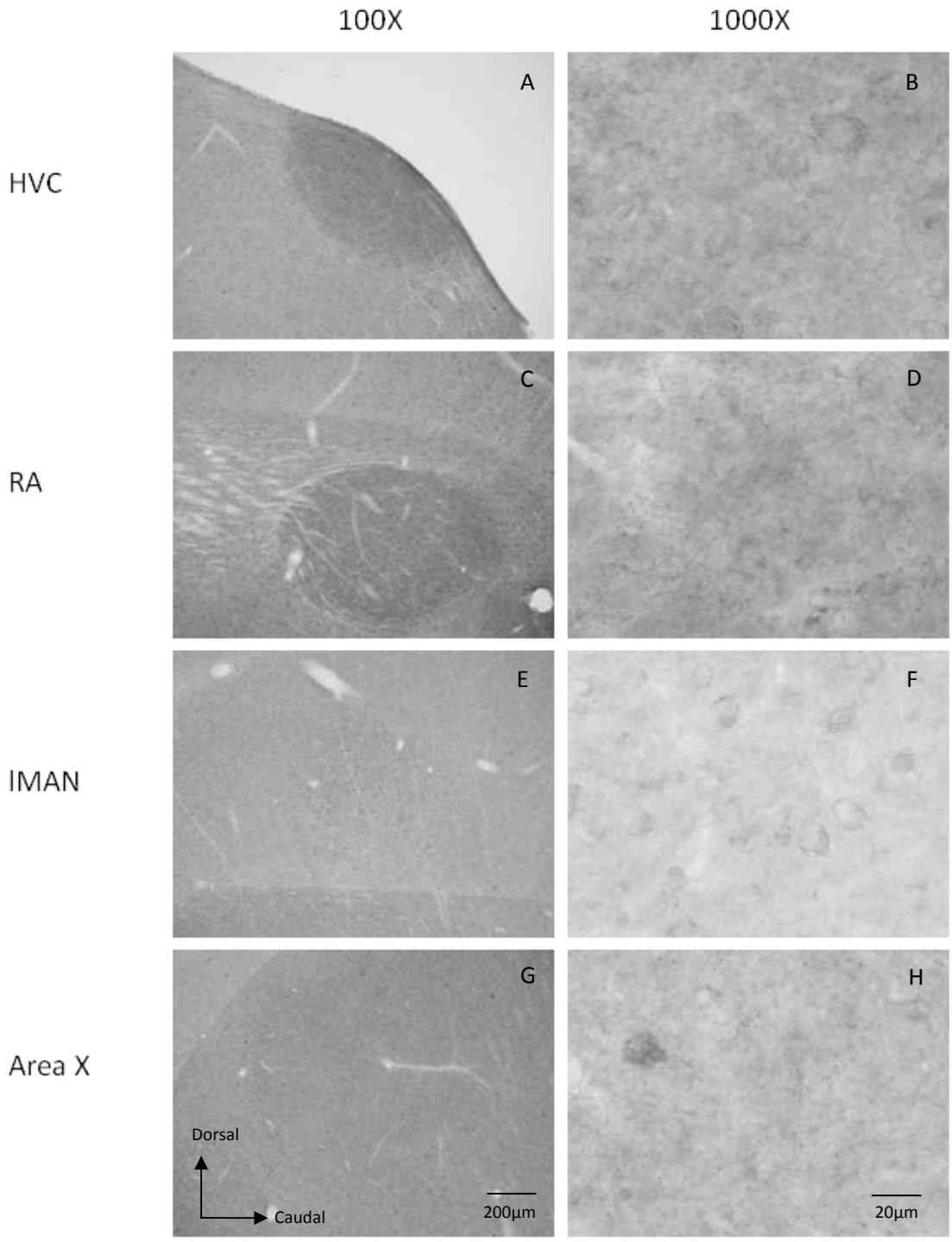

100X
1000X
HVC
RA
IMAN
Area X
A
B
C
D
E
F
G
H
Dorsal
Caudal
200µm
20µm

Figure 5.10 Anti-mesotocin receptor antibody staining of song regions of a bird treated with 300 µg/kg of oxytocin. A, B. HVC. C,D. RA (Robust Nucleus of the Acropallium). E,F. lMAN (Lateral Magnocellular Nucleus of the Anterior Nidopallium). G, H. Area X (Area X of the medial striatum).


100X
1000X
HVC
RA
IMAN
Area X
A
B
C
D
E
F
G
H
Dorsal
Caudal
200µm
20µm

CHAPTER SIX: CONCLUSIONS AND FUTURE DIRECTIONS

For this project the location of mesotocin receptors in male zebra finches within the brain at various ages of vocal development was of interest and it was determined that a zebra finch specific mesotocin antibody needed to be synthesized since commercially available antibodies proved to produce unreliable results. Traditional and in-silico cloning techniques were used to elucidate the putative cDNA sequence of the mesotocin receptor and several other proteins associated with mesotocin signaling such as the mesotocin peptide, neurophysin (carrier molecule of mesotocin, and the zebra finch like oxytocinase (enzyme responsible for the degradation of mesotocin) and this allowed for the development of nucleic acid probe and an antibody to study expression of the zebra finch mesotocin receptor. Mesotocin receptor expression pattern were determined in sections of fixed male zebra finch brain tissue during various stages of vocal development: 10 days, 25- [auditory learning], 50- [subsong], 75- [plastic song], and 100-days of age [crystallized song]. Expression of the mesotocin receptor was detected within areas associated with song production (HVC, RA), song learning (Area X, lMAN), and auditory inputs (L2) at 25, 50, 75, and 100 days of age studied. Each of these regions performs different functions in both song learning and production. For example, lesions of RA and HVC interferes with production of memorized song, while lesions of rostral forebrain song regions (Area X, lMAN) prevent vocal learning but not production of learned song (Nottebohm, Stokes et al. 1976; Bottjer, Miesner et al. 1984; Sohrabji, Nordeen et al. 1990; Scharff and Nottebohm 1991). The peak of mesotocin receptor expression was found to occur at 50 days which is significant because the peak of mesotocin receptor expression correlates to the peak period of song learning and song practice. Song control regions are known to undergo

substantial changes in neuronal number and connectivity and it has been proposed that these changes coincide with vocal learning since volumes of HVC, RA, and Area X increase dramatically in adult males compared to that of female birds between the ages of 12 to 53 days which corresponds to a time period when the male birds are actively learning song (Bottjer, Glaessner et al. 1985; Bottjer and Johnson 1997). In-situ hybridization experiments were also done to assess mesotocin receptor mRNA expression at 25, 50, 75, and 100 days and results indicated significant expression of mesotocin mRNA in the vocal motor song region HVC. As stated previously HVC is a region of the zebra finch brain essential for the production of vocalizations. It projects to RA (another vocal motor nucleus) and ultimately influences motor output to the muscles of the syrinx vocal organ. The finding of an intense pericellular staining within song regions during the sensor-motor states of vocal development (25, 50, 75 day old groups) of the mesotocin receptor, a peak of expression of this receptor during the peak period of vocal learning, and the presence of mesotocin receptor mRNA within the vocal motor song region of HVC all lend credence to a potential role for mesotocin signaling during normal vocal learning within the zebra finch.

Other regions of distinct mesotocin receptor expression include caudal striatum where it was found that receptors that were present in young male birds decreased throughout development until adulthood where they again increased. This is an intriguing finding because both ventral and dorsal striatum have been associated with reward-related learning, and in mammals these areas are known to contain both OT and dopamine receptors (Skuse and Gallagher 2008). The existence of mesotocin receptors within striatal tissue could indicate a possible function for mesotocin signaling in behaviors associated with learning and reward. Published data supports the idea that zebra finches may have a component of reward in their song learning process. The

song learning region of Area X was shown to activate dopaminergic neurons by a projection to the ventral pallidum which in turn projected to other dopaminergic regions that was activated after hearing the birds own song (Gale and Perkel 2010).

Since the exogenous administration of an agonist of cannabinoid receptors which are densely populated throughout zebra finch song control nuclei was determined to be capable of altering zebra finch vocal development and since the exogenous administration of OT was previously determined to be capable of altering the behaviors of species including humans, it was decided to investigate whether the exogenous administration of OT could alter male zebra finch vocal development (Drago, Pederson et al. 1986; Mahalati, Okanoya et al. 1991; Soderstrom and Johnson 2003; Soderstrom and Tian 2004; Bales, van Westerhuyzen et al. 2007; Domes, Heinrichs et al. 2007; Guastella, Mitchell et al. 2008). Nests of zebra finch hatchlings (5 days of age) were injected with varying doses of OT for five days based on a survey of the literature of doses of OT that were used peripherally to produce a behavioral effect (Cushing and Carter 2000; Heinrichs, Meinlschmidt et al. 2004; Petersson, Diaz-Cabiale et al. 2005; Kramer, Choe et al. 2006; Bales, Plotsky et al. 2007; Bales, van Westerhuyzen et al. 2007; de Oliveira, Camboim et al. 2007; Domes, Heinrichs et al. 2007; Hollander, Bartz et al. 2007; Pournajafi-Nazarloo, Papademeteriou et al. 2007). Young male birds were allowed learn a song from an established adult male tutor and to mature to adulthood. Using methods that were previously established in our laboratory their resulting songs were recorded, analyzed, and compared to those of the control treated group (Soderstrom and Johnson 2003; Soderstrom and Tian 2004). It was determined that the overall quality of the song learned (as expressed by song stereotypy scores) and mean note duration were significantly decreased as a function of treatment in both OT dose groups (30 μg/kg, 300 μg/kg) when compared to that of the vehicle treated group. The alteration

in the songs learned by the treated zebra finches could be a result of an effect on memory by an erroneous perception of song or an error in the encoding of song memories. Oxytocin is capable of altering memory formation in other species and thus it may affect song production in this manner in the zebra finch as well (Gimpl and Fahrenholz 2001). The song analysis data showed that the songs produced by the OT treated zebra finches shared many of the same characteristics (altered note timing and note repetition) of songs produced by immature birds (subsongs which are analogous to the vocal babbling stage of human development) when compared to that of the vehicle treated birds. Therefore it is feasible early OT treatment may persistently alter the transition from subsong production to that of the plastic song and eventually to the final adult stereotyped song.

Agonist stimulation may cause a rapid desensitization of the OT receptor as it does with most of the other GPCRs. Gimpl and Fahrenholz (2001), have reported a greater than 60% internalization of OT receptors expressed in HEK 293 fibroblasts within 5 to 10 minutes of exposure to an agonist and it was been observed that the population of internalized OT receptors is not recycled back to the surface of the cell upon agonist withdrawl (Gimpl and Fahrenholz 2001). Evidence exists to document that early environmental changes can produce persistant alterations to the OT receptor system within the central nervous system in both humans and animals. Early-life environmental effects such as handling and maternal separation was found to persistently reduce OT neurons within the parvocellular region of the paraventricular (PVN) nuclei of the hypothalamus of the rat and this reduction was associated altered affiliative social behaviors in adult males (Todeschin, Winkelmann-Duarte et al. 2009). Furthermore, alterations to OT sensitivity within the CNS have been documented in humans when OT was given to men suffering from early-parental-separation experience (Meinlschmidt and Heim 2007). Therefore

we were interested in documenting whether the alteration in vocal learning of zebra finch vocal patterns induced by early OT treatment were associated with a persistent decrease in mesotocin receptors within four song nuclei previously demonstrated to contain distinct populations of mestocin receptors. Male zebra finches treated as previously described on days 5-9 post hatch with either OT (30 μg/kg or 300 μg/kg) or vehicle (1XPBS) were perfused with 4% paraformaldehyde and their brains were subjected to immunohistochemical analysis using our zebra finch specific mesotocin receptor antibody. Four song control regions were examined and of those examined two are integral for the vocal production of song (HVC and RA) and two have been determined to by essential for song learning (Area X and lMAN). Early OT treatment had no significant persistent effect on the expression of the mesotocin receptors in any of the song control regions examined. Even though the results documented no long term effects on the population of mesotocin receptors within these song control nuclei, the possibility that there was a transient alteration in the receptor population following treatment with OT cannot be eliminated. It is possible that a short-term disruption in mesotocin signaling in a developing brain could lead to a long term alteration in behaviors. Such alterations have been observed in the prairie vole where an injection of oxytocin antagonist on the first day of life inhibited adult male alloparental behavior without affecting pair bonding but did so without altering oxytocin receptor densities (Carter, Boone et al. 2009). Another possibility is that the while the density of mesotocin receptors may not have been significantly altered in the adult bird that was treated early in its life, the ability of the receptor to bind to mesotocin may be changed. Experimental evidence has found that human myometrial cells exposed to OT for 20 hours displayed a reduction of the ability of OT to bind to the OT receptor by tenfold (Phaneuf, Asboth et al. 1997). Perhaps either a transient decrease in the mesotocin receptor or an alteration of the ability

of the receptor to bind mesotocin could have contributed to the altered vocal learning or production of learned song we documented in OT treated birds. Finally early OT treatment may have affected adult OT levels within the CNS of the zebra finch in a similar manner to that seen in the adult male rats subjected to maternal separation and a reduction in handling as described previously (Todeschin, Winkelmann-Duarte et al. 2009).

Future Directions

From the determination that distinct populations of mesotocin receptors exist in areas within the zebra finch brain known to be critical for the production of song and for the learning of song and from the results of the behavioral experiments which demonstrated that exogenous OT given to zebra finches early in life was capable of altering either the production of learned vocal patterns, it was concluded that mesotocin signaling may be integral for either the production of vocalizations or in song learning in the male zebra finch. As previously noted an abundance of experimental data exists to links OT signaling to social and repetitive behaviors in animals and humans (Van Erp, Kruk et al. 1993; Hollander, Novotny et al. 2003; Carter, Grippo et al. 2008; Andari, Duhamel et al. 2010; Kemp and Guastella 2010). Autism spectrum disorders are associated with deficits in social behaviors and communication and the presence of repetitive behaviors and emerging data may suggest a link between an alteration in OT signaling and autism spectrum disorders (Association 2000; Rojas Wahl 2004; Carter 2007; Liu, Kawamura et al. 2010). The findings presented here are important discoveries because these results not only show the receptor distribution of a member of the OT super family in CNS areas responsible for aspects of communication but that give evidence that exposure to OT can alter this species' form of communication thus providing additional support to the hypothesis that a perturbation in OT signaling may be responsible for the behavioral deficits seen in ASD patients.

There are many worthwhile experiments to do to expand on the knowledge gained by the work done on this project. First it would be beneficial to demonstrate that OT is indeed a functional ligand for the zebra finch mesotocin receptor by completing experiments designed to give full pharmacological characterization this receptor. This could be accomplished by cloning the entire zebra finch mesotocin receptor cDNA sequence using primers designed from the putative sequence reported here in Figure 3.4 and subcloning this into a mammalian expression vector. The affinity of the mesotocin receptor for OT and related ligands could be determined using equilibrium saturation radioligand binding, and the receptor could be subjected to functional assay such as the [^{35}S]GTPγS assay using varying doses of OT to verify the ability of OT to activate the zebra finch mesotocin receptor.

The behavioral experiment conducted in this project could be further expanded as well. Carter et al demonstrated that a single dose of OT on the first day of life was capable of altering the social behaviors of prairie voles and it would be intriguing to find if we could replicate the alterations in song learning seen in Chapter 5 compared to that of a control injection using only a single dose of varying concentrations of OT (3μg/kg, 30 μg/kg, 300 μg/kg, or 3000 μg/kg) on the first day post hatch (Carter, Boone et al. 2009). It would also be important to establish the effects of an OT receptor antagonist on normal zebra finch vocal development as the experiment conducted by Carter et al. (2009), also found that an OT receptor antagonist was capable of reducing adult male alloparental behavior when given once on the first day of life (Carter, Boone et al. 2009). Therefore it would be valuable to include this treatment group in the behavioral experiment described above.

Mesotocin has also been shown to be capable of altering the behavior of female zebra finches given that the central infusion of mesotocin significantly increased the amount of time spent in a

social group of female zebra finches while the peripheral administration of an OT antagonist (OTA - desGly–NH2,d(CH2)5[Tyr(Me)2, Thr4]OVT) significantly reduced the time spent with large groups and with familiar birds (Goodson, Schrock et al. 2009). Therefore it would be important to determine if early exposure to a peripheral dose of exogenous OT was capable of altering the social behavior of the female zebra finch by monitoring quantifiable social behaviors such as allopreening and clumping. Allopreening is defined as grooming of another bird's feathers while clumping is characterized as sitting in close (touching) proximity on a perch with another animal (Zann 1996). Treated female hatchlings would be group housed together, identified regarding treatment using colored leg bands, and then digitally recorded for two hours per day during peak light hours for one week at adulthood. Recordings could then be analyzed for number of allopreening incidents and time spent allopreening as well as number and duration of clumping incidents. Results would allow for a direct comparison on the effects of exogenous OT on both communication (male zebra finch song analysis) and on social behaviors (female zebra finch allopreening and clumping). Positive results would provide further evidence of a possible link between exogenous OT exposure and alterations in behaviors similar to those seen in autism spectrum disorders.

Finally since no persistent alteration in the density of mesotocin receptor was found as a consequence of OT treatment, more work is needed to determine if the alterations in vocal learning seen following OT treatment is a function of a transient perturbation of mestocin receptor densities, mRNA quantities, or both. Immunohistochemical analysis using our zebra finch specific mestocin receptor antibody of the brains of zebra finches immediately following OT treatment would illuminate whether a transient decrease in the mesotocin receptor density could exist. At ten days of age we have documented distinct populations of the mesotocin

receptor at the vocal motor control region of HVC, the auditory field of L2, the song learning nuclei of Area X, and throughout caudal striatum. Each of the regions could be subjected to optical density analysis and results could provide vital details on the early consequences within the CNS of peripheral OT exposure. Oxytocin receptor mRNA has also been found to be sensitive to exogenous OT as human myometrial cells exposed to OT for 20 hours displayed a reduction OT receptor mRNA without altering the total number of OT receptors expressed at the cell surface which the authors of the study hypothesized may be due to either transcriptional suppression or destabilization of OT receptor mRNA (Phaneuf, Asboth et al. 1997). Therefore it may be wise to investigate if the treatment of the zebra finches with exogenous OT could produce a reduction of OT receptor mRNA by using *in-situ* hybridization on treated birds immediately following the cessation of OT treatment. As discussed previously there is evidence that early-life environmental events can persistently reduce OT neurons within the parvocellular region of the paraventricular (PVN) nuclei of the hypothalamus in rats (Todeschin, Winkelmann-Duarte et al. 2009). Therefore, it would be advantageous to investigate the possibility that the number of mesotocin neurons were modified within the PVN or supraoptic nuclei (SON) of the hypothalamus as a result of treatment with OT.

The data presented here from these studies provide support for a role for the OT-related peptide mesotocin in the vocal development of the zebra finch. We have shown that mesotocin may be an important signaling molecule to in the processes of song learning, the auditory perception of song, or the vocal motor production of song because its receptors are located in song control regions previously demonstrated to be important for these processes. Furthermore we have clearly demonstrated that young zebra finch hatchling is sensitive to peripherally administered OT since OT was found to alter the production of the final adult song. This alteration could be

the result of a dysfunction in one or all of the various processes of song learning but that it is not a consequence of long term down-regulation of the mesotocin receptor in song control regions. Results of these studies illuminate a potential relationship between exogenous OT exposure and altered vocal development which is a deficit associated with autism spectrum disorders.

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APPENDIX

Animal Care and Use Committee
East Carolina University
212 Ed Warren Life Sciences Building
Greenville, NC 27834
252-744-2436 office • 252-744-2355 fax

September 10, 2007

Ken Soderstrom, Ph.D.
Department of Pharmcology
Brody 7S-10
ECU Brody School of Medicine

Dear Dr. Soderstrom:

Your Animal Use Protocol entitled, "Effect of Oxytocin Administration on Male Zebra Finch Vocal Development," (AUP #W211) was reviewed by this institution's Animal Care and Use Committee on 09/10/07. The following action was taken by the Committee:

"Approved as submitted"

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure