ABSTRACT

MCCAFFREY, KATHERINE ANNE. Exploring the Distribution and Potential Roles of the Developmental Transcription Factor *Zic2* in an Adult Protogynous Teleost, *Thalassoma bifasciatum.* (Under the direction of Dr. John Godwin.)

The bluehead wrasse (*Thalassoma bifasciatum*) is a valuable model for studying neuroendocrine processes because it displays two discrete male phenotypes, initial phase (IP) males and territorial, terminal phase (TP) males, and undergoes socially-controlled protogynous sex change. Previously generated microarray-based comparisons suggested that *zic2* was upregulated in the brains of terminal phase males relative to initial phase males. The highly conserved members of the *zic* family of zinc-finger transcription factors play critical roles in regulating cellular proliferation and differentiation, but their roles as transcription factors in adulthood are poorly understood. Studies aimed at understanding the conserved roles of *zic* transcription factors during vertebrate brain development have targeted *zic2* as one of the few genes that is independent of the hedgehog pathway and linked to the congenital malformation of the forebrain termed holoprosencephaly (HPE). In addition, *zic2* mRNA is found in a wide range of human cancers, and several researchers have found that *zic* family members may serve as a potential marker for abnormal cell growth. This study describes the localization and expression patterns across sexual phenotypes of neural *zic2* in an adult teleost and offers insight into the potential role of *zic2* as a regulatory factor in the sex-change signaling pathway.

We cloned a 727bp sequence for neural *zic2* from field-collected bluehead wrasses. *In situ* hybridization with [α-^{35}S]CTP-labeled riboprobes was utilized to localize and assess the relative abundance of brain *zic2* mRNA across sexual phenotypes. We found that *zic2* mRNA expression in the adult bluehead wrasse brain was not only extremely abundant in the granular cells of the cerebellum, but also widespread in other brain areas including in the thalamus, hypothalamus, habenula, torus semicircularis, torus longitudinalis, medial longitudinal fascicle and telencephalic areas. Quantitative autoradiography and phosphor screens showed *zic2* mRNA hybridization signal density in the preoptic area of the hypothalamus was significantly higher in terminal phase males relative to both initial phase males and females, and silver grain analysis confirmed this relationship between phenotypes. No significant difference in abundance was found in *zic2* signal across phenotypes in the habenula or cerebellum. As *zic2* has been shown to regulate tissue specific expression of the dopamine receptor D1 (DRD1) and function as a transcriptional repressor, our study illustrates *zic2* expression could potentially be acting on dopaminergic targets in the preoptic area of the hypothalamus, an area associated with sexually-motivated behavior and behavioral sex change.

Exploring the Distribution and Potential Roles of the Developmental Transcription Factor *Zic2* in an Adult Protogynous Teleost, *Thalassoma bifasciatum*

by
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DEDICATION

To my mother and grandfather.

BIOGRAPHY

Katherine “Kelly” McCaffrey was born in Lake Forest, Illinois in 1981 and raised in Scottsdale, Arizona. In 2004, she received her B.S. in zoology and minor in chemistry from Northern Arizona University in Flagstaff, Arizona. In 2005, she attended a semester of veterinary medical school but left the program with her decision to pursue graduate studies and better incorporate her broad research interests in biology alongside her background in physiology. In 2006, she came to North Carolina State University to pursue a master’s degree in zoology. Under the mentorship of Dr. John Godwin, she studied the behavioral biology and neuroendocrinology of the bluehead wrasse. Upon completion of her degree, she plans to continue at NCSU as a research technician.

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I would like to especially thank my mother for her wisdom and words of encouragement over the years, as well as the rest of my family for their support. Finally, I would like to acknowledge Benoit Guénard, who has not only offered daily feedback as a scientific colleague but also encouraged me during the most challenging of times with his optimism and thoughtfulness. I am indebted to his patience and am thankful our paths have joined.

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CHAPTER I

Mapping the molecular signaling systems underlying both sexual expression and behavioral adaptation is fundamental to our understanding of neural development, mental health, and disease pathways. The major brain areas and neuroendocrine factors in teleosts are generally conserved to those in mammals (Bolis, 2001), and teleost models have made strong contributions in numerous biological research fields, including neuropharmacology, endocrine disruptor and toxicology studies, and carcinogenesis. Many neuropeptides, hormones, and genes found in teleosts are homologous to those present in tetrapods. Teleost models offer several useful aspects to the study of neuroendocrinological function, including behavioral sex change and multiple male phenotypes. To gain insight into the neural correlates associated with behavioral, social, and gonadal changes, this project investigates the neural mechanisms associated with the behavioral and sex-changing cues in the bluehead wrasse, *Thalassoma bifasciatum* (Bloch, 1791), a protogynous Caribbean reef fish. The bluehead wrasse is a well-characterized model for studying neuroendocrine processes associated with sex and role change. Bluehead wrasses display two discrete male phenotypes, "female-mimic" initial phase (IP) males and territorial, terminal phase (TP) males, and undergo socially-induced female-to-male sex change (Warner and Swearer, 1991; Bass and Grober, 2002; Godwin et *al*., 1996). Removal of TP males induces behavioral and gonadal masculinization in the largest females in social groups. Under socially permissive conditions, initial phase males may also undergo behavioral and coloration change to TP male status. Interestingly, behavioral phenotype expression has been shown to be

independent of gonadal influences in this species (Godwin et *al*., 1996). The mechanisms by which behavioral cues are transduced into the neuroendocrine processes underlying these phenomena remain largely to be discovered.

Zic family transcription factors have multiple roles in early neural patterning (Nagai et *al.*, 2000; Inoue et *al.*, 2008) and cellular proliferation (Merzdorf, 2007). Zic family members show strong sequence conservation across vertebrates, particularly in their zinc finger domains and regions immediately surrounding these domains, as well as in early neural developmental function (Merzdorf, 2007). The expression of z*ic2* has been linked to the developmental malformation holoprosencephaly (HPE) (Benedyk et *al*., 1994; Merzdorf, 2007) as well as consistently targeted as an indicator of tumorigenesis (Yokota et *al.*, 1996; Gure et *al*., 2000; Chen et *al.,* 2004; Grinberg and Millen, 2005; Bidus et *al*., 2006). A number of proteins are known to be regulated by *zic* transcription factors (Aruga, 2004, Merzdorf, 2007). However, to date, only a few direct downstream targets of *zic2* have been identified and much remains to be learned regarding the molecular mechanisms and signaling pathways through which *zic* genes exert their function and their role into adulthood. The distribution and abundance of neural *zic2* mRNA is undocumented in the adult teleost. Few works localizing neural *zic2* in adult animals have reported *zic2* expression as widespread, and some studies have restricted adult *zic2* expression as a marker uniquely associated with cerebellar granule neurons (Nagai et *al*., 1997; Salero and Hatten, 2007). However, *zic2* was linked to the direct downstream regulation of D1 dopaminergic receptors (Yang et *al.*, 2000) and, recently, Brown and Brown (2009) found *zic2* signal overlapping with known markers

of adult neurogenesis, including the olfactory bulb, rostral migratory stream, and subventricular zone.

The dopaminergic system has been consistently implicated in the regulation of sexual behavior, and studies have looked into the involvement of related monoamines in teleost sex-change (Larson et *al.*, 2003a; Larson et *al*., 2003b). Additional works have mapped sexually dimorphic neuropeptides and monoamines linked to sexual behavior, including arginine vasotocin (AVT) (Propper et *al.*, 1992; Jurkevich et *al*., 1995; Godwin et *al*., 2000), tyrosine hydroxylase (TH) (Meek et *al*., 1989; Ball et *al*., 1995; Rink and Wullimann, 2001; Marsh et *al*., 2006), and the estrogen biosynthesis enzyme aromatase (Marsh et *al.*, 2006; Baillen and Balthazart, 2007), in the hypothalmus and looked into the co-localization, regulation, and roles in behavioral signaling pathways. The bluehead wrasse model allows the study of behavioral roles, neuroanatomical localization, and expression variation across sexual phenotypes, therefore permitting potential functional investigation into *zic2*, a transcription factor that is well-studied in developmental biology but whose roles in adulthood are poorly understood.

In Chapter II, we describe the localization of *zic2* neural mRNA in the brain of the bluehead wrasse using riboprobe-based *in situ* hybridization techniques. Maps included in this chapter diagram the neuroanatomical distribution of *zic2* gene expression, demonstrate that *zic2* mRNA localization is not restricted to the cerebellum, and suggest *zic2* may tentially be linked to the abundant neurogenesis and neural flexibility found in adult teleosts.

In Chapter III, we examine *zic2* mRNA abundance in the preoptic area of the

hypothalamus, a critical integrative region well-studied for involvement in sexual behavior and function. The preoptic area shows important variation in neurochemical signaling systems across sexual phenotypes in the bluehead wrasse. Differential expression of *zic2* in the preoptic area across sexual phenotypes, along with information from the literature regarding this area, suggests co-localization with critical hormones, neuropeptides, and other neurochemicals involved in the sex-change signaling pathway.

CHAPTER II

DISTRIBUTION OF THE DEVELOPMENTAL TRANSCRIPTION FACTOR, *ZIC2*, IN THE ADULT BLUEHEAD WRASSE BRAIN, *THALASSOMA BIFASCIATUM*

Abstract

Members of the *zic* family of zinc-finger transcription factors have important actions during neural patterning and neural crest development in regulating cellular proliferation and differentiation, but their role as transcription factors in adulthood is poorly understood. Few published works have reported *zic2* distribution in the mature brain and, to our knowledge, the distribution of neural *zic2* mRNA is undocumented in adult non-mammalian vertebrates. This study describes the distribution of neural *zic2* in an adult protogynous reef fish, the bluehead wrasse (*Thalassoma bifasciatum*). The bluehead wrasse is a well-characterized model for studying the neuroendocrine processes underlying behavioral and gonadal sex-change and exhibits three sexual phenotypes: terminal phase males, initial phase males, and females. We cloned a 727bp partial *zic2* cDNA from field-collected bluehead wrasses to generate a cDNA template for a [α-^{35}S]CTP-labeled riboprobe *in situ* hybridization. The expression of *zic2* mRNA in the adult bluehead wrasse brain is not only abundant in the granule cells of the cerebellum, as routinely described in the mouse, rat, and human, but is also present in the thalamus, hypothalamus, habenula, torus semicircularis, torus longitudinalis, medial longitudinal fascicle and telencephalic areas. Quantitative autoradiographic analysis of *zic2* in the cerebellum across bluehead phenotypes showed no significant differences in abundance. As previous microarray data generated from our lab indicated a differential expression of *zic2* across phenotypes, we propose that variation in *zic2* signal abundance is localized outside of the cerebellum in the adult bluehead wrasse. We also suggest that the widespread expression of *zic2* in the forebrain may potentially be linked to the abundant neurogenesis and neural flexibility found in teleosts.

Introduction

Members of the *zic* family of zinc-finger transcription factors act during neural patterning and neural crest development and play critical roles in regulating cellular proliferation and differentiation. However, their roles as transcription factors in adulthood are poorly understood. This study describes the distribution of neural *zic2* mRNA transcripts in adults of a protogynous reef fish, the bluehead wrasse (*Thalassoma bifasciatum*), and offers insight into *zic2's* potential role as a regulatory factor in the signaling pathway mediating sex change. The social regulation of sex change in the bluehead wrasse requires rapid activation of neuronal circuitry underlying reproductive behavior and function, thereby providing an exceptional opportunity for study. The highly conserved nature of the *zic* gene family makes it an excellent model for studying control of cell proliferation and differentiation across vertebrate and invertebrate groups. *Zic* family members each encode zinc-finger transcription factors composed of five Cys_2His_2 zinc-finger domains (Grinberg and Millen, 2005). The *Drosophila* pair-rule gene *odd-paired (opa)* is the homolog to the vertebrate *zic* gene family and not only shares a highly similar zinc finger motif, but also identical exon-intron boundary sequences to mouse *zic* genes, providing evidence that the genes originate from a common ancestral gene (Benedyk et *al.*, 1994; Aruga et *al*., 1996).

Numerous studies of *zic* transcription factors during vertebrate brain embryogenesis have found them critical for proper neurulation and morphology. Furthermore, these studies have begun to uncover the molecular and developmental mechanisms underlying *zic* mutation-linked birth defects. A wide variety of human birth defects have been associated with heterozygous mutations in *zic* gene family members. In particular, *zic2* has been

targeted as one of the few genes, independent of the hedgehog pathway, linked to a severe and prevalent congenital malformation of the forebrain termed holoprosencephaly (HPE) (Benedyk et *al.,* 1994; Merzdorf, 2007). Consistent with this association, *zic* family members, specifically *zic1*, *zic2*, and *zic3,* play essential roles in medial forebrain development (Nagai et *al.*, 2000; Inoue et *al*., 2008). Both *zic1* and *zic2* are strongly expressed in the developing telencephalon and mesencephalon, specifically the medial septal nucleus, the thalamic nuclei, and preoptic nucleus (Aruga et *al*., 2002). The regulation of *zic2* is crucial for the timing of neurulation (Nagai et *al*., 2000) and was identified as a candidate gene for HPE based on brain malformations related to specific chromosome deletions in human 13q32 (Brown et *al*., 1993;1998). The *zic2* mutation and brain malformations demonstrated in mice have been shown to be analogous to the abnormality caused by human *zic2* mutations (Brown et *al*., 1998; Nagai et *al*., 2000). Mice homozygous for the *zic2* knockdown mutation, $zic2^{kd/kd}$, are reported to show HPE (Nagai et *al.*, 2000), and in humans and mice, *zic2* has been shown essential to the formation of midline CNS structures (Nagai et *al.*, 2000). Diminished expression of *zic2* results in neural tube defects affecting both posterior neuropore closure, resulting in spina bifida, and anterior neuropore closure, resulting in exencephaly and anencephaly (Nagai et *al*., 2000).

Although it is well-known that interactions between embryonic signaling pathways ensure proper development, increasing evidence suggests that these pathways remain active in specific cells within adult organs. Deregulation of cellular activity within such regions contributes to tumor formation and its progression (Pasca di Magliano and Hebrok, 2003). Examining healthy tissue, *zic2* mRNA has been detectable in the brain and testis, but

minimal or no expression has been noted in normal skin, kidney, small intestine, pancreas, uterus, or lung (Gure et *al*., 2000). However, among tumorous tissues, *zic2* mRNA expression is found in 50% or more of a wide range of human cancers, including colon cancer, breast cancer, head and neck cancer, lung cancer, bladder cancer, melanoma, leiomyosarcoma, and synovial sarcoma (Gure et *al*., 2000). Yokota and collaborators (1996) found that *zic* may serve as a potential marker for medulloblastomas, and Gure and collaborators (2000) and Bataller and collaborators (2002, 2004) found that in most cases of abnormal cell growth, antibodies to *zic* proteins were present before a tumor was diagnosed. Such work has lead researchers to suggest that antibodies to *zic* proteins can be utilized to predict the development of certain neoplasms.

Activation of *Wnt* (Polakis, 2000; Giles et *al*., 2003; Bafico et *al*., 2004) and hedgehog (Hh) signaling pathways is commonly observed in malignant tumor onset and development. The zinc-finger domain and DNA binding specificity of the *zic* family shows significant homology to those of the *Gli* family, a family of transcription factors key to the vertebrate hedgehog pathway (Mizugishi et *al*., 2001). As shown by Mizugishi and collaborators (2001), *zic1*, *zic2*, and *zic3* were able to bind *Gli* recognition sites and activate a variety of promoters, suggesting a potential role for *zic* as a transcriptional co-activator involved in the gene expression process. Evidence in mouse models for a *zic2-Wnt* activation of pancreatic cancer indicated that *Wnt* signaling might be activated independently from hedgehog signaling (Pasca di Magliano et *al*., 2007) while work in zebrafish noted *zic2a* acted in a pathway temporally distinct from hedgehog signaling during forebrain development (Sanek and Grinblat, 2008). Other experiments directed at understanding the

conserved nature of *zic2* and its transcriptional targets have shown that both *zic2* and *zic5* mediate the proliferative function of canonical *Wnt*-signaling (Grinblat et *al*., 2008). This large body of work suggests *zic* genes play an evolutionarily conserved role in regulatory signaling that not only coordinates normal cell growth and differentiation, but also disease pathways and tumorigenesis (Logan and Nusse, 2004).

As many *zic* studies have been purely developmental, few published works have examined *zic2* distribution in the mature brain. No studies have examined *zic2* expression in the adult teleost to the authors' knowledge. In the mature mammalian brain, *zic2* is strongly expressed in cerebellar granule cells in the hindbrain, as described in mouse (Aruga et *al*., 1994; 1996; 2002; Nagai et *al*., 1997; Salero and Hatten, 2007), rat (Williams et *al*., 2008), and human (Yokota et *al.*,1996). Published studies have described adult brain expression of *zic2* as restricted to the cerebellum and functioning as a marker for granule cell neurons and their precursors (e.g., Salero and Hatten, 2007). However, there have been notable exceptions. In addition to cerebellar granule cell labeling, Aruga and collaborators (1994) detected weak *zic* mRNA signaling in the medial habenula nucleus, olfactory bulb, thalamus, and pontine nucleus in the adult mouse brain. Similarly, Brown and Brown (2000) noted mouse *zic2* expression in the olfactory bulb, the rostral migratory stream (RMS), and the sub-ventricular zone (SVZ) in addition to hindbrain labeling. Brown and Brown (2009) additionally described *zic2* expression as overlapping with known markers of adult neural stem cells and neurogenesis, suggestive of a pluripotential role. The Allen Brain Atlas, a database of mouse brain gene expression maps, shows *zic2* highly expressed in the cerebellum, and elsewhere within the adult brain to a lesser degree.

In this study, we utilize *in situ* hybridization with field-collected animals to generate a *zic2* mRNA brain expression map for the bluehead wrasse. The bluehead wrasse offers a natural model for studying neuroendocrine processes underlying behavioral and gonadal sex change. This species exhibits three sexual phenotypes: large, aggressive terminal phase (TP) males and the smaller initial phase (IP) males and females. Most TP males maintain reproductive territories during a daily spawning period where they court and spawn with females. By contrast, IP males often act as female-mimics and spawn using either 'sneaking' tactics or as part of large groups of IP males (termed group spawns; Warner, 1984; Semsar et *al.,* 2001; Semsar and Godwin, 2004). Our second goal is to quantify *zic2* signaling in the cerebellum across sexual phenotypes. Previous microarray data from our lab indicated the upregulation of *zic2* in TP males relative to IP males (Passador-Gurgel, unpublished data) and potentially suggests this gene may be important in distinct phenotypes or the sex change pathway. Our third aim is to determine if the observed difference in *zic2* regulation could potentially be localized to regions outside of the granule layer of the cerebellum, a region already known for intense *zic2* signaling in adult rodents and primates.

Materials and Methods

Study site and species: Adult bluehead wrasses (*Thalassoma bifascidum*) of the three sexual phenotypes were collected from patch reefs under permit in waters off the Florida Keys National Marine Sanctuary near Key Largo, Florida (25°13'W, 80°14'W) during July 2008. Females, IP males, and TP males were captured using a lift net as described previously (Warner and Swearer, 1991; Godwin et *al.,* 1996). Captures occurred between 0910h-1250h

over two consecutive days. Observations before capture ensured sampling was performed during the daily spawning period. Fish were sacrificed immediately after capture (within three minutes) on a small boat using an overdose of MS-222 (tricaine methanesulfonate, Sigma, St. Louis, MO). Before harvesting of tissue, the length of all fishes was measured (standard length; mean: F=69.0mm (62.3-75.8mm), IP=68.9mm (61.4-82.9mm), TP=88.9mm (84.2-94.0mm) and sex confirmed by visualization of genital papillae, gonadal dissection, and/or expression of sperm or eggs following manual pressure on the abdomen. Within approximately three minutes post-sacrifice, brain dissection was complete and tissues were snap frozen on dry ice. Brains remained frozen on dry ice during shipment to the laboratory at NCSU where they remained at -80°C until further processing. Brains were embedded in OCT compound (Tissutek, Durham, NC), cryosectioned coronally at 20µm, and transferred to Superfrost slides (Fisher Scientific, St. Louis, MO). To allow comparison of successive sections receiving different antisense and control treatments, consecutive sections were mounted on six alternating slides. All slides were stored at -80°C until used for *in situ* hybridization. All experimental methods described are in compliance with the guidelines of and were approved by the Institutional Animal Care and Use Committee of North Carolina State University (NCSU).

Preparation of riboprobe template DNA: Previous work in our lab generated bluehead wrasse cDNA plasmid stocks incorporating partial clones from the brain tissue of field-collected fish (pBluescript vector; Stratagene, La Jolla, CA; Passador-Gurgel et *al.,* unpublished). *Zic2*

cDNA was amplified with Taq Polymerase (Promega, Madison, WI) and M13 forward and reverse primers. PCR product was run on a 1% agarose gel to verify plasmid size, purified with a QIAquick PCR Purification Kit (Quiagen, Valencia, CA) via microcentrifuge, and sent to the University of Chicago Cancer Research Center (CRC) DNA Sequencing Facility (Chicago, IL) for sequencing. Sequencing results from CRC facilities allowed GenBank BLAST (NCBI) verification of a 727 bp partial *zic2* sequence and confirmed the homology of this sequence to mammalian *zic2*. This purified PCR product was transformed with JM109 competent cells (Sigma, St. Louis, MO) and plated onto LB agar plates containing ampicillin. Colonies were blue/white screened with X-Gal (Promega, Madison, WI) for insert incorporation, and selected colonies were grown in LB broth. Culture tube growth was purified via a QIAprep Spin Miniprep Kit (Quiagen, Valencia, CA), digested with ECOR1 (Promega, Madison, WI) and HINDIII (Promega, Madison, WI), and run on a gel with a 50bp ladder (Promega, Madison, WI) to observe insert size against uncut plasmid. Upon confirmation of the *zic2* insert, plasmid was either linearized with EcoR1 for anti-sense template or HindIII for sense template. Linearized template was gel purified and extracted with a QIAquick Gel Extraction Kit (Quiagen, Valencia, CA). Purified, linearized *zic2* template DNA was stored at -20°C until probe synthesis.

Probe synthesis and in situ hybridization: Riboprobe-based *in situ* hybridization was used to assess the distribution and relative abundance of brain *zic2* mRNA. The protocol followed was described previously for assessing mRNA abundances in rat brain tissue (Patisaul et *al.,*

1999) and Atlantic croaker brain tissue (Young et *al.*, 1994, as referenced in Hawkins et *al*., 2000). The *in situ* hybridization was performed using a 727 bp ^{35}S-labeled RNA probe transcribed from template *zic2* cDNA. The cRNA probe was synthesized with a MAXIscript *In Vitro* Transcription Kit (Ambion, Austin, TX) and labeled with [α-^{35}S]CTP (1250 Ci/mmol, 70mCi/ml; Perkin Elmer, Boston, MA). Antisense probe was transcribed using T7 RNA polymerase from the MAXIscript kit, while sense probe was transcribed using T3 RNA polymerase (Promega, Madison, WI). The probe was ethanol precipitated overnight and kept at -20^{o}C until hybridization. Slides went through pre-hybridization washes and were incubated at 50^{o}C with pre-hybridization buffer overnight under moist chamber conditions. Probe was added to buffer with tRNA and Tris•HCL, pH 7.5, EDTA DTT (TED) at 0.170ng/ml/kb (120ng/µl), applied to prepped slides, and incubated overnight. Following post-hybridization washes, slides were placed on Kodak BioMax MR film for 20 hours (Kodak, Rochester, NY). Within two-weeks post-processing, slides were dipped in Kodak NTB3 autoradiographic emulsion (Carestream Health, New Haven, CT), dried, and exposed in a light-sealed box at 4°C for three weeks. Exposed slides were developed for 4 minutes (Kodak D19; Kodak, Rochester, NY), rinsed briefly with running water, and fixed for 15 minutes (Kodak Fixer; Kodak, Rochester, NY) under safelight conditions. After rinsing off the fixer for approximately 20 minutes under running water, slides were cresyl violet stained and coverslipped using Permount (Fisher Scientific, St. Louis, MO) to observe tissue morphology. Control treatment with a sense-strand probe highlighted areas where nonspecific binding was likely to occur and, therefore, was used to compare with antisense slides to eliminate background and false signal. A one hour pre-digestion with RNaseA

(60µg/ml, 37°C) (Sigma, St. Louis, MO) eliminated target RNA and cold antisense probe was utilized to rule out false signal. A probe dilution test at 0.340ng/ml/kb (240ng/µl), 0.170ng/ml/kb (120ng/µl), and 0.085ng/ml/kb (60ng/µl) was utilized to ensure a saturating quantity of probe was being used by examining the amount bound in the cerebellum, a region of high *zic2* mRNA expression. Cerebellar sections from one animal were employed for comparison across dilution treatments.

Localization of signal and autoradiographic densitometry analysis: Distribution maps for *zic2* mRNA based on a terminal phase (TP) male were created by comparison of antisense and sense-strand control slides from the same animals. Sections were compared against other animals of the TP male, IP male, and female phenotypes to assess consistency of *zic2* mRNA localization (24 animals; eight of each phenotype). Complementary sections were compared microscopically while mapping regions of hybridization signal against morphological features in cresyl stained slides. Neuroanatomical nomenclature is consistent with Wullimann et *al.* (1996) and previous work from our laboratory (Marsh et *al.,* 2006). Bright field and dark field images were obtained using a Leica digital microscope (Leica Microsystems, Bannockburn, IL) with a Retiga 2000R, 12 Bit Color Camera (QImaging, Surry, BC, Canada) and MCID image analysis software (InterFocus Imaging Ltd., Linton, Cambridge, UK). Images were adjusted for light and contrast in Windows Photo Gallery (Microsoft Corporation, Redmond, Washington). Quantitative regional autoradioautography of film exposed to hybridization slides was employed for phenotypic comparisons of signal in the cerebellum using MCID software. Labeling density in the cerebellum was quantified

and compared across twenty-four animals: eight animals from each of the three phenotypes. Based on morphology, the density was quantified in ten comparable cerebellar sections from each animal. A rotatable ellipse (100x75μm) drawn using MCID Film Densitometry software was utilized for the quantification procedure. The elliptically-shaped template was designed to lie within the confines of the cerebellum, and template placement was kept constant across all sections and animals. Density readings were entered into JMP software and analyzed via ANOVA (SAS, Cary, NC). A similar protocol was used to compare relative labeling densities from film exposure of probe dilution test slides. Background density measurements, taken immediately adjacent to the template outside cerebellar tissue, were subtracted from each reading, and density readings across comparable sections from the same animal were submitted to a Wilcoxon signed-rank nonparametric test (SAS, Cary, NC). ClustalW version 2.0.10 (European Bioinformatics Institute, Cambridge, UK) was utilized for aligning multiple nucleotide and amino acid sequences.

Results

Cloning of zic2 cDNA plasmid stocks: We amplified cDNA plasmid stock for *zic2* originally isolated from field-collected bluehead wrasses and incorporating a sequence most similar to teleost *zic2* in the NCBI database: *zic2* in *Gasterosteus aculeatus* (three-spined stickleback; e-value = 0.0) and *zic2a* in *Danio rerio* (zebrafish; e-value = 0.0). Figure 1 shows *zic2* plasmid digestion and resulting insert size against uncut 3.0kb plasmid and 50bp ladder. The insert falls close to the 700bp band, and the supercoiled, uncut plasmid is distinguishable

from the digested plasmid. Although teleosts possess two *zic2* homologs, *zic2a (zic2.1)* and *zic2b* (*zic2.2),* due to genome duplication (Toyama et *al.,* 2004; Merzdorf, 2007), *zic2a* is highly similar to the previously reported zebrafish *zic2* gene and shares a more conserved amino acid structure than *zic2b* when compared to other vertebrate *zic2* sequences (Toyama et *al.*, 2004). The 727 bp nucleotide sequence isolated from the bluehead wrasse is shown in Figure 3.

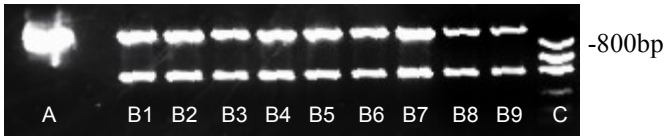


Figure 1. Double-digestions of plasmid with ECOR1 and HINDIII indicating *zic2* cDNA insert length (B1-9) compared against 50bp ladder (C) and uncut 3.0kb plasmid (A). Note alignment of the third band of the step ladder (C) with *zic2* insert (second band; B) at approximately 700-750bp mark.

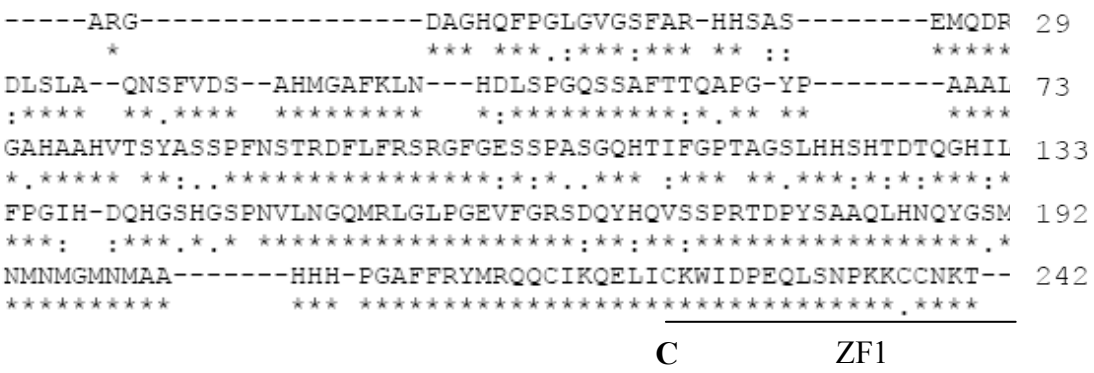


Figure 2. Predicted *zic2* partial amino acid sequence from the bluehead wrasse brain. Columns indicated with a "*" signify identical amino acids when our clone was aligned with the translated nucleotide sequences of *Danio rerio* and *Mus musculus*. A partial C2H2 zinc-finger domain, zinc-finger domain 1 (ZF1), and the presence of a conserved cysteine residue (**C**) is shown. In accordance with ClustalW2 programming (European Bioinformatics Institute, 2009), conserved substitutions are designated as ":" and semi-conserved substitutions ".". Dashes were made for alignment purposes, and substitutions are based on physio-chemical properties and structure as further specified in ClustalW2.

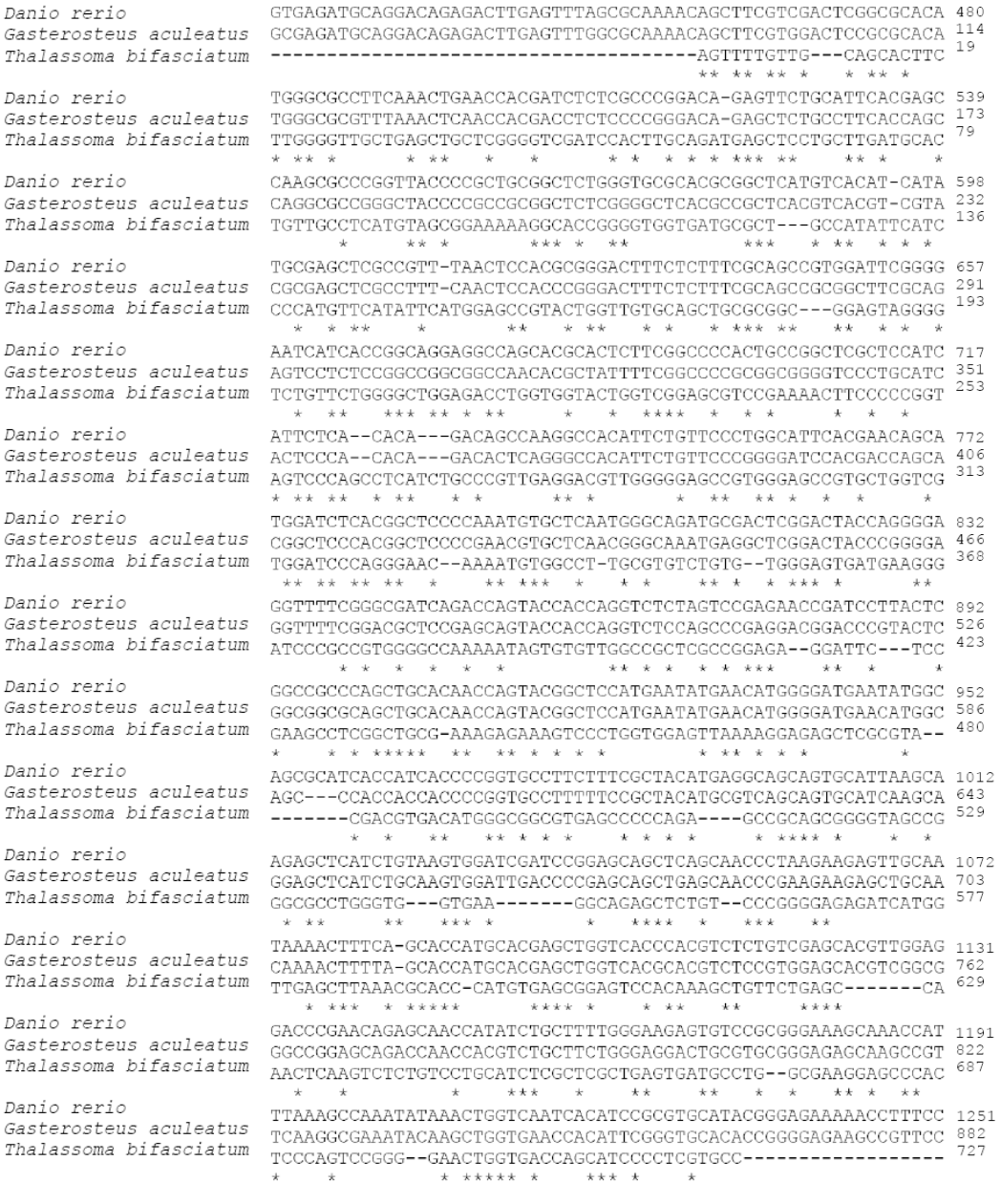


Figure 3. Confirmed *zic2* partial nucleotide sequence isolated from *Thalassoma bifasciatum* (bluehead wrasse) brain aligned with *Danio rerio* (zebrafish) and *Gasterosteus aculeatus* (three-spined stickleback). Nucleotides indicated with "*" represent identical nucleotides when our clone was aligned with zebrafish and stickleback *zic2* sequences from the NCBI database. Dashes were made for alignment purposes in ClustalW2 program (European Bioinformatics Institute, 2009).

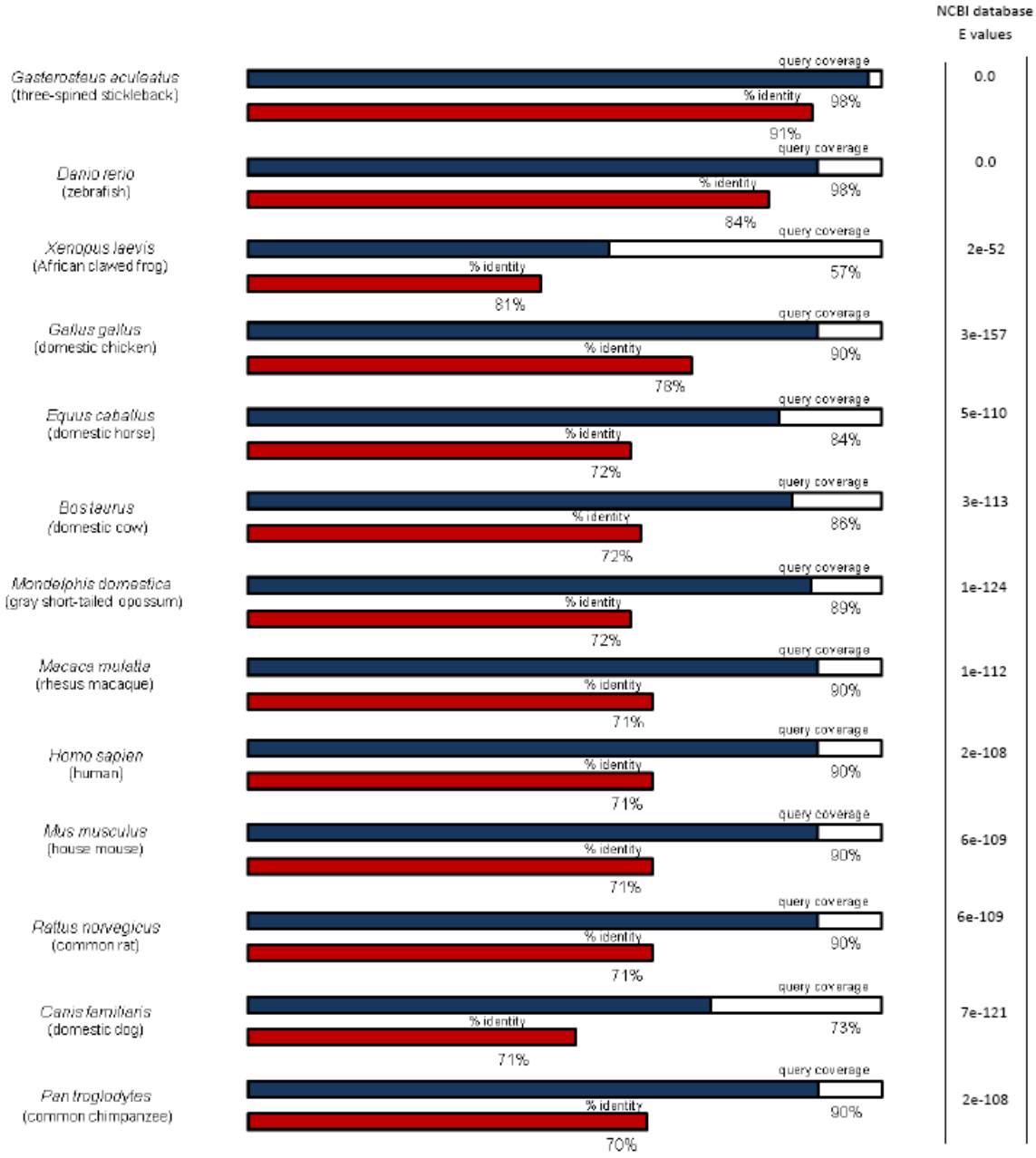


Figure 4. Cloned bluehead wrasse (*Thalassoma bifasciatum*) brain *zic2* nucleotide sequence compared to other vertebrate species. Query coverage bar (blue) indicates the percentage of our bluehead sequence that was aligned with a specific sequence in the NCBI GenBank database. The % identity bar (red) indicates the percentage of shared nucleotides. GenBank e-values for sequence similarity are provided.

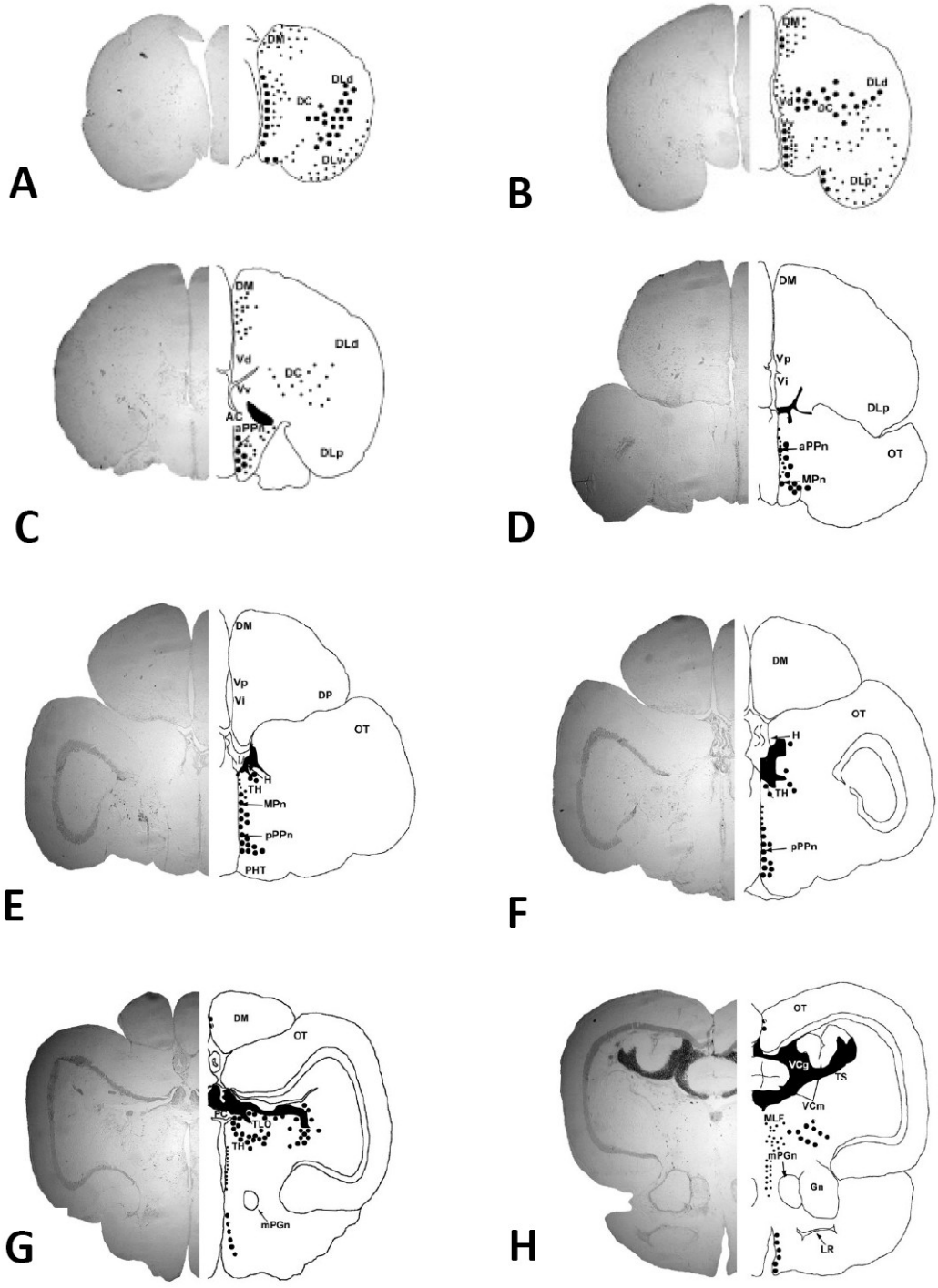
The highest nucleotide sequence homology of the bluehead wrasse *zic2* clone was with teleost species (three-spined stickleback, zebrafish). A comparison of the alignment and sequence similarity of bluehead brain *zic2* with other vertebrate brain *zic2* is shown in Figure 4. Initial NCBI BLAST results of the *Thalassoma bifasciatum* sequence, indicated 84% homology to *Danio rerio* (AF151535.1) and 91% homology to *Gasterosteus aculeatus* (BT027912.1). Bluehead neural *zic2* had 78% homology to *Gallus gallus* (XR_026941.1), 81% homology to *Xenopus laevis* (BC042229.1), and 71% homology to *Homo sapiens* (AF193855.1), *Rattus norvegicus* (NM_001108392.1), *Macaca mulatta* (XM_001093759.1), and *Mus musculus* (NM_009574.3). Comparison of amino acid sequences using ClustalW2 software estimated our cloned bluehead sequence had an alignment score of 95% with *Danio rerio* and 82% with *Mus musculus* and that our bluehead sequence included a partial component of zinc-finger domain 1, one the five C2H2 zinc-finger motifs present across *zics* (Figure 2).

Zic2 mRNA localization and mapping: Hybridization signal indicating *zic2* mRNA was visualized in cells and found in great abundance throughout the forebrain, midbrain, and hindbrain. In control slides, a *zic2* sense probe showed non-specific labeling in the strongly Nissl-positive glomerular nucleus and optic tectum and was utilized to rule out false signal.

Hybridization signal was widespread and diffuse in the telencephalon. Signal was found in the central nucleus, medial zone, and posterior portions of the lateral zone of the telencephalic area dorsalis, the ventral and dorsal zone of the telencephalic area ventralis, and the dorsal ventral division of the lateral zone of the area dorsalis (Figure 5, A-C). In the

telencephalon, large labeled neurons were distributed from the central nucleus of the telencephalic area dorsalis to the ventral division of the lateral zone of the telencephalic area dorsalis, as well as along the walls of the telencephalic ventricle (Figure 5, A-B). Signaling was visible spanning from the posterior telencephalon towards the olfactory bulb in the rostral migratory stream. In the preoptic area of the hypothalamus, numerous prominent neurons expressing *zic2* were observed throughout the magnocellular and anterior and posterior parvocellular preoptic nuclei (Figure 5, C-F). Just lateral to the anterior commissure and anterior preoptic area and ventral to the ventral zone of the telencephalic area ventralis, solid hybridization signal in the entopeduncular nucleus was mirrored in both hemispheres (Figure 5, C). An intense layer of hybridization signal was noted throughout the habenula and along its boundaries extending into thalamic nuclei (Figure 5, D-F). Further into the midbrain, a dense layer of hybridization signal was noted in the torus semicircularis and torus longitudinalis, as well the presence of more discrete labeling just ventral to the torus semicircularis (Figure 5, G-H). In these regions, the ventral portion of the posterior commissure exhibited large *zic2*-labeled cells (Figure 5, G-H). Medial to the preglomerular nuclei and along the midline, diffuse signal stretched into the medial longitudinal fasicle (Figure 5H). In the hindbrain, a strongly-labeled layer of signal extended throughout the granule cell layer of the valvula cerebelli and cerebellum, as well as the crista cerebellaris (Figure 5N). *Zic2* cells were found lining the ventral tegmental commissure and inferior lobe of the hypothalamus (Figure 5L), and signal was observed in the medial longitudinal fascicle ventral to the cerebellum (Figure 5N).

Figure 5. Neuroanatomical distribution of *zic2* mRNA on representative coronal sections (A–N) of the brain of the bluehead wrasse. Relative abundance and localization of *zic2* signal is indicated on the right side of the midline along with structure labels. Black-shaded areas indicate a solid layer of signaling, large circles indicate distinct cell body labeling, while smaller circles indicate the presence of diffuse hybridization signal not clearly restricted to distinct cells. See abbreviations in Table 1.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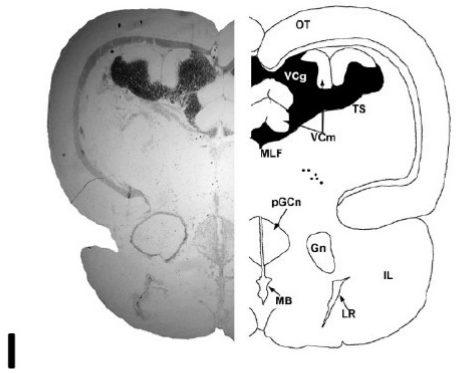
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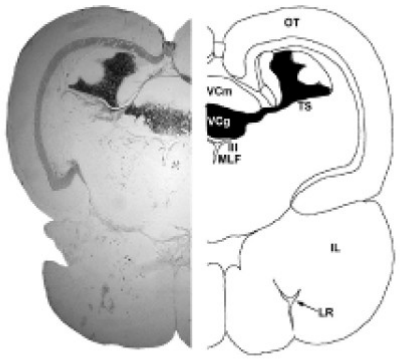
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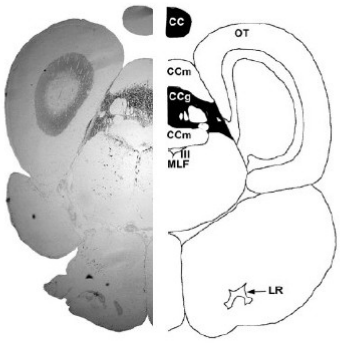
CC
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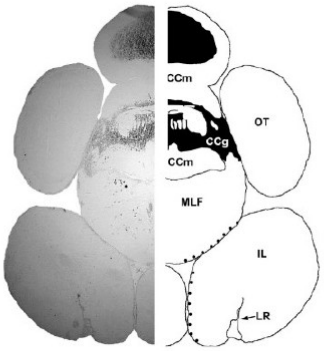
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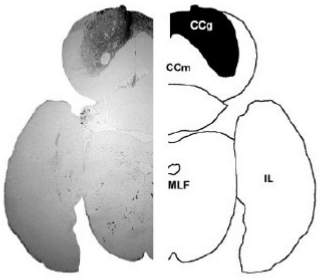
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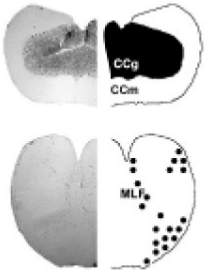
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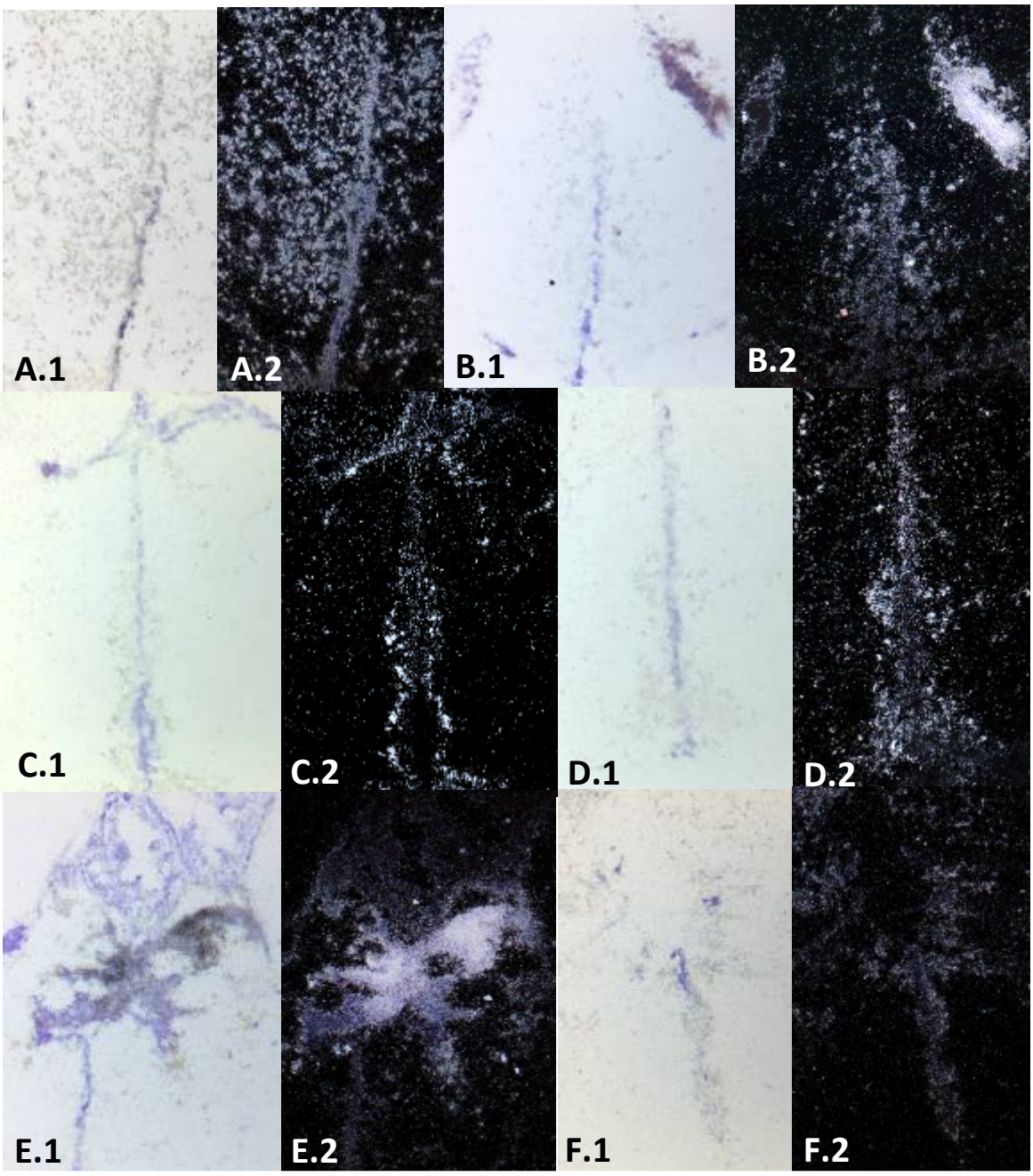
Table 1. Abbreviations for neuroanatomical structures referred to in text and Figures 5 and 6.

AC	anterior commissure
CC	crista cerebellaris
CCg	granule cell layer of the cerebellum
CCm	molecular cell layer of the cerebellum
DC	central nucleus of telencephalic area dorsalis
DLp	posterior part of lateral zone of telencephalic area dorsalis
DLd	dorsal division of lateral zone of telencephalic area dorsalis
DLv	ventral division of lateral zone of telencephalic area dorsalis
DM	medial zone of telencephalic area dorsalis
DP	posterior zone of telencephalic area dorsalis
gMP	gigantocellular portion of the magnocellular preoptic nucleus
Gn	glomerular nucleus
H	habenula
IL	inferior lobe of the hypothalamus
LR	lateral recess
MB	mammillary body
MLF	medial longitudinal fascicle
MPn	magnocellular preoptic nucleus
mPGn	medial preglomerular nucleus
OT	optic tectum
PC	posterior commissure
PHT	preopticohypophysial tract
PGCn	preglomerular commissural nucleus
aPPn	anterior parvocellular preoptic nucleus
pPPn	posterior parvocellular preoptic nucleus
TH	thalamus
TLO	torus longitudinalis
TS	torus semicircularis
VCg	granule cell layer of the valvula cerebelli
VCm	molecular cell layer of the valvula cerebelli
Vd	dorsal zone of telencephalic area ventralis
Vi	intermediate zone of telencephalic area ventralis
Vp	postcommissural nucleus of telencephalic area ventralis
Vv	ventral zone of telencephalic area ventralis

Cerebellar phenotypic comparison and autoradiographic densitometry: A dilution test aimed at optimizing probe concentration and confirming saturation of endogenous *zic2* mRNA indicated comparable hybridization signal levels in the cerebellum with probe concentrations at 0.340ng/ml/kb, 0.170ng/ml/kb, and 0.085ng/ml/kb. At 0.340ng/ml/kb,

mean relative density across five alternating cerebellar sections was 0.71752, at 0.170ng/ml/kb density was 0.71752, and at 0.085ng/ml/kb density readings were 0.77178 (Wilcoxon signed-rank test, $p = 0.6977$). Analysis of cerebellar hybridization signal intensity across phenotypes showed no significant difference in *zic2* mRNA labeling density (ANOVA; $F_{2,238} = 2.3089$, $p = 0.1016$).

Figure 6. *Zic2* mRNA labeling with corresponding images of silver grains taken in both bright and dark field at 100X total magnification. Representative location of signaling referenced to Figure 4 maps. Label shown extending into the central nucleus of telencephalic area dorsalis from the mid-telencephalon (A.1-2; map A); anterior parvocellular preoptic nucleus and entopeduncular nucleus (B.1-2; map C); parvocellular and magnocellular preoptic nuclei extending into the anterior habenula (C.1-2; map D); magnocellular and posterior preoptic nuclei (D.1-2; map F); habenula (E.1-2; map F); thalamus extending to posterior commissure (F1-2; map G); thalamus, posterior commissure, and torus semicircularis and longitudinalis (G.1-2; map G); neurons in the anterior medial longitudinal fascicle (H.1-2; map H); granule cell layer of the valvula cerebella (I.1-2; map H); granule cell layer of the cerebellum (J.1-2, map K); medial longitudinal fascicle (K.1-2, map N)


A.1
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D.1
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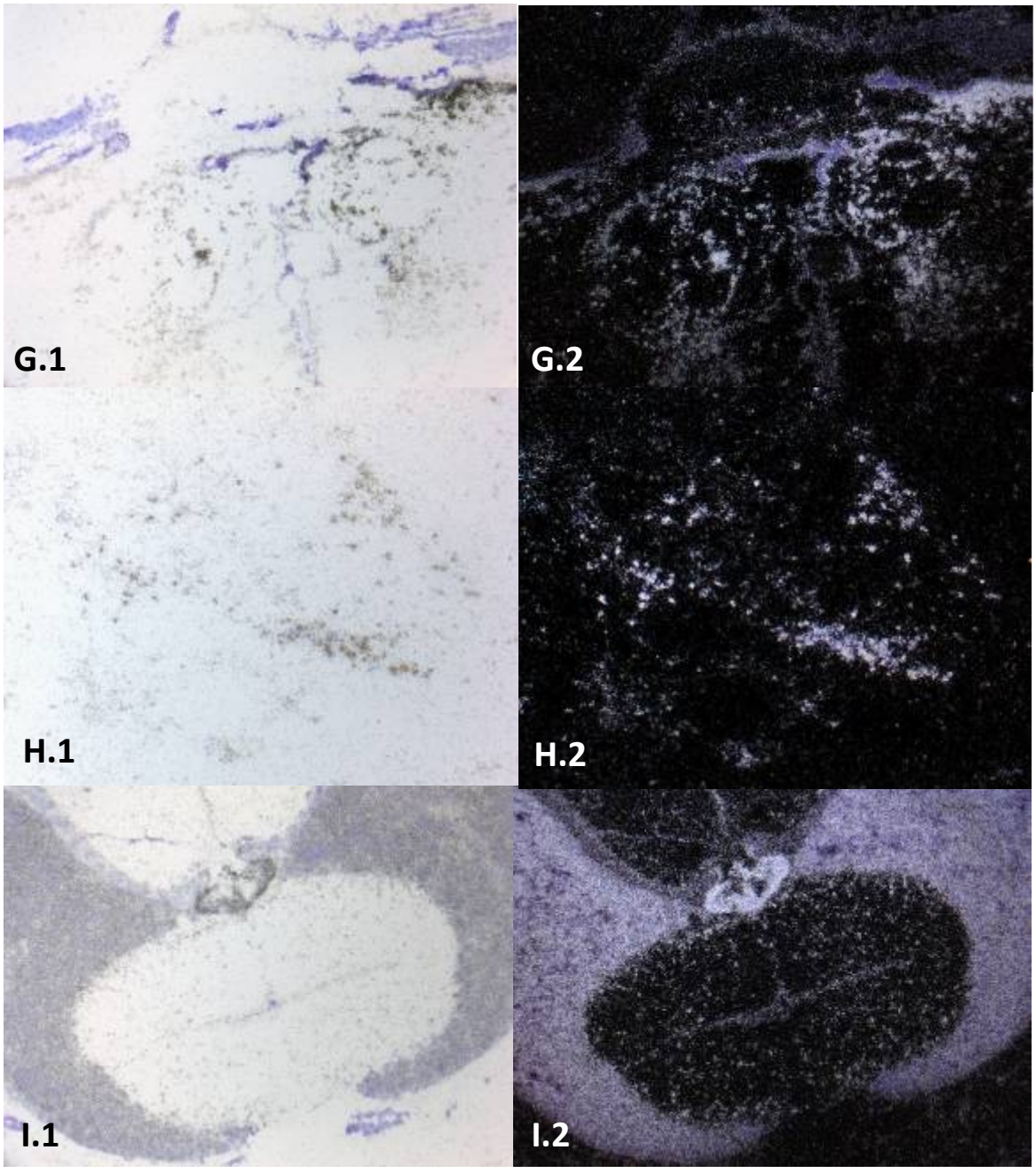
F.1
F.2


G.1
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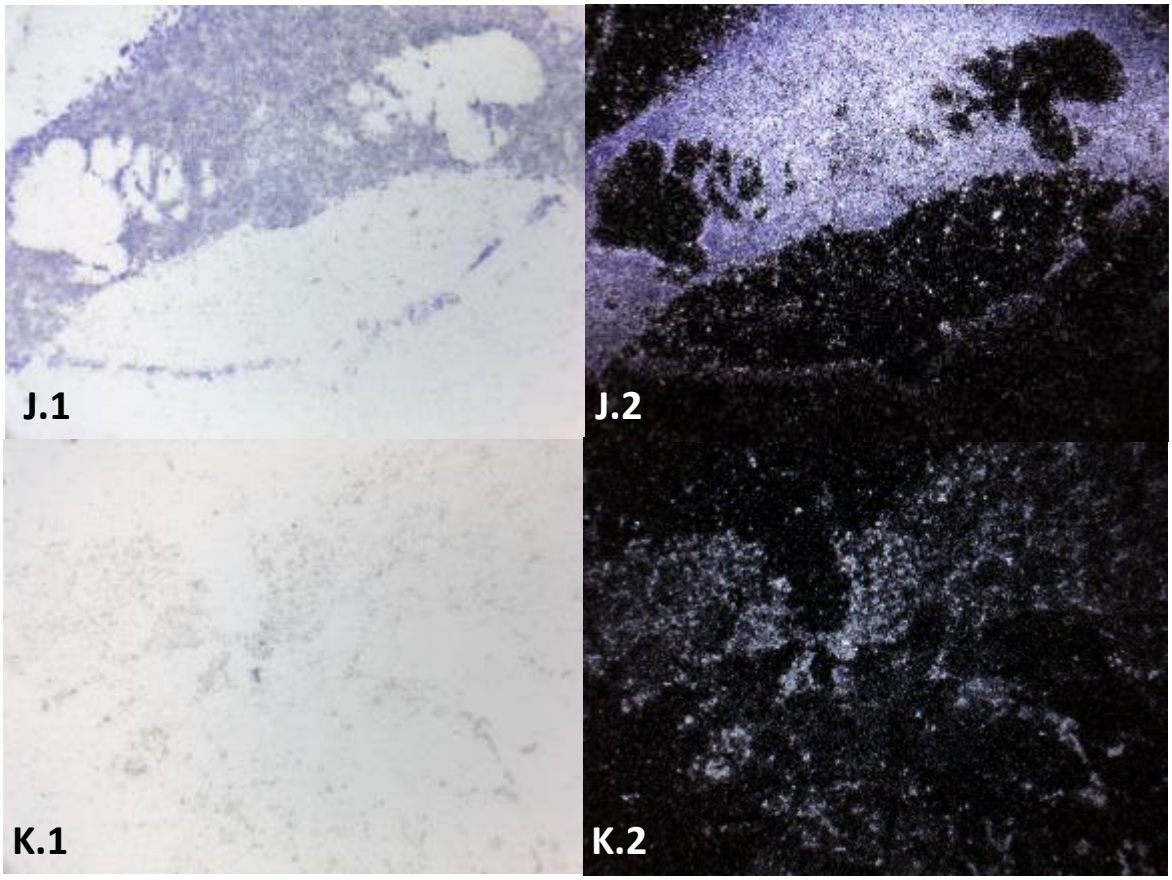

Discussion

Zic family transcription factors play multiple roles in early neural development and differentiation (Merzdorf, 2007), and *zic* homologs have been identified in urochordates, cephalochordates, nematodes, arthropods, and vertebrates (Aruga, 2004). Although studied for its role in development, this is the first report of the distribution of neural *zic2* mRNA in the adult teleost.

We successfully cloned neural *zic2* from field-collected bluehead wrasse. The 727 bp sequence showed higher nucleotide sequence identity with the three-spined stickleback and zebrafish, 91% (e-value = 0.0) and 84% (e-value = 0.0), respectively, than to non-teleost vertebrates. All vertebrate species found within the NCBI database showed 71% or greater homology to our bluehead *zic2* clone at the nucleotide level, and comparison of the amino acid sequence for our partial *zic2* clone to *Danio rerio* (zebrafish) estimated 95% sequence similarity. Zinc-finger region amino acid identity has been described as greater than 93% between *Danio rerio* and *Mus musculus*, *Xenopus laevis*, and *Homo sapiens* (Toyama et *al*., 2004), further demonstrating the highly conserved nature of *zic2* across vertebrates.

We utilized a partial *zic2* sequence from the bluehead wrasse to generate a cDNA template for riboprobe synthesis and *in situ* hybridization. Our cloned bluehead *zic2* sequence showed a high level of homology to other vertebrate *zic2* sequences. We found that *zic2* brain mRNA was not only expressed in the adult cerebellum, but also widely across the mature bluehead brain. Observed expression patterns in the ventral telencephalon and preoptic area of the hypothalamus may be associated with sexual phenotype differentiation and possibly with the sex change pathway as these regions have been implicated in sex-typical behaviors and function.

Zic2 distribution was generally in accord with that described by Brown and Brown (2009), Aruga and collaborators (1994), and online mouse *zic2* maps found at Allen Brain Atlas. In addition to the cerebellar labeling well documented in other species, *zic2* expression was found widely in the bluehead wrasse brain, including the telencephalon and diencephalon. We found that *zic2* is expressed in the thalamus, hypothalamus, habenula, and

anterior telencephalon. In accord with findings in Aruga et *al*., 2002, we found dense *zic2* labeling throughout the granule cell layer of the cerebellum. Additional signal was noted in the granule layer of the valvula cerebella and crista cerebellaris. The valvula cerebella and crista cerebellaris are structures supplied with mechanosensory input in teleosts (Wullimann et *al*., 1991) and, as shown in zebrafish, contain proliferating cells that give rise to cerebellar granule neurons (Chapouton et *al.*, 2007). In the forebrain, *zic2* mRNA was located in the adult bluehead hypothalamus among other structures. Specifically, hybridization signal was located in large nuclei within magnocellular and anterior and posterior parvocellular bodies in the preoptic area, similar to the distribution of AVT (arginine vasotocin) neurons described in the bluehead wrasse (Godwin et *al*., 2000; Marsh et *al*., 2006). The preoptic area is a key integrative region for reproductive behavior and function and exhibits sexual dimorphisms in a variety of species including bluehead wrasses (Gorski et *al.*, 1980; Elofsson et *al*., 1997; Cooke et *al*., 1998; Foran and Bass, 1999; Godwin et *al*., 2000). Previous findings from our laboratory show differences in the expression of behaviorally and reproductively important aromatase and AVT genes in the preoptic area across sexual phenotypes (Godwin et *al.*, 2000; Marsh et *al.*, 2006).

Consistent with Aruga and collaborators (1994) and Brown and Brown (2009), we found *zic2* expression in the telencephalon. The lateral and posterior zones of the telencephalon in teleosts potentially represent a functional homolog of the mammalian hippocampus, and there are descriptions of cellular proliferation and neurogenesis in these regions in fishes (Chapouton et *al*., 2007). Specifically, *zic2* signaling was noted extending from the telencephalon toward the olfactory bulb. The input of new neurons in the olfactory

bulb is thought to allow the plasticity in circuitry necessary to adjust to environmental and odor association changes (Alvarez-Buyella and Garcia-Verdugo, 2002). Strong *zic2* signaling was also noted in the entopeduncular nucleus, located dorso-laterally to the anterior preoptic nucleus in both hemispheres. In trout, significant habenular afferent fibers and some thalamic afferent fibers have been shown to arise in the entopeduncular nucleus (Yanez and Anadon, 1996). As described in zebrafish, the teleost entopeduncular nucleus may be homologous to the entopeduncular nucleus of nonprimate mammals and the internal segment of the globus pallidus of primates (Mueller and Guo, 2009). Dopamine, a neurotransmitter involved in motivated behavior, differentially regulates the output pathway from the striatum to the entopeduncular nucleus/internal globus pallidus of primates (Gerfern et *al*., 1990). Striatal neurons that express dopamine receptor D1 (DRD1) have been shown to provide inhibitory input to the entopeduncular nucleus (Gerfern et *al.*, 1990), and nigrostriatal lesion and dopamine agonists affect neuron firing patterns of the rodent entopeduncular nucleus (Ruskin et *al*., 2002).

The presence of *zic2* signal in the torus semicircularis, torus longitudinalis, and medial longitudinal fascicle may indicate a role associated with auditory, mechanosensory, and electrosensory lateral-line information processing (Echteler, 1985; Striedter, 1991; Coughlin and Hawryshyn, 1995), visual processing (Northmore et *al*., 1983), and oculomotor processing (Pola and Robinson, 1978), respectively. Behavioral interactions, chemical, and visual cues have been shown to be essential for mediating sex-change in female bluebanded gobies, a protogynous reef fish (Lorenzi et *al*., 2006). Socially-mediated sex-changing cues were shown processed via the visual system in *Thalassoma duperrey*, a congener to the

bluehead wrasse (Ross et *al*., 1983). Robust signaling was noted throughout the habenula extending into thalamic and hypothalamic nuclei. Signaling in the thalamus could indicate a function in sensory integration and gating. The presence of *zic2* mRNA in the habenula, which receives inputs from both the hypothalamus and thalamus and outputs to regions containing dopaminergic neurons, is potentially of interest as dopaminergic pathways are important in motivated behaviors (Ellison, 1994). As the habenula has outputs to both dopaminergic and serotonergic targets, it is regarded as an important link between the limbic and striatal forebrain in at least mammals (Ellison, 1994; Wang and Aghajanian, 1977). Although *zic2* expression is found in the medial septal nucleus, the thalamic nuclei, and preoptic nucleus in the developing mammalian brain (Aruga et *al*., 2002), we observed a similar pattern of signaling in the adult bluehead wrasse brain. This expression pattern in adult bluehead wrasse is intriguing and may partially reflect the neural plasticity that teleosts retain to a greater degree into adulthood than mammals. Regions of cellular proliferation and neurogenesis in the adult zebrafish, as described by Chapouton and collegues (2007), are not only consistent with those observed during development in mammals, but also our observed patterns of *zic2* signaling in the adult bluehead wrasse. These include labeling in the telencephalon, preoptic area, habenula, thalamus, ventricular domain of the hypothalamus, torus longitudinalis, corpus cerebella, valvula cerebella, and cerebellum (Chapouton et *al*., 2007).

Our comparison of *zic2* mRNA abundances in the adult cerebellum showed no significant differences across TP, IP male, or female animals. A previous microarray study did indicate differences in *zic2* mRNA abundance across sexual phenotypes in the bluehead

wrasse brain (Passador-Gurgel, unpublished data), but the results here suggest such a difference in *zic2* signaling would be expressed in brain regions other than the cerebellum. *Zic2* expression occurs from the hypothalamus and thalamus into the habenula, a structure targeting dopaminergic regions, in the adult brain of the bluehead wrasse. Alterations in dopaminergic signaling have been implicated in controlling sex change in *T. duperrey* (Larson et *al.*, 2003a, 2003b), and tyrosine-hydroxylase positive neurons are found in the preoptic area in close proximity to aromatase-immunoreactive cells as well as AVT neurons in bluehead wrasses. Of interest with respect to a potential regulatory role for *zic2* in sex change and sexual phenotype differentiation is that *zic2* protein functions as a transcriptional repressor and has been shown to regulate tissue specific expression of the dopamine receptor D1 (DRD1) (Yang et *al*., 2000). DRD1 and *zic2* genes have contrasting brain regional distributions, with DRD1 found highly expressed in the mammalian corpus striatum, including the putamen, caudate nucleus, and nucleus accumbens (Jung et *al*., 1996; Sun et *al*., 2000). Dopamine is closely associated with reward-seeking behaviors, and is therefore believed to signal to brain regions involved in acquiring new behavior (Arias-Carrion and Poppel, 2007). The non-specific dopamine receptor inhibitor haloperidol was sufficient to bring about protogynous sex change in the saddleback wrasse (Larson et *al*, 2003) and preliminary findings indicate similar effects under field conditions in bluehead wrasses (Heinz and Godwin, unpublished data). Therefore, *zic2* could play a role in mediating the dopaminergic pathway via downregulation of DRD1 in this species. DRD1 and *zic2* are therefore of particular interest for future studies.

This study of the distribution of *zic2* mRNA in the adult bluehead wrasse remains one of relatively few studies to examine the expression of this gene in the adult brain and the first in the adult teleost. We found *zic2* expressed throughout the brain in the adult bluehead wrasse, a distribution that is consistent with a potential role for this protein in regulating sexual phenotype differentiation, neurogenesis, and possibly sex change. Future studies to explore these possibilities should include mapping *zic2* protein expression and conducting co-localization work with *zic* genes across the three distinct bluehead phenotypes.

Application of a *zic2* gene knockdown agent (e.g., antisense oligonucleotide, RNAi) or administration of an upstream pathway inhibitor (i.e.,*Wnt* pathway inhibitor) to bluehead wrasses in the natural environment would clarify *zic2's* functions. Investigations centered on *zic2's* potential influence on dopaminergic signaling and the sex change pathway should explore the localization and quantification of DRD1 receptors and undertake behavioral field tests with DRD1-specific anatagonists. Future research incorporating such mechanistic approaches is central to unraveling and understanding the circuitry by which behavioral cues are transduced to neuroendocrine processes.

References

Anderson, S.L., Thompson, A.P, Krenzel, E., & Teicher, M.H. (2002) Pubertal changes in gonadal hormones do not underlie adolescent dopamine receptor overproduction. *Psychoneuroendocrinology*, **27**, 683-691

Andersen, S.L. & Teicher, M.H. (2000) Sex differences in dopamine receptors and their relevance to adhd. *Neuroscience & Biobehavioral Reviews*, **24**, 137-141

Alvarez-Buylla, A. & Garcia-Verdugo, J.M. (2002) Neurogenesis in adult subventricular zone. *The Journal of Neuroscience*, **22**(3), 629-634

Arias-Carrión, O.O. & Pŏppel, E.E. (2007) Dopamine, learning, and reward-seeking behavior. *Acta Neurobiologiae Experimentalis*, **67**, 481-488

Aruga, J. (2004) The role of zic genes in neural development. *Moelcular and Cellular Neuroscience,* **26**, 205-221

Aruga, J., Inoue, T., Hoshino, J., & Mikoshiba, K. (2002) Zic2 controls cerebellar development in cooperation with Zic1. *Journal of Neuroscience*, **22**, 218-225

Aruga, J., Nagai, T., Tokuyama, T., Hayashizaki, Y., Okazaki, Y., Chapman, V.M., & Mikoshiba, K. (1996) The mouse *zic* gene family: Homologues of the *Drosophila* pair-rule gene *odd-paired*. *The Journal of Biological Chemistry*, **271** (2) 1043-1047

Aruga, J., Yokota, N., Hashimoto, M., Furuichi, T., Fukuda, M., & Mikoshiba, K.(1994) A novel zinc finger protein, zic, is involved in neurogenesis, especially in the cell lineage of cerebellar granule cells. *Journal of Neurochemistry*, **63**, 1880–1890

Bafico, A., Liu, G., Goldin, L., Harris, V., & Aaronson, S.A. (2004) An autocrine mechanism for constitutive Wnt pathway activation in human cancer cells. *Cancer Cell*, **6**, 497-506

Ball, G.F., Casto, J.M. & Balthazart, J. (1995) Autoradiographic localization of di-like dopamine receptors in the forebrain of male and female japanese quail and their relationship with immunoreactive tyrosine hydroxylase. *Journal of Chemical Neuroanatomy*, **9**, 121-133

Bathazart, J., Baillien, M., & Ball, G.F. (2001) Phosphorylation processes mediate rapid changes of brain aromatase activity. *The Journal of Steroid Biochemistry and Molecular Biology,* **79**, 261-277

Bataller, L., Wade, D.F., Fuller, G.N., Rosenfeld, M.R., & Dalmau, J. (2002) Cerebellar degeneration and autoimmunity to zinc-finger proteins of the cerebellum. *Neurology* **59**, 1985-1987

Bataller, L., Wade, D.F., Graus, F., Stacey, H.D., Rosenfeld, M.R.,& Dalmau, J. (2004) Antibodies to Zic4 in paraneoplastic neurologic disorders and small-cell lung cancer. *Neurology*, **62**, 778-782

Benedyk, M.J., Mullen, J.R., & DiNardo, S. (1994) Odd-paired: a zinc finger pair-rule protein required for the timely activation of engrailed and wingless in Drosophila embryos. *Genes and Development*, **8**, 105-117

Bhanot, P., Marcel, B., Samos, C.H., Hsieh, C., Wang, Y., Macke, J.P., Andrew, D., Nathans, J., & Nusse, R. (1996) A new member of the *frizzled* family from *Drosophila* functions as a Wingless receptor. *Nature*.382, 225-230

Bidus, M.A., Risinger, J.I., Chandramouli, G.V.R., Dainty, L.A., Litzi, T.J., Berchuck, A., Barrett J.C., Maxwell, G.L. (2006) Prediction of lymph node metastasis in patients with endometrioid endometrial cancer using expression microarray. *Clinical Cancer Research,* **12,** 83-88

Blazquez, M., Bosma, P.T., Fraser, E.J., Van Look, K.J.W., & Trudeau, V.L. (1998) Fish as models for the neuroendocrine regulation of reproduction and growth. *Comparative Biochemistry and Physiology*, **119**, 345-364

Bolis, C.L., Piccolella, M., Dalla Valle, A.Z., & Rankin, J.C. (2001) Fish as model in pharmacological and biological research. *Pharmacological Research,* **44**, 265-280

Brown, L. & Brown, S.(2009) Zic2 is expressed in pluripotent cells in the blastocyst and adult brain expression overlaps with makers of neurogenesis, *Gene Expression Patterns,* **9**, 43-49

Brown, S., Gersen, S., Anyane-Yeboa, K. and Warburton, D. (1993) Preliminary definition of a 'critical region' of chromosome 13 in q32: report of 14 cases with 13q deletions and review of the literature. *American Journal of Medical Genetics,* **45**, 52-59

Brown, S., Russo, J., Chitayat, D., & Warburton, D. (1995) The 13q-syndrome: the molecular definition of a critical seletion in band 13q32. *American Journal of Human Genetics,* **57**, 859-866

Brown, S.S.A., Warburton, D.D., Brown, L.L.Y., Yu, C.C.Y., Roeder, E.E.R., Stengel-Rutkowski, S.S., Hennekam, R.R.C. & Muenke, M.M. (1998) Holoprosencephaly due to mutations in zic2, a homologue of drosophila odd-paired. *Nature genetics*, **20**, 180-183

Chang, J.P., Yu, K.L., Wong, A.O.L., & Peter, R.E. (1990) Differential Actions of Dopamine Receptor Subtypes on Gonadotropin and Growth Hormone Release in vitro in Goldfish. *Neuroendocrinology,* **51**, 664-674.

Chapouton P., Jagasia, R., & Bally-Cuif, L. (2007) Adult neurogenesis in non-mammalian vertebrates. *BioEssays,* **29**, 745-757

Chen, C.M. & Struhl, G. (1999) Wingless transduction by the Frizzled and Frizzled2 proteins of *Drosophila*. *Development*, **126**, 5441-5452

Chen, L.C., Vural, B., Ustuner, Z., Saip, P., Ozbek, M., Gonen, L., Old, Y.T., & Gure, A. (2004) Immunoreactivity to SOX-1, -2, -3 and ZIC2 antigens and correlation to survival in small cell lung cancer (SCLC). *Journal of Clinical Oncology*, **22***,* 7159

Cooke, B., Hegstrom, C.D., Villeneuve, L.S., & Breedlove, S.M. (1998) Sexual differentiation of the vertebrate brain: principles and mechanisms, *Frontiers in Neuroendocrinology*, **19**, 323-362

Conacci-Sorrell, M., Zhurinsky, J., & Ben-Ze'ev, A. (2002) The cadherin-catenin adhesion system in signaling and cancer *Journal of Clinical Investigation*, **109**, 987–991

Coughlin, D.J. & Hawryshyn, C.W. (1995) A cellular basis for polarized-light vision in rainbow trout. *Journal of Comparative Physiology*,**176,**, 261-272

Demeret, C., Yaniv, M. & Thierry, F. (1994) The e2 transcriptional repressor can compensate for sp1 activation of the human papillomavirus type 18 early promoter. *Journal of Virology*, **68**, 7075-7082

Echteler, S.M (1985) Organization of central auditory pathways in a teleost fish*, Cyprinus carpio*. *Journal of Comparative Physiology*, **156**, 267-280

Ellison, G. (1994) Stimulant-induced psychosis, the dopamine theory of schizophrenia, and the habenula. *Brain Research Brain Research Reviews,***19**(2), 223-239

Elofsson, U, Winburg, S., & Francis, R.C. (1997) Number of preoptic GnRH-immunoreactive cells correlates with sexual phase in a protandrously hermaphroditic fish, the dusky anemonefish (*Amphiprion melanopus*), *Journal of Comparative Physiology*,**181**, 484-492

Foran, C.M & Bass, A.H. Preoptic GnRH and AVT: Axes for sexual plasticity in teleost fish. *General and Comparative Endocrinology*, **116**, 141-152

Fujimura, N., Vacik, T., Machon, O., Vlcek, C., Scalabrin, S., Speth, M., Diep, D., Krauss, S., Kozmik, Z. (2007) Wnt-mediated down-regulation of Sp1 target genes by a transcriptional repressor Sp5. *Journal of Biological Chemistry,* **282**(2), 1225-1237

Gainetdinov, R.R., Mohn, A.R. & Caron, M.G. (2001) Genetic animal models: Focus on schizophrenia. *Trends in Neurosciences*, **24**, 527-533

Gerfern, C., Engber, T., Mahan, L., Susel, Z., Chase, T., Monsma, F. & Sibley, D. (1990) D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. *Science*, **250**, 1429-1432

Giles, R.H., van Es, J.H., & Clevers, H. (2003) Caught up in a Wnt storm: Wnt signaling in cancer. *Biochimica et Biophysica Acta*, **1653**, 1-24

Godwin, J., Crews, D. & Warner, R.R. (1996) Behavioural sex change in the absence of gonads in a coral reef fish. *Proceedings: Biological Sciences*, **263**, 1683-1688

Godwin, J., Sawby, R., Warner, R.R., Crews, D., & Grober, M.S. (2000) Hypothalamic arginine vasotocin mRNA abundance variation across sexes and with sex change in a coral reef fish. *Brain, Behavior, and Evolution*, **55**, 77–84

Goldman-Rakic, P.S.P.S., Castner, S.A.S.A., Svensson, T.H.T.H., Siever, L.J.L.J. & Williams, G.V.G.V. (2004) Targeting the dopamine d1 receptor in schizophrenia: Insights for cognitive dysfunction. *Psychopharmacology*, **174**, 3-16

Gorski, R.A, Harlan, R.E., Jacobson, C.D, Shryne, J.E., & Southam A.M. (1980) Evidence for the existence of a sexual dimorphic nucleus in the preoptic area of the rat. *Comparative Neurology*, **193**, 529-539

Grinberg, I. & Millen, K.J. (2005) The ZIC gene family in development and disease. *Clinical Genetics*, **67**, 290-296.

Grinblat, Y., Nyholm, M., Taylor, A., & Burgess, S. (2008) A role for zic genes during neural tube morphogenesis in zebrafish *Abstract from 67th Annual Meeting/ Developmental Biology*, **319**(2), 514-515

Grober, M.S., Jackson, I.M., Bass, A.H. (1991) Gonadal steroids affect LHRH preoptic cell number in a sex/role changing fish. *Journal of Neurobiology,* **22**, 734-741

Grober, M.S. & Bass, A.H. (2002) Life history, neuroendocrinology, and behavior in fish. *Hormones, Brain and Behavior,* **2**, 331-347

Gure, A.O., Stockert, E., Scanlan, M.J., Keresztes, R.S., Jager, D., Altorki, N.K., Old, L.J., & Chen, Y.T.(2000) Serological identification of embryonic neural proteins as highly immunogenic tumor antigens in small cell lung cancer. *Proceedings of the NationalAcademy of Science*, **97**, 4198-4203

Hirvonen, J.J., Van Erp, T.G.T.G.M., Huttunen, J.J., Aalto, S.S., Nagren, K.K., Huttunen, M.M., Lönnqvist, J.J., Kaprio, J.J., Cannon, T.D.T.D. & Hietala, J.J. (2006) Brain dopamine d1 receptors in twins discordant for schizophrenia. *The American Journal of Psychiatry*, **163**, 1747-1753

Huang, H.C. & Klein, P.S. (2004) The Frizzled family: receptors for multiple signal transduction pathways. *Genome Biology*, **5**, 234

Imai K.S., Satou Y., & Satoh, N. (2002) Multiple functions of Zic-like gene in the differentiation of notochord, central nervous system and muscle in *Ciona savignyi* embryos. *Development***, 129***,* 2723-2732

Inoue, T., Ogawa, M., Mikoshiba, K. & Aruga, J. (2008) *Zic* deficiency in the cortical marginal zone and meninges results in cortical lamination defects resembling those in Type II Lissencephaly**.** *The Journal of Neuroscience*, **28**(18), 4712-4725

Johansson, V. & Johansson (2005) Growth hormone-induced stimulation of swimming and feeding behaviour of rainbow trout is abolished by the d1 dopamine antagonist sch23390. *General and Comparative Endocrinology*, **141**, 58

Jung, A.B. & Bennett, J.P. (1996) Development of striatal dopaminergic function. I. Pre- and postnatal development of mRNAs and binding sites for striatal D1 (D1a) and D2 (D2a) receptors. *Developmental Brain Research*, **94**, 109-120.

Kitaoka, S.S., Furuyashiki, T.T., Nishi, A.A., Shuto, T.T., Koyasu, S.S., Matsuoka, T.T., Miyasaka, M.M., Greengard, P.P. & Narumiya, S.S. (2007) Prostaglandin e2 acts on ep1 receptor and amplifies both dopamine d1 and d2 receptor signaling in the striatum. *The Journal of Neuroscience*, **27**, 12900-12907

Larson, E.T., Norris, D.O., Gordon Grau, E. & Summers, C.H. (2003a) Monoamines stimulate sex reversal in the saddleback wrasse. *General and Comparative Endocrinology*, **130**, 289-298

Larson, E.T., Norris, D.O. & Summers, C.H. (2003b) Monoaminergic changes associated with socially induced sex reversal in the saddleback wrasse. *Neuroscience*, **119**, 251-263

Levavi-Sivan, B., Biran, J. & Fireman, E. (2006) Sex steroids are involved in the regulation of gonadotropin-releasing hormone and dopamine d2 receptors in female tilapia pituitary. *Biology of Reproduction*, **75**, 642-650

Li, P.P., Shah, S.S., Huang, L.L., Carr, A.L.A.L., Gao, Y.Y., Thisse, C.C., Thisse, B.B. & Li, L.L. (2007) Cloning and spatial and temporal expression of the zebrafish dopamine d1 receptor. *Developmental dynamics*, **236**, 1339-46

Lidow, M.S. & Goldman-Rakic, P.S. (1994) A common action of clozapine, haloperidol, and remoxipride on D1- and D2-dopaminergic receptors in the primate cerebral cortex. *Proceedings of the National Academy of Science*. **91**, 4353–4356.

Logan, C.Y. & Nusse, R. (2004) The wnt signaling pathway in development and disease. *Annual Review of Cell and Developmental Biology.* **20**, 781–810

Lorenzi, V., Earley, R.L., & Grober, M.S. (2006) Preventing behavioural interactions with a male facilitates sex change in female bluebanded gobies. *Lythrypnus dalli, Behavior, Ecology, and Sociobiology*, **59**, 715–722

Marsh, K.E., Creutz, L.M., Hawkins, M.B. & Godwin, J.J. (2006) Aromatase immunoreactivity in the bluehead wrasse brain, *Thalassoma bifasciatum*: Immunolocalization and co-regionalization with arginine vasotocin and tyrosine hydroxylase. *Brain Research*, **1126**, 91-101

Marsh, K.E. (2007) Neuroendocrine transduction of social cues in the bluehead wrasse, *Thalassoma bifasciatum*. PhD thesis, Zoology, North Carolina State University, Raleigh, NC, 57-78

McMahon, A., Junette, K., & Merzdorf, C. (2007) The expression of zic1, zic2, zic3, and zic4 in early chick embryos. *Developmental Biology*, **306**, 355-356

Merzdorf, C.S. (2007) Emerging roles for zic genes in early development. *Developmental Dynamics*, **236**, 922-940

Merzdorf, C.S. & Sive, H. (2006) The zic1 gene is an activator of Wnt signaling. *The International Journal of Developmental Biology*, **50,** 611-617

Miyata, H., Ikawa, E., & Ohama, E. (1998) Medulloblastoma in an adult suggestive of external granule cells as its origin: a histological and immunohistochemical study. *Brain Tumor Pathology*, **15**, 31-**3**5

Mizugishi, K., Aruga, J., Nakata, K. & and Mikoshiba, K. (2001) Molecular properties of zic proteins as transcriptional regulators and their relationship to GLI proteins. *The Journal of Biological Chemistry*, **276** (3), 2180-2188

Montague, D.M., Lawler, C.P., Mailman, R.B., & Gilmore, J.H. (1999) Developmental regulation of the dopamine d1 receptor in human caudate and putamen - a new frontier in developmental neurobiology. *Neuropsychopharmacology*, **21**, 641-649

Moon, R.T., Bowerman, B., Boutros, M., & Perrimon, N. The promise and perils of wnt signaling through ß-catenin, *Science*, **296**, 1644-1646

Mueller, T. & Guo, S. (2009) The distribution of GAD67-mRNA in the adult zebrafish (teleost) forebrain reveals a prosomeric pattern and suggests previously unidentified homologies to tetrapods, *The Journal of Comparative Neurology*, **516**, 553-568

Munro, C.A., Mccaul, M.E., Wong, D.F., Oswald, L.M., Zhou, Y., Brasic, J., Kuwabara, H., Kumar, A., Alexander, M., Ye, W. & Wand, G.S. (2006) Sex differences in striatal dopamine release in healthy adults. *Biological Psychiatry*, **59**, 966-974

Nagai T., Aruga J., Minowa O., Sugimoto, T., Ohno, Y., Noda, T., & Mikoshiba, K. (2000) *Zic2* regulates the kinetics of neurulation. *Proceedings of the National Academy of Science*, **97**, 1618–1623

Northmore, D.P.M., Williams, B., & Vanegas, H. (1983) The teleostean torus longitudinalis: response related to eye movements, visuotopic mapping, and functional relations with the optic tectum. *Journal of Comparative Physiology*, **150**, 39-50

Pasca di Magliano, M. & Hebrok M. (2003) Hedgehog signaling in cancer formation and maintenance. *Nature Reviews Cancer*, **3** (12), 903–911

Pasca di Magliano, M., Biankin, A.V., Heiser, P.W., Cano, D.A., Gutierrez, P.J.A., Deramaudt, T., Segara, D., Dawson, A.C., Kench, J.G., Henshall, S.M., Sutherland, R.L., Dlugosz, A., Rustgi, A.K., Hebrok, M. (2007) Common activation of canonical wnt signaling in pancreatic adenocarcinoma. *PlosOne*, **11,** 1155

Peter, R.E., Chang, J.P., Nahorniak, C.S., Omeljaniuk, R.J., Sokolowska, M., Shih, S.H., & Billard, R. (1986) Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. *Recent Progress in Hormone Research,* **42**, 513-548

Pola, J. & Robinson, D.A. Oculomotor signals in medial longitudinal fasciculus of the monkey. *Journal of Neurophysiology*, **41**(2), 245-259

Polakis, P. (2000) Wnt signaling and cancer. *Genes and Development*, **14**, 1837-1851

Rodriguiz, R.M., Chu, R., Caron, M.G. & Wetsel, W.C. (2004) Aberrant responses in social interaction of dopamine transporter knockout mice. *Behavioural Brain Research*, **148**, 185-198

Ross, M.R., Losey, G.S., & Diamond, M. (1983) Sex change in a coral reef fish: dependence of stimulation and inhibition on relative size. *Science,* **221**, 574-575

Ruskin, D., Bergstrom, D., & Walters, J. (2002) Nigrostriatal lesion and dopamine agonists affect firing patterns of rodent entopeduncular nucleus neurons. *Journal of Neurophysiology*, **88**, 487-496

Saegusa, M., Hashimura, M., Yoshida, T., & Okayasu, I. (2001) beta- Catenin mutations and aberrant nuclear expression during endometrial tumorigenesis. *British Journal of Cancer,* **84**, 209-217

Sanek, N.A. & Grinblat, Y. (2008) A novel role for zebrafish zic2a during forebrain development. *Developmental Biology*, **317**, 325-335

Seeman, P.P. (1987) Dopamine receptors and the dopamine hypothesis of schizophrenia. *Synapse*, **1**, 133-152

Semsar, K. & Godwin, J. (2004) Multiple mechanisms of phenotype development in the bluehead wrasse. *Hormones and Behavior,* **45**, 345-353

Semsar, K., Kandel, F.L.M., & Godwin, J. (2001)Manipulations of the AVT system shift social status and related courtship and aggressive behavior in the bluehead wrasse. *Hormones and Behavior*, **40,** 21–31

Striedter, G.F. (1991) Auditory, elecctrosensory,and mechanosensory lateral line pathways through the forebrain in catfishes. *Journal of Comparative Neurology*, **312**, 311-331

Sun, Z. & Reiner, A. (2000) Localization of dopamine d1a and d1b receptor mrnas in the forebrain and midbrain of the domestic chick. *Journal of Chemical Neuroanatomy*, **19**, 211-224

Toyama, R., Gomez, D.M., Mana, M.D., & Dawid, I.B. (2004) Sequence relationships and expression patterns of zebrafish zic2 and zic5 genes. *Gene Expression Patterns*, **4**, 345–350

Tetsu, O. & McCormick, F. (1999) ß-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature*, **398**, 422-428.

Thorpe, C.J., Weidinger, G., and Moon, R.T. (2005) Wnt/ß-catenin regulation of the Sp1-related transcription factor sp5l promotes tail development in zebrafish. *Development,* **132**, 1763-1772

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C. D'Amico, M., Pestell, R., & Ben-Ze'ev, A. (1999) The cyclin D1 gene is a target of the ß-catenin/LEF-1 pathway. *Proceedings of the National Academy of Science*, **96**, 5522-5527

Wang, J.J. & Bannon, M.J.M.J. (2005) Sp1 and sp3 activate transcription of the human dopamine transporter gene. *Journal of Neurochemistry*, **93**, 474-82

Wang, R.Y. and Aghajanian, G.K. (1977) Physiological evidence for habenula as major link between forebrain and midbrain raphe. *Science*, **197**(4298), 89-91

Warner, R.R. (1984) Mating behavior and hermaphroditism in coral reef fishes. *American Scientist,* **72**, 128-136

Warner, R.R. and Swearer, S.E. (1991) Social control of sex change in the bluehead wrasse, *Thalassoma bifasciatum* (Pisces: Labridae). *Biological Bulletin*, **181**, 199-204

Wilczynski, W., Yang, W.J., & Simmons, D. Sex differences and hormone influences on tyrosine hydroxylase immunoreactive cells in the leopard frog. *Journal of Neurobiology,* **56***,* 54-65

Wong, D.F., Broussolle, E.P., Wand, G., Villemagne, V., Danals, R.F., Links, J.M., Zacur, H.A., Harris, J., Naidu, S., Braestrup, C., Wagner Jr., W.N., & Gjedde, A. (1987) In vivo measurement of dopamine receptors in human brain by positron emission tomography, age and sex differences. *Annuals of the New York Academy of Sciences*, **515**, 203-214

Wullimann, M.F., Hofmann, M.H., & Meyer, D.L. (1991) The valvula cerebelli of the spiny eel, *Macrognathus aculeatus,* receives primary lateral-line afferents from the rostrum of the upper jaw. *Cell and Tissue Research*, **266**, 285-293

Yanez, J. & Anadon, R. (1996) Afferent and efferetnt connections of the habenula in the rainbow trout (*Oncorhynchus mykiss*): an indocarbocyanine dye (DiI) study. *The Journal of Comparative Neurology*, **372**, 529-543

Yang, C.C., Kim, H.H.S., Seo, H.H. & Kim, K.K.S. (1998) Identification and characterization of potential cis-regulatory elements governing transcriptional activation of the rat tyrosine hydroxylase gene. *Journal of Neurochemistry*, **71**, 1358-1368

Yang, Y., Hwang, C.K., Junn, E., Lee, G., & Mouradian, M.M. (2000) Zic2 and sp3 repress sp1-induced activation of the human D_{1A} dopamine receptor gene. *Journal of Biological Chemistry*, **275**, 38863-38869

Yokota, N., Aruga, J., Takai, S., Yamada, K., Hamazaki, M., Iwase, T., Sugimura, H., & Mikoshiba, K. (1996) Predominant expression of human zic in cerebellar granule cell lineage and medulloblastoma. *Cancer Research*, **56**, 377-383

CHAPTER III

SEXUAL PHENOTYPE DIFFERENCES IN *ZIC2* MRNA ABUNDANCE IN THE PREOPTIC AREA OF A PROTOGYNOUS TELEOST

Abstract

The highly conserved members of the *zic* family of zinc-finger transcription factors are primarily known for their roles in embryonic signaling pathways and regulation of cellular proliferation and differentiation. Studies aimed at understanding the conserved functions of *zic* transcription factors during vertebrate brain development have targeted z*ic2* as one of the few genes, independent of the hedgehog pathway, linked to the congenital malformation of the forebrain termed holoprosencephaly (HPE). This study describes sexual phenotype differences in abundances of *zic2* mRNA in the preoptic area of the hypothalamus in an adult teleost, the bluehead wrasse (*Thalassoma bifasciatum*). The bluehead wrasse is a well-studied model for exploring neuroendocrine processes associated with socially-induced protogynous sex change and also displays intrasexual dimorphism with two discrete male phenotypes, female-mimic initial phase (IP) males and territorial, terminal phase (TP) males.

We cloned a 727bp sequence for neural *zic2* from field-collected animals and previously demonstrated widespread localization of *zic2* signal in bluehead brain tissue. Microarray-based findings generated from our laboratory suggested *zic2* was upregulated in terminal phase males relative to females, but recent work found no significant difference in cerebellar *zic2* mRNA abundance across sexual phenotypes. *In situ* hybridization with [α-^{35}S]CTP-labeled riboprobe was employed to further assess the relative abundance of brain *zic2* mRNA across sexual phenotypes. Hybridization signal density was assessed in the preoptic area of the hypothalamus, a region strongly implicated in sexual behavior and function. Autoradiographic and phosphor screen quantification methods showed z*ic2* mRNA hybridization signal intensity was significantly elevated in terminal phase males relative to

both initial phase males and females. Silver grain counts in the preoptic area confirmed this relationship across phenotypes. By contrast, no significant difference in hybridization signal was found across phenotypes in the habenula, a brain region not implicated in the control of sexual behavior. Although not directly tested in this study, work in other systems has linked *zic2* to the direct regulation of DRD1 receptors as well as found *zic2* signal overlapping with known markers of neurogenesis in adult animals. This suggests at least a potential role for *zic2* in mediating behavioral sex change and neural plasticity.

Introduction

The bluehead wrasse, *Thalassoma bifasciatum*, is a well-studied model for studying sex change and neuroendocrine processes associated with sex and role reversal. This species displays two discrete male phenotypes, initial phase (IP) males and territorial, terminal phase (TP) males, and undergoes socially-induced sex change (Warner, 1984; Godwin et *al*., 1996; Bass and Grober, 2002). Terminal phase (TP) males are large and brilliantly colored and most defend reef spawning sites aggressively. Defense of a spawning site provides TP males with access to females, who visit the site where TP males court and spawn. Females and initial phase (IP) males share a similar external morphology consisting of less vivid yellow and brown coloration and smaller body size. Initial phase (IP) males may act as female-mimics to enable "sneak spawning" and to gain access to a female within a TP male's territory (Warner, 1984; Semsar et *al.*, 2001; Semsar and Godwin, 2004). Initial phase (IP) males are not territorial or aggressive, but may undergo sex role change to TP male status. Likewise, the largest female within a social group will undergo sex-change to become a TP

male if social conditions permit (large relative size and social dominance – Warner and Swearer, 1991; Godwin et *al*., 1996). Behavioral changes including aggression and TP-male typical courtship behavior occur in minutes in these females while development of secondary sexual characteristics, including color change, and gonad modification is complete in less than two weeks (Warner and Swearer, 1991). The bluehead wrasse is therefore a model system that allows the study of neuroendocrinological processes underlying both rapid behavioral and gonadal sex change within a natural context.

Zic family members encode zinc-finger transcription factors composed of five tandem Cys2His2 zinc-finger domains (Grinberg and Millen, 2005). The highly conserved members of the *zic* family of zinc-finger transcription factors play critical roles in upregulating proliferation factors and delaying differentiation during development (Merzdorf, 2007), but we know little about their role in mature tissues. Several works have found that *zic* may serve as a potential marker for abnormal cell growth (Yokota et *al.*, 1996; Grinberg and Millen, 2005), and *zic2* mRNA is expressed in at least 50% of a wide range of human cancers (Gure et *al.*, 2000).The overexpression of *zic2* may influence node metastasis (Chen et *al.,* 2004; Bidus et *al*., 2006), and *zic2* is one of several transcription factors that bind to the β-catenin gene (CTNNB1; Li et *al*., 2007) whose overexpression has been observed in up to 38% of endometrial cancer cases (Saegusa et *al.*, 2001). With increasing evidence suggesting that molecular pathways important to development remain active in specific adult tissues and that cellular deregulation within such regions contributes to tumor formation (Pasca di Magliano and Hebrok, 2003), understanding the function of *zic* transcription factors in mature animals

is of great fundamental and applied importance. *Zic* genes are evolutionarily conserved and appear to hold important roles in regulatory signaling that not only coordinates normal cell growth and differentiation, but also disease pathways and tumorigenesis (Logan and Nusse, 2004).

Studies aimed at understanding the conserved roles of *zic* transcription factors during vertebrate brain development and patterning have found them critical to proper mammalian neurulation and morphology (Nagai et *al.*, 2000; Inoue et *al.*, 2008). Both *zic1* and *zic2* are strongly expressed in the developing telencephalon and mesencephalon, specifically the medial septal nucleus, the thalamic nuclei, and preoptic nucleus (Aruga et *al.*, 2002), and *zic2* has been shown to be essential to formation of midline central nervous system structures (Nagai et *al.*, 2000). In particular, *zic2* has been targeted as one of the few genes, independent of the hedgehog pathway, linked to the severe and prevalent congenital malformation of the forebrain termed holoprosencephaly (HPE) (Benedyk et *al*., 1994; Merzdorf, 2007). Related studies work towards the critical goal of uncovering the molecular basis underlying *zic* mutation-linked birth defects.

A number of proteins are known to be regulated by *zic* transcription factors (Aruga, 2004, Merzdorf, 2007). To date, however, only a few direct downstream targets of *zic2* have been identified: *ApoE (*Salero et *al.*, 2001*)*, α*CAM kinase II* (Sakurada et *al.*, 2005), and activator region 1 (AR1) of dopamine receptor D1 (Yang et *al.*, 2000) (cited from Merzdorf, 2007). These targets may suggest functions for *zic2* in differentiated tissues as the early developmental roles for *ApoE*, α*CAM kinase II*, and dopamine receptor D1 remain unclear (Merzdorf, 2007). Although the activities of *zic* genes in differentiated tissues outside of the

cerebellum are poorly understood, Herrera and collaborators were able to show a necessity of *zic2* in the specification and guidance of RGCs projecting ipsilaterally at the midline (2004). Many describe adult brain expression of *zic2* as restricted to the cerebellum and functioning as a marker for granule cell neurons and their precursors (e.g., Salero and Hatten, 2007). However, Aruga and collaborators (1994), Brown and Brown, (2009), and our recent localization work on neural *zic2* in the bluehead wrasse have described additional labeling in the forebrain. This study compares neural *zic2* mRNA abundances across sexual phenotypes in the adult bluehead wrasse (*Thalassoma bifasciatum*) and offers insight into *zic2's* potential role as a regulatory factor in sex change. Teleost genomes appear to encode seven *zic* genes, perhaps from a duplication event, but *zic2/zic2a* is one of the five highly conserved *zics* among vertebrate groups (Merzdorf, 2007).

Zic2 functions as a transcriptional repressor and has been shown to regulate tissue specific expression of the dopamine receptor D1 (DRD1, D1A) by binding to activator region 1 (AR1), one of its few direct downstream targets (Yang et *al*., 2000). Yang and collaborators (2000) noted that the absence of *zic2* in the striatum appeared to permit high DRD1 mRNA expression, whereas high levels of *zic2* in the cerebellum may be repressing DRD1 gene transcription. Consistent with this hypothesis, z*ic2* and DRD1 genes have contrasting brain region distributions (Jung et *al.*, 1996; Sun et *al*., 2000), and our recent localization study illustrated *zic2* expression could potentially be co-localized with dopaminergic targets in the preoptic area, a region known to be associated with sexual behavior and reproductive function. Our objective in this study is to examine the abundance of neural *zic2* mRNA expression across the three sexual phenotypes of the bluehead wrasse.

In considering the relative abundance differences in neural *zic2* across phenotypes, we also discuss the potential implications of a possible interaction between *zic2* and the dopaminergic system in the brain of this protogynous teleost. In the bluehead wrasse, *zic2* signaling may play a regulatory role in the neural flexibility and endocrine signaling adaptation necessary for sex change.

The cloning and use of a 727bp sequence most similar to teleost *zic2/zic2a* was previously utilized to map *zic2* mRNA expression in the adult bluehead wrasse brain (Chapter II). We found *zic2* mRNA expression in the adult bluehead wrasse brain not only extremely abundant in the granular cells of the cerebellum, but expression was also widespread throughout the brain, including in the thalamus, hypothalamus, habenula, torus semicircularis, torus longitudinalis, medial longitudinal fascicle, and telencephalic areas (Chapter II). Microarray data generated from our lab suggested that *zic2* was upregulated in terminal phase males relative to females (Passador-Gurgel, unpublished data), and our prior localization study revealed z*ic2* mRNA to be present in regions known to possess dopaminergic innervation and implicated in the sex-change pathway. The previous study showed no significant difference in *zic2* mRNA abundance in the cerebellum across phenotypes, suggesting differences in other areas might account for the microarray results. Here, we investigated the possibility that *zic2* mRNA abundance is elevated in the preoptic area of terminal phase male blueheads relative to females and initial phase males.

Materials and Methods

Study site and species: Adult bluehead wrasses (*Thalassoma bifascidum*) of all three phenotypes were collected from patch reefs in the Florida Keys National Marine Sanctuary near Key Largo, Florida (25°13'W, 80°14'W) under permit during July 2008. Females, initial phase (IP) males, and terminal phase (TP) males were captured using a lift net as described previously (Warner and Swearer, 1991; Godwin et *al.*, 1996). In order to ensure accurate behavioral phenotyping, captures occurred between 0900h-1300h over two consecutive days. Fish were observed before capture to verify spawning activity. Captured fish were sacrificed immediately (within three minutes) using an overdose of MS-222 (tricaine methanesulfonate, Sigma, St. Louis, MO). Before harvesting of tissue, the length of all fishes was measured (standard length; mean: F=69.0mm (62.3-75.8mm), IP=68.9mm (61.4-82.9mm), TP=88.9mm (84.2-94.0mm)) and sex confirmed by visualization of genital papillae, gonadal dissection, and/or expression of sperm or eggs following manual pressure on the abdomen. Within approximately three minutes post-sacrifice, brain dissection was complete and samples snap frozen. Brains were transported on dry ice to the laboratory at NCSU where they remained at -80°C until further processing. Brain tissue was embedded in OCT compound (Tissutek, Durham, NC), coronally cryosectioned at 20μm, and transferred onto Superfrost slides (Fisher Scientific, St. Louis, MO). In order to allow comparison of antisense and control treatments across adjacent sections, consecutive sections were positioned on six alternating slides. All slides were stored at -80°C until used in *in situ* hybridization. All experimental methods were in compliance with the guidelines of the Institutional Animal Care and Use Committee of North Carolina State University (NCSU).

Preparation of riboprobe template DNA: Previous work in our lab generated a bluehead microarray database and cDNA plasmid stocks (pBluescript vector; Stratagene, La Jolla, CA) incorporating partial clones of cDNA generated from the brain tissue of field-collected fishes. *Zic2* cDNA was amplified with Taq Polymerase (Promega, Madison, WI) and M13 forward and reverse primers. The PCR product was run on a 1% agarose gel to verify plasmid size, purified with a QIAquick PCR Purification Kit (Quiagen, Valencia, CA) according to the manufacturer's direction, and sequenced. Comparison of the 727 bp sequence using BLAST (NCBI) showed greatest homology to *Gasterosteus aculeatus* (three-spined stickleback; 91%; BT027912.1) and *Danio rerio* (zebrafish; 84%; AF151535.1) and supported the identification as *zic2*. Confirmed and purified PCR product was transformed with JM109 competent cells (Sigma, St. Louis, MO) and plated onto LB agar plates with ampicillin. Colonies were blue/white screened with X-Gal (Promega, Madison, WI) for insert incorporation, and selected colonies were grown in LB broth. Plasmid was purified via a QIAprep Spin Miniprep Kit (Quiagen, Valencia, CA), digested with ECOR1 (Promega, Madison, WI) and HINDIII (Promega, Madison, WI), and run on a gel to observe insert size against uncut plasmid. Upon confirmation of the *zic2* insert, plasmid was either linearized with ECOR1 for antisense template or HINDIII for sense template. To additionally control for probe specificity, an alternate 315bp antisense *zic2* template was cut from the same plasmid and sequence via BAMHI digestion (Promega, Madison, WI). Linearized template was gel purified and extracted with a QIAquick Gel Extraction Kit (Quiagen, Valencia, CA). Purified and linearized *zic2* template DNA was stored at -20°C until probe reaction.

Probe reaction and in situ hybridization: Riboprobe *in situ* hybridization was used to assess the relative abundance of brain *zic2* mRNA across sexual phenotypes. The protocol followed was described previously for assessing mRNA abundances in rat brain tissue (Patisaul et *al.*, 1999). The *in situ* hybridization was performed using a 727 bp ^{35}S-labeled RNA probe transcribed from template *zic2* DNA. The RNA probe was synthesized with a MAXIscript In Vitro Transcription Kit (Ambion, Austin, TX) and labeled with [α-^{35}S]CTP (1250 Ci/mmol, 70mCi/ml; Perkin Elmer, Boston, MA). Antisense probe from both 727bp and 315bp *zic2* template was transcribed using T7 RNA polymerase within the MAXIscript kit, while sense probe was transcribed using T3 Polymerase (Promega, Madison, WI). Probe reactions were ethanol precipitated overnight and kept at -20^{o}C until addition to hybridization buffer. Slides were washed and then incubated at 50^{o}C with pre-hybridization buffer overnight under moist chamber conditions. RNAse control slides went through an additional one hour ribonuclease A digestion step (60µg/ml, 37°C) (Sigma, St. Louis, MO) during standard washes that followed overnight pre-hybridization. Probes were denatured at 95°C for 2-3 minutes and immediately chilled in ice before addition to warmed hybridization buffer. Hybridization buffer contained tRNA and Tris•HCL, pH 7.5, EDTA DTT (TED) at protocol concentrations; 15/20 buffer, 4/20 probe +TED, 1/20 tRNA (Patisaul et *al*., 1999), and probe was added at 190ng/µl/kb (138ng/µl) for each reaction. A probe concentration level of 170ng/µl/kb was previously shown saturating in quantification of the strongly *zic2* expressing cerebellum, and in a probe concentration control, levels were shown saturating down to 85ng/µl/kb (Chapter II). Hybridization buffer solution was applied to prepped slides and incubated overnight.

Following post-hybridization washes, slides were placed on Kodak BioMax MR film for 73 hours at room temperature (Kodak, Rochester, NY) for autoradiographic quantification. After film exposure, slides were placed on Kodak phosphor screens (Carestream Health, New Haven, CT) for five days following which radioisotopic hybridization signal was read on a Bio-Rad Molecular Imager FX scanner (Bio-Rad Laboratories, Inc., Hercules, CA). Within two-weeks post-processing, slides were dipped in Kodak NTB3 autoradiographic emulsion (Carestream Health, New Haven, CT), dried, and exposed in a light-sealed box at 4°C for three weeks. Exposed slides were developed for four minutes (Kodak D19; Kodak, Rochester, NY), rinsed briefly with running water, and fixed for 12 minutes (Kodak Fixer; Kodak, Rochester, NY) under safelight conditions. After rinsing for approximately 20 minutes under running water, slides were cresyl violet stained and coverslipped with Permount (Fisher Scientific, St. Louis, MO) to observe morphology. All images were taken using a Retiga 2000R, 12 Bit Color Camera (QImaging, Surrey, BC, Canada) and MCID Image Capture software (InterFocus Imaging Ltd., Linton, Cambridge, UK).

Quantification of hybridization signal and comparisons across sexual phenotypes: Quantitative regional autoradiography using MCID software (InterFocus Imaging Ltd., Linton, Cambridge, UK) was employed for a high resolution measurement of signal density (indicating relative hybridization signal) in the preoptic area of the hypothalamus across phenotypes. Typically, four sections from each animal were used for quantification of optical density, but this ranged from two to six, with sample sizes for the different sexual phenotypes totaling five females (19 sections; 3-5 sections per animal), eight initial phase

males (29 sections; 2-5 sections per animal), and seven terminal phase males (39 sections; 3-6 sections per animal). A rotatable ellipse (120 X 75μm) drawn using MCID Densitometry software was utilized for the quantification procedure. The elliptically-shaped template was designed to tightly border visible signal in the preoptic area of female phenotypes. The template was placed centrally along the midline in a position encompassing the entire preoptic area but more tightly aligned to boundaries of signaling in female animals. The area of quantification and placement of the template was kept constant across all sections and animals to ensure accuracy of quantification procedures. Background density measures, taken immediately adjacent to the template with the same ellipse, were subtracted from each reading, and density values were recorded for all sections exhibiting hybridization signal in the preoptic area. Sections from individual animals were averaged and mean density readings were entered into JMP 7.0.0 (SAS Institute, Cary, NC) and analyzed via Oneway ANOVA and Tukey-Kramer tests to localize differences. Prior to autoradiographic quantification, Quantity One 4.6.6 analysis software (Bio-Rad Laboratories, Inc., Hercules, CA) allowed measurement of hybridization signal from phosphor screen scans. A 203μm^2 circular region was applied over sections showing signal in the pre-optic area of the hypothalamus and habenula (following the quantification procedure described), and the hybridization signal of each target area was computed in counts/mm^2 by the analysis software. Typically, three to four sections from each animal were used for this lower resolution quantification procedure. Phenotype sample sizes for preoptic area quantification totaled 5 females (16 sections; 2-4 sections per animal), 8 initial phase males (25 sections; 2-5 sections per animal), and 7 terminal phase males (23 sections; 2-4 sections per animal).

Phenotype sample sizes for habenula quantification totaled 5 females (15 sections; 2-4 sections per animal), 8 initial phase males (27 sections; 2-5 sections per animal), and 7 terminal phase males (29 sections; 3-5 sections per animal). Hybridization signals taken throughout the preoptic area were averaged across sections for each animal, and statistical analysis of hybridization signal across phenotypes was performed using JMP 7.0.0 (SAS Institute, Cary, NC). Hybridization signal for *zic2* was compared in the preoptic area and habenula across sexual phenotypes using analysis of variance (ANOVA) followed by Tukey-Kramer procedure for post hoc comparisons where ANOVA indicated a significant difference overall. Density values collected from the two screens utilized were standardized based on a comparison of background measurements, and sexual phenotypes were randomly distributed across both screens. The density values for the phenotypes were independent from the screens (Two-way ANOVA; $F_{1, 14} = 0.3479$, $p = 0.5647$) and from the interaction between the screens and phenotypes (Two-way ANOVA; $F_{2, 14} = 0.4223$, $p = 0.6636$).

Quantification of individual silver grains in the preoptic area was conducted for signal comparison across phenotypes at the cellular-level using MCID software (InterFocus Imaging Ltd., Linton, Cambridge, UK). Typically, four of five sections from each animal were used for quantification purposes, but ranged from three to six, with sample sizes for the different sexual phenotypes totaling four females (17 sections; 4-6 sections per animal), five initial phase males (22 sections; 3-6 sections per animal), and six terminal phase males (34 sections; 5-7 sections per animal). A rectangle (245 X 65μm) drawn using MCID software was utilized for the quantification procedure. Grain counts were recorded from one hemisphere of all animals. The template was designed to tightly border visible signal on one

side of the preoptic area of female phenotypes and was placed parallel to the midline in a fixed anatomical position across all animals. The area of quantification and placement of the template was kept constant across all sections and animals to ensure accuracy of quantification procedures. Silver grain background taken adjacent to the area sampled was subtracted from each reading taken. Sections from individual animals were averaged and mean silver grain count readings were entered into JMP 7.0.0 (SAS Institute, Cary, NC) and analyzed via Oneway ANOVA followed by a Tukey-Kramer test. For silver grain quantification data, a Wilcoxon signed-rank test was additonally conducted to address concerns of variance homogeneity. To assess the importance of standard length and phenotype on observed mRNA abundance, a multivariate stepwise regression was utilized. This analysis was repeated for film, phosphor screen, and silver grain quantification techniques.

Results

Zic2 mRNA sexual phenotype comparison: Control treatment with a sense-strand probe showed non-specific labeling in the glomerular nucleus and optic tectum. Comparison of adjacent sense strand sections to antisense sections from the same animal was utilized to correct for background and identify potential non-specific labeling (Figure 3). The alternate, 315bp antisense *zic2* probe (BAMH1digested clone; Promega, Madison, WI) showed identical patterns of labeling to the probe generated from the 727bp *zic2* template, and pre-digestion with RNaseA eliminated target RNA.

Hybridization signaling was clearly apparent in the preoptic area of the hypothalamus across all three phenotypes, but appeared stronger in terminal phase males (Figure 1). Signaling was assessed through phosphor imaging screens, autoradiographic film optical density, and silver grain quantification techniques. Hybridization signal density was significantly different in the preoptic area of the hypothalamus between the phenotypes by each method. Both phosphor screen analysis and autoradiographic optical density quantification procedures demonstrated higher abundance of *zic2* mRNA in terminal phase males relative to initial phase males and females (Tukey-Kramer, $p<0.05$) (Figure 5; ANOVA, autoradiographic quantification: $F_{2, 17} = 5.6930$, $p=0.0128$; phosphorimaging screens: $F_{2, 17} = 4.3873$, $p=0.0291$). Both a significant correlation between mean absolute density values from autoradiographic film optical density and phosphor screen quantification and with ranking of values from individuals demonstrated consistency in relative values between measurement techniques (Figure 8, absolute value: $R = 0.6976$, $p = 0.0006$; ranking: $R = 0.7097$, $p = 0.0005$). Correlation of autoradiographic film optical density and silver grain quantification via mean absolute density measures and ranking of individuals again showed consistency in relative values between density-based and grain counting techniques (Figure 9, absolute value: $R = 0.7650$, $p = 0.0023$; ranking: $R = 0.7692$, $p = 0.0021$). Silver grain quantification in the preoptic area shared a consistent relationship between phenotypes to that demonstrated with density comparisons. Grain counts in the preoptic area of terminal phase males were significantly higher than both females and initial phase males (Figure 6; ANOVA; $F_{2, 12} = 15.2390$, $p= 0.0005$; Wilcoxon signed-rank test, $p = 0.0063$). No significant difference was found in *zic2* signal across phenotypes in the habenula

on phosphorimaging screens ($F_{2, 17} = 2.1764$, $p = 0.1440$). Comparison across silver grain, autoradiographic film optical densities, and phosphor screen techniques demonstrated a consistent relationship between phenotypes (ratios of means silver grain/film/screens; F:IP = 0.9939/0.8827/0.9057; TP:IP = 1.7600/1.4066/1.4072; TP:F = 1.7703/1.5934/1.5535; Figure 7). For the three techniques, phenotype was the only variable which entered into the stepwise model and explained part of the variation observed in quantification. No correlation was observed between standard length (silver grain, $p = 0.4835$; film, $p = 0.6143$; screen, $p = 0.8735$) and mRNA abundance. The model considered values for initial phase males and females together (silver grain, $p = 0.9730$; film, $p = 0.4907$; screen, $p = 0.6167$) and demonstrated that only the relationship between females and initial phase males to terminal phase males explained part of the variation observed (silver grain, $p=0.0001$, R = 0.8471; film, $p = 0.0036$, R = 0.6194; screen, $p = 0.0080$, R = 0.5748).

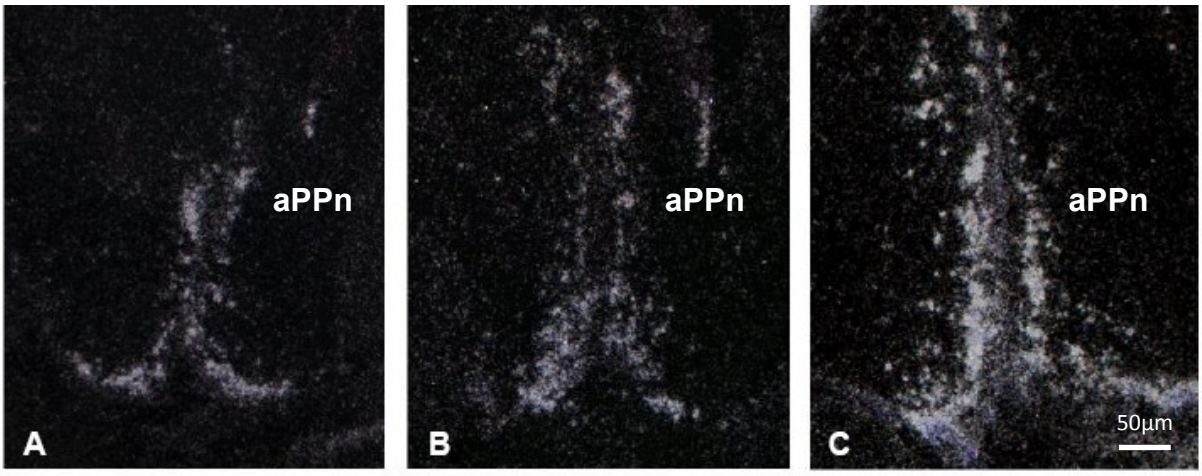

Figure 1. Expression of zic2 mRNA in the anterior parvocellular preoptic nucleus (aPPn) of the hypothalamus across female (A), initial phase male (B), and terminal phase male bluehead wrasse (C). Darkfield images of silver grain localization and density were taken at 100X total magnification.

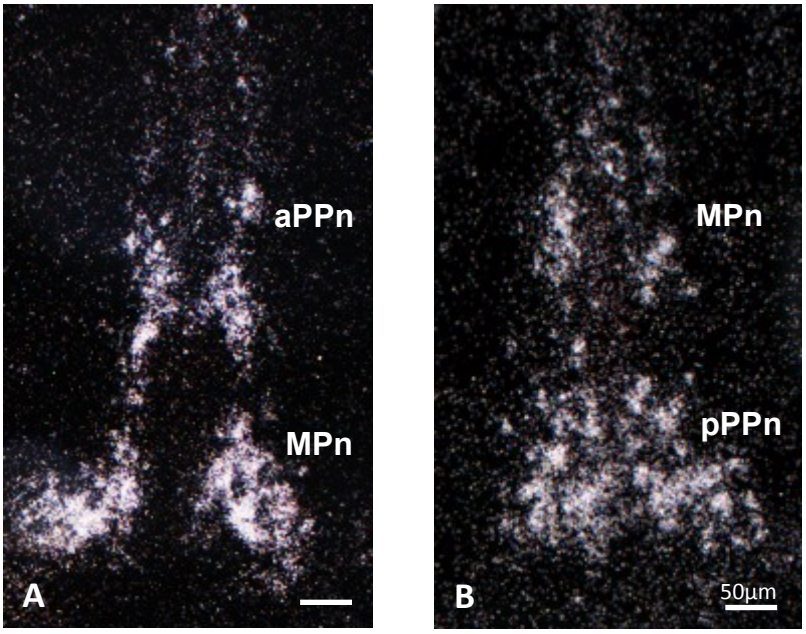

Figure 2. *Zic2* mRNA signaling in the anterior parvocellular (aPPn) and magnocellular preoptic nuclei (MPn) (A) and magnocellular (MPn) and posterior parvocellular preoptic nuclei (pPPn) of the hypothalamus (B). Darkfield images of silver grain localization and density were taken at 100X total magnification.

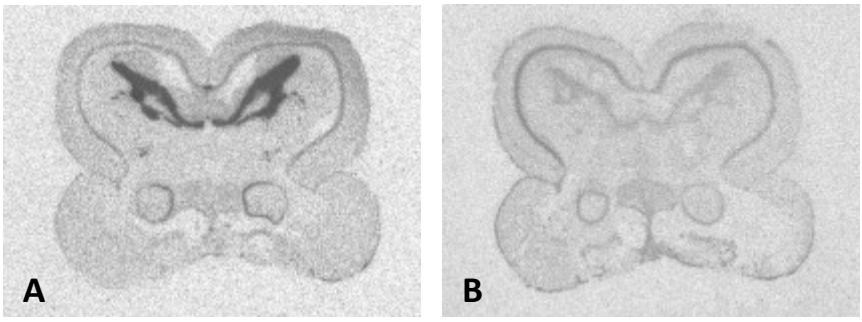

Figure 3. Control treatment demonstrating *zic2* antisense probe (A) compared to sense strand probe (B). Images were taken on autoradiographic film.

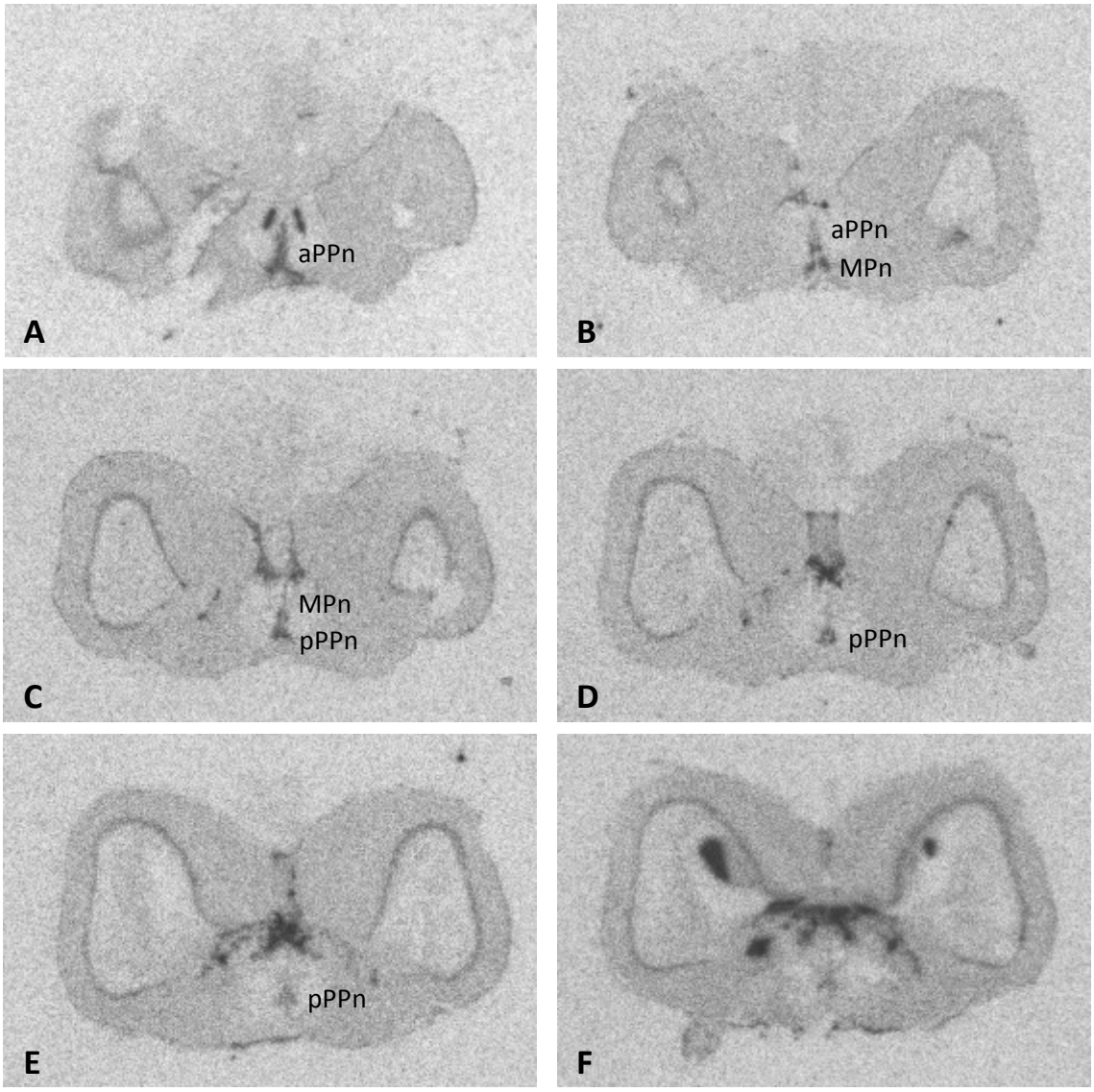

Figure 4. Series of autoradiographic images demonstrating *zic2* signal throughout the preoptic area of the hypothalamus and forebrain (A-F); anterior parvocellular (aPPn), magnocellular preoptic nuclei (MPn), and posterior parvocellular preoptic nuclei (pPPn) of the hypothalamus. Note signal localization in regions known to exhibit tyrosine hydroxylase expression in zebrafish (Rink and Wullimann, 2001) and bluehead wrasses (Marsh et *al*., 2006).

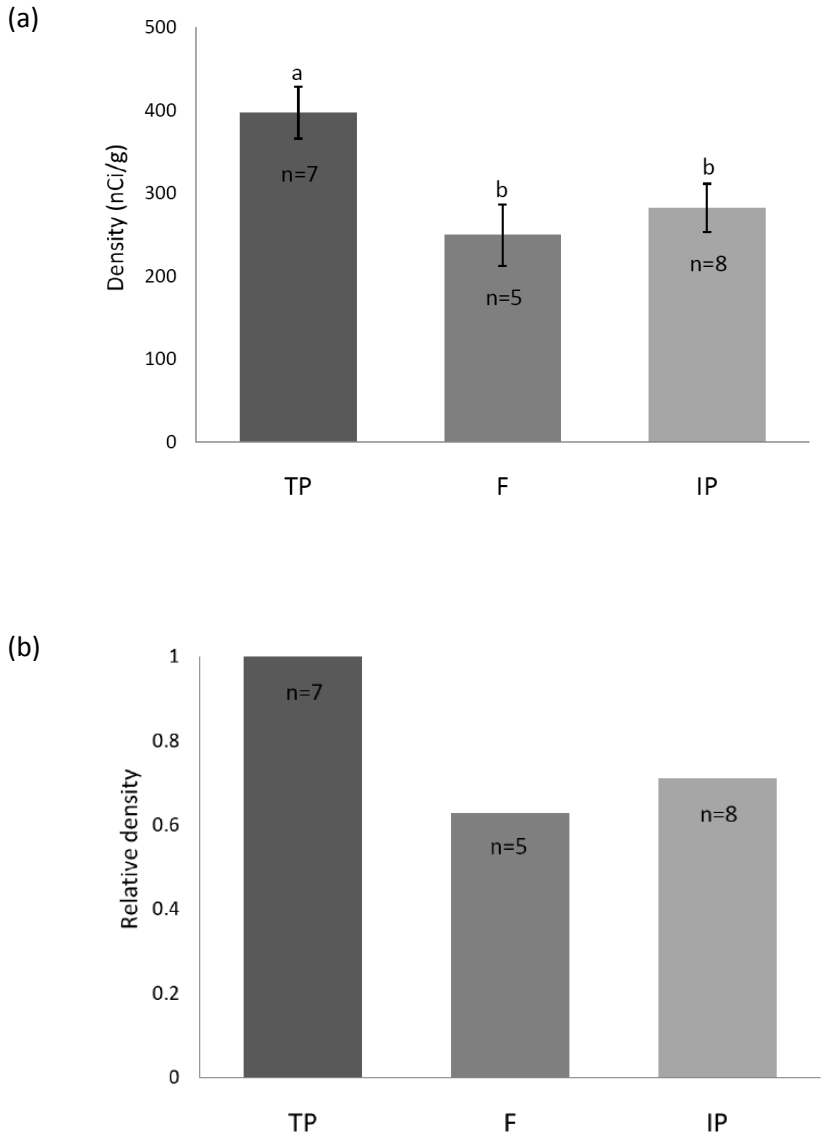

Figure 5. (a) Mean density of *zic2* mRNA signal abundance and (b) relative density of *zic2* mRNA in the preoptic area of the hypothalamus across phenotypes using autoradiographic quantification; terminal phase male (TP) (n=7; mean = 397.64; SE = 31.20), initial phase male (IP) (n=8; mean = 282.69; SE = 29.18), and female (F) (n=5; mean = 249.55; SE = 36.91). A higher abundance of *zic2* mRNA was observed in terminal phase males relative to initial phase males and females.

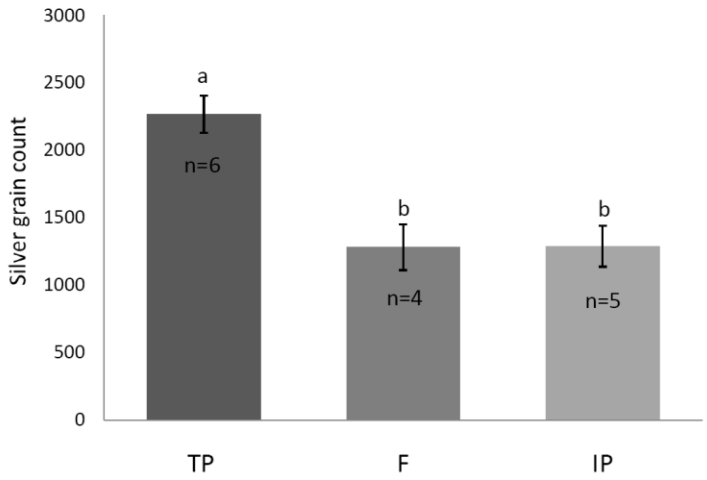

Figure 6. Mean silver grain counts of *zic2* mRNA quantified in the preoptic area of the hypothalamus across phenotypes; terminal phase male (TP) (n=6; mean = 2268.39; SE = 137.89), initial phase male (IP) (n=5; mean= 1289.16; SE = 151.05), and female (F) (n=4; mean = 1281.33; SE = 168.87). Grain counts in the preoptic area of terminal phase males were significantly higher than both females and initial phase males.

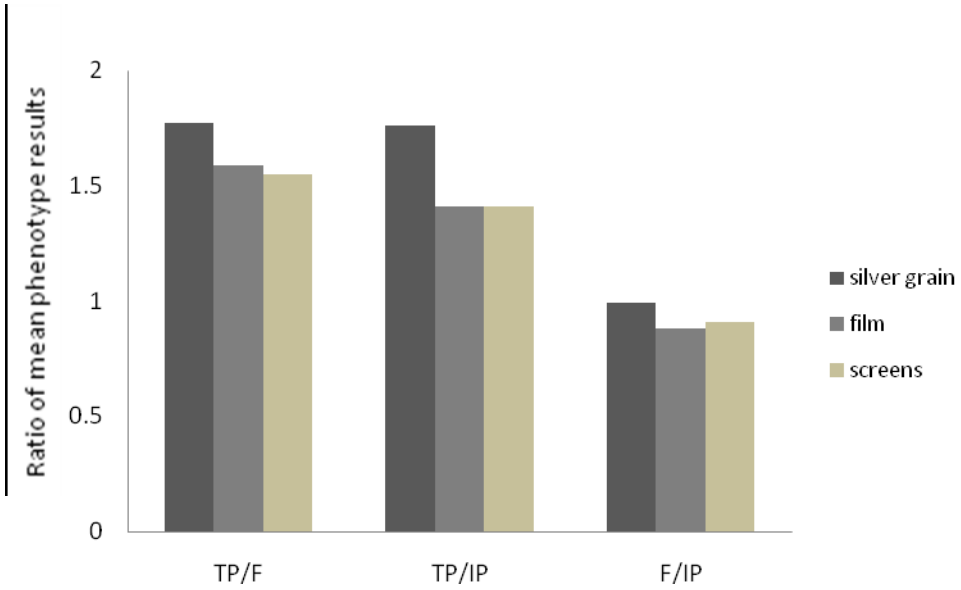

Figure 7. Comparison of quantification procedures for *zic2* mRNA localized in the preoptic area of the hypothalamus. Ratios of mean signal density or mean grain totals for each phenotype was examined across techniques: silver grain counts and autoradiographic film and phosphor screen density readings. Silver grain data is based on a smaller sample size: terminal phase male (TP) (n=6), initial phase male (IP) (n=5), and female (F) (n=4). Film and screen data sample size: terminal phase male (TP) (n=7), initial phase male (IP) (n=8), and female (F) (n=5).

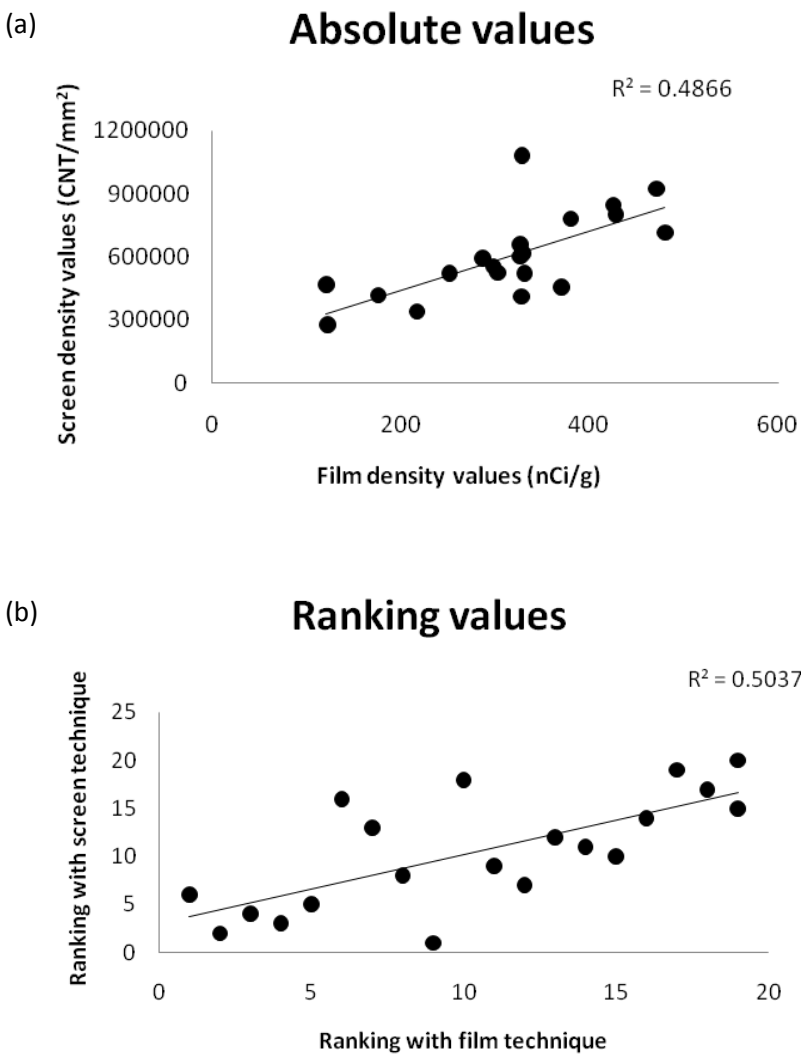

Figure 8. (a) Absolute mean density values and (b) ranking of individual animals used in autoradiographic film and phosphor screen density quantification; (TP) (n=7), initial phase male (IP) (n=8), and female (F) (n=5).

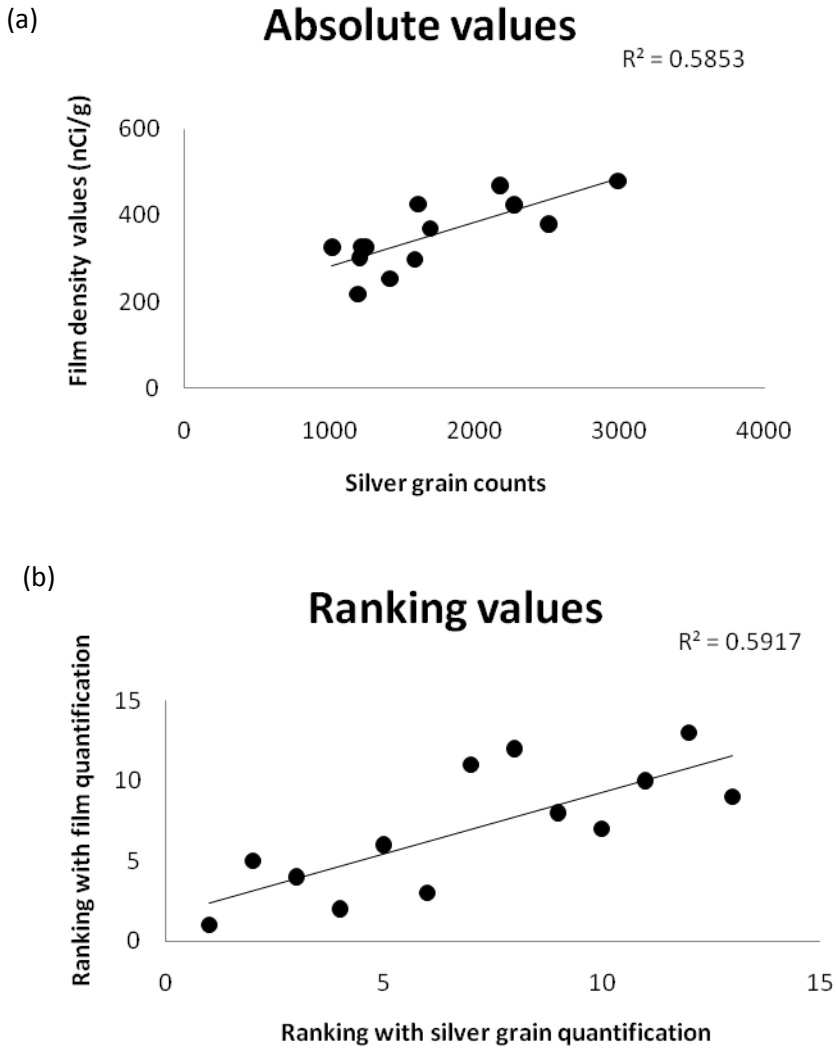

Figure 9. (a) Absolute mean density values and (b) ranking of individual animals used in autoradiographic film density and silver grain quantification; terminal phase male (TP) (n=6), initial phase male (IP) (n=5), and female (F) (n=4).

Discussion

This study describes the phenotypic distribution of neural *zic2* abundance in the preoptic area of the hypothalamus of a protogynous teleost, the bluehead wrasse (*Thalassoma bifasciatum*). We confirmed that *zic2* mRNA was more abundant in the preoptic area of terminal phase (TP) males relative to both initial phase (IP) males and females, but found no significant difference in *zic2* mRNA in the habenula across phenotypes. We previously reported that *zic2* mRNA expression in the adult bluehead wrasse brain was not only extremely strong in the granular cells of the cerebellum, but also widespread throughout the brain, including in the thalamus, hypothalamus, habenula, torus semicircularis, torus longitudinalis, medial longitudinal fascicle, and telencephalic areas (Chapter II). Consistent with the comparisons in habenula here, phenotype comparisons found no significant differences in *zic2* mRNA abundances in the cerebellum (Chapter II).

Most studies on *zic* genes are focused on their central roles as transcription factors during the embryonic developmental period or related to cellular proliferation and the potential tumorigenic aspects of the z*ic* family. Salero and Hatten (2007) describe the neural localization of *zic2* in mature animals as restricted to the cerebellum and as a marker for granule cell neurons and their precursors. However, consistent with mouse studies by Aruga and collaborators (1994) and Brown and Brown (2009), our work on the bluehead wrasse describes *zic2* mRNA expression as distributed more widely in the adult animal brain. To our knowledge, no studies have looked at mature teleost *zic2* neural localization or compared *zic2* abundance across phenotypes. Interestingly, z*ic2* expression in the developing mouse brain (Aruga et *al.*, 2002) shows a somewhat similar pattern of labeling to that observed in

the adult bluehead wrasse brain. Regions of cellular proliferation and neurogenesis in the adult zebrafish, as described by Chapouton and collegues (2007), are in agreement with our observed patterns of adult bluehead *zic2* signaling. Recently, it has also been demonstrated that *zic2* expression overlaps with known markers of neurogenesis in the adult mouse brain (Brown and Brown, 2009). Therefore, we suggest that the differential expression of *zic2* mRNA in the preoptic area of bluehead wrasses may potentially be tied to the neural plasticity critical to environmentally-mediated sex change.

The preoptic area of the hypothalmus is a critical area for reproductive behavior and function. In addition to disrupting maternal behavior (Jacobson et *al.*, 1980) and facilitating female sexual receptivity (Powers and Valenstein, 1972), bilateral lesions to the preoptic area have been shown to severely impair patterns of male sexual behavior across vertebrate classes. Specific lesion studies have shown preoptic area dependence on male goldfish following, butting, and spawning activity (Koyama et *al.*, 1984), socio-sexual behavior in male rats (Paredes et *al*., 1993), and courtship maneuvers in male starlings (Riters and Ball, 1999). Similarly, electrical stimulation of preoptic and hypothalamic areas has been shown to produce sexual behavioral responses in several vertebrate species, including male sunfish (Demski, 1983), male rhesus monkeys (Perachio et *al*,, 1979), and male rats (Giulano et *al*., 1996). Upon finding phenotype differences in *zic2* mRNA in the preoptic area, we discuss potential regulatory roles *zic2* may play in neuroendocrine signaling underlying the sex-change pathway and what may be promising directions for future research.

As z*ic2* has been shown to directly target and downregulate tissue specific expression of the human dopamine receptor D1 (also referred to as DRD1 or D1A; Yang et *al*., 2000), it is possible that *zic2* acts on dopaminergic innervation within the hypothalamus. The dopaminergic system has been consistently implicated in the regulation of sexual behavior in other models and two studies have examined the involvement of monoamines in sex change in a congener of the bluehead wrasse, *Thalassoma duperrey* (Larson et *al.,* 2003a; Larson et *al.*, 2003b). In many gonochoristic fishes, as in other vertebrates, monoamines control gonadotrophin-releasing hormone (GnRH) and gonadotropes (GtH), which in turn control the release of gonadal steriods (Peter et *al*., 1986; Peter and Yu, 1997). In some teleosts, dopamine (DA) serves as an important regulator of reproduction via inhibition of GnRH secretion, acting to decrease circulating levels of LH and FSH (Peter et *al*., 1986). Dopamine (DA) is closely associated with reward-seeking and sexual behaviors in vertebrates (Bathazart et *al.*, 2002). The nigrostriatal, mesolimbic, and mesocortical dopamine systems appear critical to learning (Arias-Carrion and Poppel, 2007), and studies specifically suggest dopamine projections to the striatum and frontal cortex are essential in mediating effects of reward and acquiring new behavior (Schultz, 2007). Pharmacologically-induced increases in extracellular dopamine are often associated with increased aggressive behavior (De Almeida et *al*., 2005).

The DRD1 AR promoter is one of only a few known direct targets of *zic2*. In cell culture, *zic2* prevents the binding of Sp1 and Sp3 transcriptional activators to the DRD1 AR promoter (Yang et al., 2000). In the saddleback wrasse, *Thalassoma duperrey*, non-specific dopaminergic receptor inhibition via haloperidol induced protogynous sex change in a

socially inhibitory environment (i.e., with a larger individual present; Larson et *al*, 2003a). In the Japanese quail, localization of the rate-limiting enzyme in dopamine synthesis, tyrosine hydroxylase, has shown that neural areas containing high densities of tyrosine hydroxylase fibers also contain high densities of DRD1 receptors (Ball et *al*., 1995). Tyrosine hydroxylase-ir cell bodies and fibers have been described in the preoptic area of teleosts, including the electric fish, zebrafish, and the bluehead wrasse (Meek et *al*., 1989; Rink and Wullimann, 2001; Marsh et *al*., 2006). Based on previous studies of dopaminergic innervation and the known presence of dopamine receptors in the teleostean preoptic area (Rink and Wullimann, 2001), we believe future co-localization studies of *zic2* and DRD1, in addition to field tests with specific dopamine receptor antagonists, will help to further test a potential role of *zic2* and the dopaminergic system in sex change.

The expression of *zic2* appears localized to similar regions of the preoptic area of the bluehead as previous studies mapping neuropeptides and monoamines linked to behavioral sex change, including arginine vasotocin (AVT) and tyrosine hydroxylase (TH), as well as the estrogen biosynthesis enzyme, aromatase. Estrogen levels have been shown to have organizational effects as well as activate sex specific behaviors (Bakker et *al*., 2004; Bathazart et *al*., 2002; Balthazart and Ball, 2006); the aromatization of testosterone to estradiol has been associated with inhibition of teleost sex-change (Black et al., 2005; Marsh, 2007). Estradiol implants inhibited female-to-male sex-change in blueheads under socially-permissive conditions and implanted females showed higher aromatase mRNA expression in the preoptic area than females or IP males (Marsh, 2007). In addition, the co-regionalization of aromatase-ir cell bodies and fibers with tyrosine hydroxylase-ir neurons has been

described in the preoptic area of the bluehead wrasse (Marsh et *al*., 2006), and aromatase activity has been shown to be downregulated by dopamine in quail (Baillen and Balthazart, 2007). Of further note, Lee and Mouradian (1999) observed an estrogen induced upregulation of DRD1 gene transcription in humans. Interestingly, the DRD1 receptor subtype has also been implicated as being responsible for contributing to progestin receptor activation, and DRD1 agonists have been shown to facilitate female sexual behavior in rats, thus mimicking the effects of progesterone (Mani et *al*., 1997). Recent work in male European starlings has found breeding context dependent densities of DRD1 in the medial preoptic area (Heimovics et *al*., 2009). It is possible that regulation of dopamine receptors in the preoptic area and alterations in dopamine responsiveness are closely linked to the expression of socio-sexual behavior and sex change in the bluehead wrasse.

Although the potential role of *zic2* in the sex-change pathway is not directly tested in this study, *zic2* is clearly localized in regions known to be involved in regulating phenotypic plasticity and sexual function. This initial study describing a difference of *zic2* mRNA in the preoptic area of the hypothalmus across sexual phenotypes suggests a potential role in neural plasticity. The function of *zic2* remains relatively unstudied in the adult animal, and this work is therefore novel in adult teleosts. We have provided evidence of sexual phenotype difference in *zic2* expression in the preoptic area of the adult bluehead wrasse, a critical region for male-typical sexual behavior and reproductive function generally. This is also consistent with microarray data from our laboratory that indicate elevated expression of *zic2* in terminal phase males (Passador-Gurgel and Godwin, unpublished data). Although more work is needed, our findings suggest that *zic2,* acting as a transcription factor, could

potentially play a role in mediating sex change through effects on neurogenesis or dopamine signaling. The observed sexual phenotype differences in preoptic area abundance suggest *zic2* may play a role in mediating endocrine signaling necessary for socially-controlled sex change.

References

Arias-Carrión, O.O. & Pŏppel, E.E. (2007) Dopamine, learning, and reward-seeking behavior. *Acta Neurobiologiae Experimentalis*, **67**, 481-488

Aruga, J. (2004) The role of *zic* genes in neural development. *Moelcular and Cellular Neuroscience,* **26**, 205-221

Aruga, J., Inoue, T., Hoshino, J., & Mikoshiba, K. (2002) Zic2 controls cerebellar development in cooperation with Zic. *Journal of Neuroscience*, **22**, 218-225

Aruga, J., Yokota, N., Hashimoto, M., Furuichi, T., Fukuda, M., & Mikoshiba, K.(1994) A Novel Zinc Finger Protein, Zic, Is Involved in Neurogenesis, Especially in the Cell Lineage of Cerebellar Granule Cells. *Journal of Neurochemistry*, **63**, 1880–1890

Baillen, M. & Balthazart, J. (1997) A direct dopaminergic control of aromatase activity in the quail preoptic area. *Journal of Biochemistry and Molecular Biology,* **63**, 99- 113

Bakker, J., Honda, S., Harada, N., & Balthazart, J. (2004). Restoration of male sexual behavior by adult exogenous estrogens in male aromatase knockout mice. *Hormones and Behavior*. **16**, 483-490

Ball, G.F., Casto, J.M. & Balthazart, J. (1995) Autoradiographic localization of D1-like dopamine receptors in the forebrain of male and female japanese quail and their relationship with immunoreactive tyrosine hydroxylase. *Journal of Chemical Neuroanatomy*, **9**, 121-133

Balthazart, J., Baillien, M., & Ball, G.F., (2002) Interactions between aromatase (estrogen synthase) and dopamine in the control of male sexual behavior in quail. *Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology.* 132, 37-55.

Benedyk, M.J., Mullen, J.R., & DiNardo, S. (1994) Odd-paired: a zinc finger pair-rule protein required for the timely activation of engrailed and wingless in Drosophila embryos. *Genes and Development*, **8**, 105-117

Bidus, M.A., Risinger, J.I., Chandramouli, G.V.R., Dainty, L.A., Litzi, T.J., Berchuck, A., Barrett J.C., Maxwell, G.L. (2006) Prediction of Lymph Node Metastasis in Patients with Endometrioid Endometrial Cancer Using Expression Microarray. *Clinical Cancer Research,* **12,** 83-88

Black, M.P., Balthazart, J., Baillien, M., & Grober, M.S. (2005) Socially induced and rapid increases in aggression are inversely related to brain aromatase activity in a sex-changing fish, *Lythrypnus dalli*. *Proceedings of the Royal Society,* **272**, 2435-2440

Brown, L. & Brown, S.(2009) Zic2 is expressed in pluripotent cells in the blastocyst and adult brain expression overlaps with makers of neurogenesis, *Gene Expression Patterns,* **9**, 43-49

Chen, L.C., Vural, B., Ustuner, Z., Saip, P., Ozbek, M., Gonen, L., Old, Y.T., & Gure, A. (2004) Immunoreactivity to SOX-1, -2, -3 and ZIC2 antigens and correlation to survival in small cell lung cancer (SCLC). *Journal of Clinical Oncology*, **22***,* 7159

De Almeida, R.M.M., Ferrari, P.F., Parmigiani, S. & Miczek, K.A. (2005) Escalated aggressive behavior: Dopamine, serotonin and gaba. *European Journal of Pharmacology*, **526**, 51-64

Demski, L.S. (1983) Behavioral effects of electrical stimulation of the brain. *Fish Neurobiology*, vol. 2, Higher Brain Areas and Functions (ed. R. E. Davis and R. G. Northcutt), Ann Arbor: Univeristy of Michigan Press, 317–359

Giuliano, F., Rampin, O., Brown, K., Courtois, F., Benoit, G., & Jardin, A. (1996) Stimulation of the medial preoptic area of the hypothalamus in the rat elicits increases in intracavernous pressure. *Neuroscience Letters,* **209**, 1-4

Godwin, J., Crews, D. & Warner, R.R. (1996) Behavioural sex change in the absence of gonads in a coral reef fish. *Proceedings: Biological Sciences*, **263**, 1683-1688

Grinberg, I. & Millen, K.J. (2005) The ZIC gene family in development and disease. *Clinical Genetics*, **67**, 290-296.

Grober, M.S. & Bass, A.H. (2002) Life history, neuroendocrinology, and behavior in fish. *Hormones, Brain and Behavior,* **2**, 331-347

Gure, A.O., Stockert, E., Scanlan, M.J., Keresztes, R.S., Jager, D., Altorki, N.K., Old, L.J., & Chen, Y.T.(2000) Serological identification of embryonic neural proteins as highly immunogenic tumor antigens in small cell lung cancer. *Proceedings of the NationalAcademy of Science*, **97**, 4198-4203

Heimovics, S.A., Cornil, C.A., Ball, G.F., & Riters, L.V. (2009) D1-like dopamine receptor density in nuclei involved in social behavior correlates with song in a context-dependent fashion in male European starlings *Neuroscience,* **159**, 962-973

Herrera, E, Marcus, R., Li, S., Williams, S.E., Erskine, L., Lai, E., & Mason, C. (2004) Foxd1 is required for proper formation of the optic chiasm. *Development,* **131**, 5727-5739

Inoue, T., Ogawa, M., Mikoshiba, K. & Aruga, J. (2008) *Zic* deficiency in the cortical marginal Zone and meninges results in cortical lamination defects resembling those in Type II Lissencephaly**.** *The Journal of Neuroscience*, **28**(18), 4712-4725

Jacobson, C., Terkel, J., Gorski, R., & Sawyer, C. (1980) Effects of small medial preoptic area lesions on maternal behavior: Retrieving and nest building in the rat. *Brain Research*, **194** (2), 471-478

Jung, A.B. & Bennett, J.P. (1996) Development of striatal dopaminergic function. I. Pre- and postnatal development of mRNAs and binding sites for striatal D1 (D1a) and D2 (D2a) receptors. *Developmental Brain Research*, **94**, 109-120.

Koyama, Y., Satou, M.,Oka Y., & Ueda, K. (1984) Involvement of telencephalic hemispheres and the preoptic area in sexual behavior of the male goldfish, *Carassius auratus*: a brain lesion study. *Behavioral and Neural Biology*, **1**, 70-86

Larson, E.T., Norris, D.O., Gordon Grau, E. & Summers, C.H. (2003a) Monoamines stimulate sex reversal in the saddleback wrasse. *General and Comparative Endocrinology*, **130**, 289-298

Larson, E.T., Norris, D.O. & Summers, C.H. (2003b) Monoaminergic changes associated with socially induced sex reversal in the saddleback wrasse. *Neuroscience*, **119**, 251-263

Lee, S.H. & Mouradian, M.M. (1999) Up-regulation of D1A dopamine receptor gene transcription by estrogen. *Molecular and Cellular Endocrinology*, **156**, 151-157

Mani, S.K., Blaustein, J.D., & O'Malley, B.W. (1997) Progesterone receptor function from a behavioral perspective, *Hormones and Behavior*, **31**, 244-255

Marsh, K.E., Creutz, L.M., Hawkins, M.B. & Godwin, J.J. (2006) Aromatase immunoreactivity in the bluehead wrasse brain, *Thalassoma bifasciatum*: Immunolocalization and co-regionalization with arginine vasotocin and tyrosine hydroxylase. *Brain Research*, **1126**, 91-101

Marsh, K.E. (2007) Neuroendocrine transduction of social cues in the bluehead wrasse, *Thalassoma bifasciatum*. PhD thesis, Zoology, North Carolina State University, Raleigh, NC, 57-78

Meek, J., Joosten, H.W., & Steinbusch, H.W.M. (1989) Distribution of dopamine immunoreactivity in the brain of the mormyrid teleost *Gnathonemus petersii. The Journal of Comparative Neurology*, **282** (3), 362-383

Merzdorf, C.S. (2007) Emerging roles for *zic* genes in early development. *Developmental Dynamics*, **236**, 922-940

Nagai T., Aruga J., Minowa O., Sugimoto, T., Ohno, Y., Noda, T., & Mikoshiba, K. (2000) *Zic2* regulates the kinetics of neurulation. *Proceedings of the National Academy of Science*, **97**, 1618–1623

Paredes, R., Highland,L., & Karam, P. (1993) Socio-sexual behavior in male rats after lesions of the medial preoptic area: Evidence for reduced sexual motivation. *Brain Research,* **618** (2), 271-276

Pasca di Magliano, M. & Hebrok M. (2003) Hedgehog signaling in cancer formation and maintenance. *Nature Reviews Cancer*, **3** (12), 903–911

Patisaul, H.B., Whitten, P.L., & Young, L.J. (1999) Regulation of estrogen receptor beta mRNA in the brain: opposite effects of 17b-estradiol and the phytoestrogen, coumestrol. *Molecular Brain Research,* **67**, 165–171

Peter, R.E., Chang, J.P., Nahorniak, C.S., Omeljaniuk, R.J., Sokolowska, M., Shih, S.H., & Billard, R. (1986) Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. *Recent Progress in Hormone Research,* **42**, 513-548

Peter, R.E. & Yu, K.L. (1997) Neuroendocrine regulation of ovulation in fishes: basic and applied aspects. *Reviews in Fish Biology and Fisheries***, 7,** 173-197

Perachio, A., Marr, L., & Alexander, M. (1979) Sexual behavior in male rhesus monkeys elicited by electrical stimulation of preoptic and hypothamic areas. *Bran Research,* **177** (1), 127-144

Powers, B.& Valenstein, E. (1972) Sexual receptivity: Facilitation by medial preoptic lesions in female rats. *Science*, **175** (4025), 1003-1005

Rink, E. & Wullimann, M.F. (2001) The teleostean (zebrafish) dopaminergic system ascending to the subpallidum (striatum) is located in the basal diencephalon (posterior tuberculum). *Brain Research,* **889**, 316-330

Riters, L. and Ball, G. (1999) Lesions to the medial preoptic area affect singing in the male european starling (*Sturnus vulgaris*). *Hormones and Behavior,* **36**, 276-286

Saegusa, M., Hashimura, M., Yoshida, T., & Okayasu, I. (2001) beta- Catenin mutations and aberrant nuclear expression during endometrial tumorigenesis. *British Journal of Cancer,* **84**, 209-217

Sakurada, T., Mima, K., Kurisaki, A., Sugino, H. & Yamauchi, T. (2005) Neuronal cell type-specific promoter of the *alpha CaM kinase II* gene is activated by *zic2*, a Zic family zinc finger protein. *Neuroscience Research*, **53**, 323-330

Salero, E. & Hatten, M.E. (2007) Differentiation of ES cells into cerebellar neurons. *Proceedings of the National Academy of Science,* **104**(8), 2997-3002

Salero, E., Perez-Sen, R., Aruga, J., Gimenez, C., & Zafra, F. (2001) Transcription factors *zic1* and *zic2* bind and transactivate the *apolipoprotein E* gene promoter. *Journal of Biological Chemistry*, **276**, 1881-1888

Schultz, W. (2007) Multiple dopamine functions at different time courses. *Annual Review of Neuroscience*, **30**, 359-388

Semsar, K. & Godwin, J. (2004) Multiple mechanisms of phenotype development in the bluehead wrasse. *Hormones and Behavior,* **45**, 345-53

Semsar, K., Kandel, F.L.M., & Godwin, J. (2001) Manipulations of the AVT system shift social status and related courtship and aggressive behavior in the bluehead wrasse. *Hormones and Behavior*, **40,** 21–31

Sun, Z. & Reiner, A. (2000) Localization of dopamine D1a and D1b receptor mrnas in the forebrain and midbrain of the domestic chick. *Journal of Chemical Neuroanatomy*, **19**, 211-224

Warner, R.R. (1984) Mating behavior and hermaphroditism in coral reef fishes. *American Scientist,* **72**, 128-136

Warner, R.R. and Swearer, S.E. (1991) Social control of sex change in the bluehead wrasse, *Thalassoma bifasciatum* (Pisces: Labridae). *Biological Bulletin*, **181**, 199-204

Yang, Y., Hwang, C.K., Junn, E., Lee, G., & Mouradian, M.M. (2000) Zic2 and Sp3 repress Sp1-induced activation of the human D_{1A} dopamine receptor gene. *Journal of Biological Chemistry*, **275**, 38863-38869

Yokota, N., Aruga, J., Takai, S., Yamada, K., Hamazaki, M., Iwase, T., Sugimura, H., & Mikoshiba, K. (1996) Predominant expression of human *zic* in cerebellar granule Cell lineage and medulloblastoma. *Cancer Research*, **56**, 377-383

Young, L.J., Lopreato, G.F., Horan, K., & Crews, D. (1994) Cloning and in situ hybridization analysis of estrogen receptor, progesterone receptor, and androgen receptor expression in the brain of whiptail lizards *(Cnemidophorus uniparens* and *C. inornatus). The Journal of Comparative Neurology*, **347**, 288-300

LITERATURE CITED

Anderson, S.L., Thompson, A.P, Krenzel, E., & Teicher, M.H. (2002) Pubertal changes in gonadal hormones do not underlie adolescent dopamine receptor overproduction. *Psychoneuroendocrinology*, **27**, 683-691

Andersen, S.L. & Teicher, M.H. (2000) Sex differences in dopamine receptors and their relevance to adhd. *Neuroscience & Biobehavioral Reviews*, **24**, 137-141

Alvarez-Buylla, A. & Garcia-Verdugo, J.M. (2002) Neurogenesis in adult subventricular zone. *The Journal of Neuroscience*, **22**(3), 629-634

Arias-Carrión, O.O. & Pŏppel, E.E. (2007) Dopamine, learning, and reward-seeking behavior. *Acta Neurobiologiae Experimentalis*, **67**, 481-488

Aruga, J. (2004) The role of zic genes in neural development. *Molecular and Cellular Neuroscience,* **26**, 205-221

Aruga, J., Inoue, T., Hoshino, J., & Mikoshiba, K. (2002) Zic2 controls cerebellar development in cooperation with Zic1. *Journal of Neuroscience*, **22**, 218-225

Aruga, J., Nagai, T., Tokuyama, T., Hayashizaki, Y., Okazaki, Y., Chapman, V.M., & Mikoshiba, K. (1996) The mouse *zic* gene family: homologues of the *Drosophila* pair-rule gene *odd-paired*. *The Journal of Biological Chemistry*, **271** (2) 1043-1047

Aruga, J., Yokota, N., Hashimoto, M., Furuichi, T., Fukuda, M., & Mikoshiba, K.(1994) A novel zinc finger protein, zic, is involved in neurogenesis, especially in the cell lineage of cerebellar granule cells. *Journal of Neurochemistry*, **63**, 1880–1890

Bafico, A., Liu, G., Goldin, L., Harris, V., & Aaronson, S.A. (2004) An autocrine mechanism for constitutive wnt pathway activation in human cancer cells. *Cancer Cell*, **6**, 497-506

Baillen, M. & Balthazart, J. (1997) A direct dopaminergic control of aromatase activity in the quail preoptic area. *Journal of Biochemistry and Molecular Biology,* **63**, 99- 113

Bakker, J., Honda, S., Harada, N., & Balthazart, J. (2004). Restoration of male sexual behavior by adult exogenous estrogens in male aromatase knockout mice. *Hormones and Behavior*. **16**, 483-490

Ball, G.F., Casto, J.M. & Balthazart, J. (1995) Autoradiographic localization of di-like dopamine receptors in the forebrain of male and female japanese quail and their relationship with immunoreactive tyrosine hydroxylase. *Journal of Chemical Neuroanatomy*, **9**, 121-133

Bathazart, J., Baillien, M., & Ball, G.F. (2001) Phosphorylation processes mediate rapid changes of brain aromatase activity. *The Journal of Steroid Biochemistry and Molecular Biology,* **79**, 261-77

Balthazart, J., Baillien, M., & Ball, G.F. (2002) Interactions between aromatase (estrogen synthase) and dopamine in the control of male sexual behavior in quail. *Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology.* **132**, 37-55

Bataller, L., Wade, D.F., Fuller, G.N., Rosenfeld, M.R., & Dalmau, J. (2002) Cerebellar degeneration and autoimmunity to zinc-finger proteins of the cerebellum. *Neurology* **59**, 1985-1987

Bataller, L., Wade, D.F., Graus, F., Stacey, H.D., Rosenfeld, M.R.,& Dalmau, J. (2004) Antibodies to Zic4 in paraneoplastic neurologic disorders and small-cell lung cancer. *Neurology*, **62**, 778-782

Benedyk, M.J., Mullen, J.R., & DiNardo, S. (1994) Odd-paired: a zinc finger pair-rule protein required for the timely activation of engrailed and wingless in Drosophila embryos. *Genes and Development*, **8**, 105-117

Bhanot, P., Marcel, B., Samos, C.H., Hsieh, C., Wang, Y., Macke, J.P., Andrew, D., Nathans, J., & Nusse, R. (1996) A new member of the *frizzled* family from *Drosophila* functions as a Wingless receptor. *Nature*.382, 225-30

Bidus, M.A., Risinger, J.I., Chandramouli, G.V.R., Dainty, L.A., Litzi, T.J., Berchuck, A., Barrett J.C., Maxwell, G.L. (2006) Prediction of Lymph Node Metastasis in Patients with Endometrioid Endometrial Cancer Using Expression Microarray. *Clinical Cancer Research,* **12,** 83-8

Black, M.P., Balthazart, J., Baillien, M., & Grober, M.S. (2005) Socially induced and rapid increases in aggression are inversely related to brain aromatase activity in a sex-changing fish, *Lythrypnus dalli*. *Proceedings of the Royal Society,* **272**, 2435-2440

Blazquez, M., Bosma, P.T., Fraser, E.J., Van Look, K.J.W., & Trudeau, V.L. (1998) Fish as models for the neuroendocrine regulation of reproduction and growth. *Comparative Biochemistry and Physiology*, **119**, 345-64

Bolis, C.L., Piccolella, M., Dalla Valle, A.Z., & Rankin, J.C. (2001) Fish as model in pharmacological and biological research. *Pharmacological Research,* **44**, 265-280

Brown, L. & Brown, S.(2009) Zic2 is expressed in pluripotent cells in the blastocyst and adult brain expression overlaps with makers of neurogenesis, *Gene Expression Patterns,* **9**, 43-49

Brown, S., Gersen, S., Anyane-Yeboa, K. and Warburton, D. (1993) Preliminary definition of a 'critical region' of chromosome 13 in q32: report of 14 cases with 13q deletions and review of the literature. *American Journal of Medical Genetics,* **45**, 52-59

Brown, S., Russo, J., Chitayat, D., & Warburton, D. (1995) The 13q-syndrome: the molecular definition of a critical seletion in band 13q32. *American Journal of Human Genetics,* **57**, 859-866.

Brown, S.S.A., Warburton, D.D., Brown, L.L.Y., Yu, C.C.Y., Roeder, E.E.R., Stengel-Rutkowski, S.S., Hennekam, R.R.C. & Muenke, M.M. (1998) Holoprosencephaly due to mutations in zic2, a homologue of drosophila odd-paired. *Nature genetics*, **20**, 180-183

Chang, J.P., Yu, K.L., Wong, A.O.L., & Peter, R.E. (1990) Differential actions of dopamine receptor subtypes on gonadotropin and growth hormone release in vitro in goldfish. *Neuroendocrinology,* **51**, 664-74.

Chapouton P., Jagasia, R., & Bally-Cuif, L. (2007) Adult neurogenesis in non-mammalian vertebrates. *BioEssays,* **29**, 745-757

Chen, C.M. & Struhl, G. (1999) Wingless transduction by the frizzled and frizzled2 proteins of *drosophila*. *Development*, **126**, 5441-52

Chen, L.C., Vural, B., Ustuner, Z., Saip, P., Ozbek, M., Gonen, L., Old, Y.T., & Gure, A. (2004) Immunoreactivity to SOX-1, -2, -3 and ZIC2 antigens and correlation to survival in small cell lung cancer (SCLC). *Journal of Clinical Oncology*, **22***,* 7159

Conacci-Sorrell, M., Zhurinsky, J., & Ben-Ze'ev, A. (2002) The cadherin-catenin adhesion system in signaling and cancer *Journal of Clinical Investigation*, **109**, 987–991

Cooke, B., Hegstrom, C.D., Villeneuve, L.S., & Breedlove, S.M. (1998) Sexual differentiation of the vertebrate brain: principles and mechanisms, *Frontiers in Neuroendocrinology*, **19**, 323-362

Cornil, C.A., Seutin, V., Motte, P., & Balthazart, J. (2004) Electrophysiological and neurochemical characterization of neurons of the medial preoptic area in Japanese quail (*Coturnix japonica*). *Brain Research,* **1029**, 224-240.

Coughlin, D.J. & Hawryshyn, C.W. (1995) A cellular basis for polarized-light vision in rainbow trout. *Journal of Comparative Physiology*, **176,**, 261-272

De Almeida, R.M.M., Ferrari, P.F., Parmigiani, S. & Miczek, K.A. (2005) Escalated aggressive behavior: Dopamine, serotonin and gaba. *European Journal of Pharmacology*, **526**, 51-64

Demeret, C., Yaniv, M. & Thierry, F. (1994) The e2 transcriptional repressor can compensate for sp1 activation of the human papillomavirus type 18 early promoter. *Journal of Virology*, **68**, 7075-7082

Demski, L.S. (1983) Behavioral effects of electrical stimulation of the brain. *Fish Neurobiology*, vol. 2, Higher Brain Areas and Functions (ed. R. E. Davis and R. G. Northcutt), Ann Arbor: Univeristy of Michigan Press, 317–359

Echteler, S.M (1985) Organization of central auditory pathways in a teleost fish*, Cyprinus carpio*. *Journal of Comparative Physiology*, **156**, 267-280

Ellison, G. (1994) Stimulant-induced psychosis, the dopamine theory of schizophrenia, and the habenula. *Brain Research Brain Research Reviews,***19**(2), 223-239

Elofsson, U, Winburg, S., & Francis, R.C. (1997) Number of preoptic GnRH-immunoreactive cells correlates with sexual phase in a protandrously hermaphroditic fish, the dusky anemonefish (*Amphiprion melanopus*), *Journal of Comparative Physiology*,**181**, 484-492

Foran, C.M & Bass, A.H. Preoptic GnRH and AVT: Axes for sexual plasticity in teleost fish. *General and Comparative Endocrinology*, **116**, 141-152

Fujimura, N., Vacik, T., Machon, O., Vlcek, C., Scalabrin, S., Speth, M., Diep, D., Krauss, S., Kozmik, Z. (2007) Wnt-mediated down-regulation of Sp1 target genes by a transcriptional repressor Sp5. *Journal of Biological Chemistry,* **282**(2), 1225-1237

Gainetdinov, R.R., Mohn, A.R. & Caron, M.G. (2001) Genetic animal models: Focus on schizophrenia. *Trends in Neurosciences*, **24**, 527-533

Gerfern, C., Engber, T., Mahan, L., Susel, Z., Chase, T., Monsma, F. & Sibley, D. (1990) D1 and D2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. *Science*, **250**, 1429-1432

Giles, R.H., van Es, J.H., & Clevers, H. (2003) Caught up in a wnt storm: Wnt signaling in cancer. *Biochimica et Biophysica Acta*, **1653**, 1-24

Giuliano, F., Rampin, O., Brown, K., Courtois, F., Benoit, G., & Jardin, A. (1996) Stimulation of the medial preoptic area of the hypothalamus in the rat elicits increases in intracavernous pressure. *Neuroscience Letters,* **209**, 1-4

Godwin, J., Crews, D. & Warner, R.R. (1996) Behavioural sex change in the absence of gonads in a coral reef fish. *Proceedings: Biological Sciences*, **263**, 1683-1688

Godwin, J., Sawby, R., Warner, R.R., Crews, D., & Grober, M.S. (2000) Hypothalamic Arginine Vasotocin mRNA Abundance Variation Across Sexes and with Sex Change in a Coral Reef Fish. *Brain, Behavior, and Evolution*, **55**, 77–84

Goldman-Rakic, P.S.P.S., Castner, S.A.S.A., Svensson, T.H.T.H., Siever, L.J.L.J. & Williams, G.V.G.V. (2004) Targeting the dopamine d1 receptor in schizophrenia: Insights for cognitive dysfunction. *Psychopharmacology*, **174**, 3-16

Gorski, R.A, Harlan, R.E., Jacobson, C.D, Shryne, J.E., & Southam A.M. (1980) Evidence for the existence of a sexual dimorphic nucleus in the preoptic area of the rat. *Comparative Neurology*, **193**, 529-539

Grinberg, I. & Millen, K.J. (2005) The ZIC gene family in development and Disease. *Clinical Genetics*, **67**, 290-296.

Grinblat, Y., Nyholm, M., Taylor, A., & Burgess, S. (2008) A role for zic genes during neural tube morphogenesis in zebrafish *Abstract from 67th Annual Meeting/ Developmental Biology*, **319**(2), 514-515

Grober, M.S., Jackson, I.M., Bass, A.H. (1991) Gonadal steroids affect LHRH preoptic cell number in a sex/role changing fish. *Journal of Neurobiology,* **22**, 734-741

Grober, M.S. & Bass, A.H. (2002) Life history, neuroendocrinology, and behavior in fish. *Hormones, Brain and Behavior,* **2**, 331-347

Gure, A.O., Stockert, E., Scanlan, M.J., Keresztes, R.S., Jager, D., Altorki, N.K., Old, L.J., & Chen, Y.T.(2000) Serological identification of embryonic neural proteins as highly immunogenic tumor antigens in small cell lung cancer. *Proceedings of the NationalAcademy of Science*, **97**, 4198-4203

Hawkins, M.B., Thornton, J.W., Crews, D., Skipper, J.K., Dotte, A. & Thomas, P. (2000) Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. *Proceedings of the National Academy of Sciences*, **97** (20), 10751- 10756

Heimovics, S.A., Cornil, C.A., Ball, G.F., & Riters, L.V. (2009) D1-like dopamine receptor density in nuclei involved in social behavior correlates with song in a context-dependent fashion in male European starlings *Neuroscience,* **159**, 962-973

Herrera, E, Marcus, R., Li, S., Williams, S.E., Erskine, L., Lai, E., Mason, C. (2004) Foxd1 is required for proper formation of the optic chiasm. *Development,* **131**, 5727-5739

Hirvonen, J.J., Van Erp, T.G.T.G.M., Huttunen, J.J., Aalto, S.S., Nagren, K.K., Huttunen, M.M., Lönnqvist, J.J., Kaprio, J.J., Cannon, T.D.T.D. & Hietala, J.J. (2006) Brain dopamine d1 receptors in twins discordant for schizophrenia. *The American Journal of Psychiatry*, **163**, 1747-1753

Huang, H.C. & Klein, P.S. (2004) The Frizzled family: receptors for multiple signal transduction pathways. *Genome Biology*, **5**, 234

Imai K.S., Satou Y., & Satoh, N. (2002) Multiple functions of Zic-like gene in the differentiation of notochord, central nervous system and muscle in *Ciona savignyi* embryos. *Development***, 129***,* 2723-2732

Inoue, T., Ogawa, M., Mikoshiba, K. & Aruga, J. (2008) *Zic* deficiency in the cortical marginal zone and meninges results in cortical lamination defects resembling those in Type II Lissencephaly**.** *The Journal of Neuroscience*, **28**(18), 4712-4725

Jacobson, C., Terkel, J., Gorski, R., & Sawyer, C. (1980) Effects of small medial preoptic area lesions on maternal behavior: Retrieving and nest building in the rat. *Brain Research*, **194** (2), 471-478

Johansson, V. & Johansson (2005) Growth hormone-induced stimulation of swimming and feeding behaviour of rainbow trout is abolished by the d1 dopamine antagonist sch23390. *General and Comparative Endocrinology*, **141**, 58

Jung, A.B. & Bennett, J.P. (1996) Development of striatal dopaminergic function. I. Pre- and postnatal development of mRNAs and binding sites for striatal D1 (D1a) and D2 (D2a) receptors. *Developmental Brain Research*, **94**, 109-120.

Jurkevich, A., Barth,S.W., Aste,N., Panzica, G.C. & Grossmann, R. (1996) Intracerebral sex differences in the vasotocin system in birds: possible implication in behavioral and autonomic functions. *Hormones and Behavior,* **30**, 673-681

Kitaoka, S.S., Furuyashiki, T.T., Nishi, A.A., Shuto, T.T., Koyasu, S.S., Matsuoka, T.T., Miyasaka, M.M., Greengard, P.P. & Narumiya, S.S. (2007) Prostaglandin e2 acts on ep1 receptor and amplifies both dopamine d1 and d2 receptor signaling in the striatum. *The Journal of Neuroscience*, **27**, 12900-12907

Koyama, Y., Satou, M.,Oka Y., & Ueda, K. (1984) Involvement of telencephalic hemispheres and the preoptic area in sexual behavior of the male goldfish, *Carassius auratus*: a brain lesion study. *Behavioral and Neural Biology*, **1**, 70-86

Larson, E.T., Norris, D.O., Gordon Grau, E. & Summers, C.H. (2003a) Monoamines stimulate sex reversal in the saddleback wrasse. *General and Comparative Endocrinology*, **130**, 289-298

Larson, E.T., Norris, D.O. & Summers, C.H. (2003b) Monoaminergic changes associated with socially induced sex reversal in the saddleback wrasse. *Neuroscience*, **119**, 251-263

Lee, S.H. & Mouradian, M.M. (1999) Up-regulation of D1A dopamine receptor gene transcription by estrogen. *Molecular and Cellular Endocrinology*, **156**, 151-157

Levavi-Sivan, B., Biran, J. & Fireman, E. (2006) Sex steroids are involved in the regulation of gonadotropin-releasing hormone and dopamine d2 receptors in female tilapia pituitary1. *Biology of Reproduction*, **75**, 642-650

Li, P.P., Shah, S.S., Huang, L.L., Carr, A.L.A.L., Gao, Y.Y., Thisse, C.C., Thisse, B.B. & Li, L.L. (2007) Cloning and spatial and temporal expression of the zebrafish dopamine d1 receptor. *Developmental dynamics*, **236**, 1339-1346

Lidow, M.S. & Goldman-Rakic, P.S. (1994) A common action of clozapine, haloperidol, and remoxipride on D1- and D2-dopaminergic receptors in the primate cerebral cortex. *Proceedings of the National Academy of Science*. **91**, 4353–4356.

Logan, C.Y. & Nusse, R. (2004) The wnt signaling pathway in development and disease. *Annual Review of Cell and Developmental Biology.* **20**, 781–810

Lorenzi, V., Earley, R.L., & Grober, M.S. (2006) Preventing behavioural interactions with a male facilitates sex change in female bluebanded gobies. *Lythrypnus dalli, Behavior, Ecology, and Sociobiology*, **59**, 715–722

Mani, S.K., Blaustein, J.D., & O'Malley, B.W. (1997) Progesterone receptor function from a behavioral perspective, *Hormones and Behavior*, **31**, 244-255

Marsh, K.E., Creutz, L.M., Hawkins, M.B. & Godwin, J.J. (2006) Aromatase immunoreactivity in the bluehead wrasse brain, *Thalassoma bifasciatum*: Immunolocalization and co-regionalization with arginine vasotocin and tyrosine hydroxylase. *Brain Research*, **1126**, 91-101

Marsh, K.E. (2007) Neuroendocrine transduction of social cues in the bluehead wrasse, *Thalassoma bifasciatum*. PhD thesis, Zoology, North Carolina State University, Raleigh, NC, 57-78

McMahon, A., Junette, K., & Merzdorf, C. (2007) The expression of *zic1*, *zic2*, *zic3*, and *zic4* in early chick embryos. *Developmental Biology*, **306**, 355-56

Meek, J., Joosten, H.W., and Steinbusch, H.W.M. (1989) Distribution of dopamine immunoreactivity in the brain of the mormyrid teleost *Gnathonemus petersii. The Journal of Comparative Neurology*, **282** (3), 362-383

Merzdorf, C.S. (2007) Emerging roles for zic genes in early development. *Developmental Dynamics*, **236**, 922-40

Merzdorf, C.S. & Sive, H. (2006) The zic1 gene is an activator of wnt signaling *The International Journal of Developmental Biology*, **50,** 611-17

Miyata, H., Ikawa, E., & Ohama, E. (1998) Medulloblastoma in an adult suggestive of external granule cells as its origin: a histological and immunohistochemical study. *Brain Tumor Pathology*, **15**, 31-5

Mizugishi, K., Aruga, J., Nakata, K. & and Mikoshiba, K. (2001) Molecular properties of zic proteins as transcriptional regulators and their relationship to GLI proteins. *The Journal of Biological Chemistry*, **276** (3), 2180-2188

Montague, D.M., Lawler, C.P., Mailman, R.B., & Gilmore, J.H. (1999) Developmental regulation of the dopamine d1 receptor in human caudate and putamen - a new frontier in developmental neurobiology. *Neuropsychopharmacology*, **21**, 641-649

Moon, R.T., Bowerman, B., Boutros, M., & Perrimon, N. The promise and perils of wnt signaling through ß-catenin, *Science*, **296**, 1644-1666

Mueller, T. & Guo, S. (2009) The distribution of GAD67-mRNA in the adult zebrafish (teleost) forebrain reveals a prosomeric pattern and suggests previously unidentified homologies to tetrapods, *The Journal of Comparative Neurology*, **516**, 553-568

Munro, C.A., Mccaul, M.E., Wong, D.F., Oswald, L.M., Zhou, Y., Brasic, J., Kuwabara, H., Kumar, A., Alexander, M., Ye, W. & Wand, G.S. (2006) Sex differences in striatal dopamine release in healthy adults. *Biological Psychiatry*, **59**, 966-974

Nagai T., Aruga J., Minowa O., Sugimoto, T., Ohno, Y., Noda, T., & Mikoshiba, K. (2000) Zic2 regulates the kinetics of neurulation. *Proceedings of the National Academy of Science*, **97**, 1618–1623

Nagai, T., Aruga, J., Takada, S., Gunther, T., Sporle, R., Schughart, K., & Mikoshiba, K. (1997) The expression of the mouse *zic1*, *zic2*, and *zic3* gene suggests an essential role for zic genes in body pattern formation. *Developmental Biology*, **182**, 299-313

Northmore, D.P.M., Williams, B., & Vanegas, H. (1983) The teleostean torus longitudinalis: response related to eye movements, visuotopic mapping, and functional relations with the optic tectum. *Journal of Comparative Physiology*, **150**, 39-50

Paredes, R., Highland,L., & Karam, P. (1993) Socio-sexual behavior in male rats after lesions of the medial preoptic area: Evidence for reduced sexual motivation. *Brain Research,* **618** (2), 271-276

Pasca di Magliano, M. & Hebrok M. (2003) Hedgehog signaling in cancer formation and maintenance. *Nature Reviews Cancer*, **3** (12), 903–911

Pasca di Magliano, M., Biankin, A.V., Heiser, P.W., Cano, D.A., Gutierrez, P.J.A., Deramaudt, T., Segara, D., Dawson, A.C., Kench, J.G., Henshall, S.M., Sutherland, R.L., Dlugosz, A., Rustgi, A.K., Hebrok, M. (2007) Common activation of canonical wnt signaling in pancreatic adenocarcinoma. *PlosOne*, **11,** 1155

Patisaul, H.B., Whitten, P.L., & Young, L.J. (1999) Regulation of estrogen receptor beta mRNA in the brain: opposite effects of 17b-estradiol and the phytoestrogen, coumestrol. *Molecular Brain Research,* **67**, 165–171

Perachio, A., Marr, L., & Alexander, M. (1979) Sexual behavior in male rhesus monkeys elicited by electrical stimulation of preoptic and hypothamic areas. *Bran Research,* **177** (1), 127-144

Peter, R.E., Chang, J.P., Nahorniak, C.S., Omeljaniuk, R.J., Sokolowska, M., Shih, S.H., & Billard, R. (1986) Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. *Recent Progress in Hormone Research,* **42**, 513-48

Pola, J. & Robinson, D.A. Oculomotor signals in medial longitudinal fasciculus of the monkey. *Journal of Neurophysiology*, **41**(2), 245-259

Polakis, P. (2000) Wnt signaling and cancer. *Genes and Development*, **14**, 1837-1851

Powers, B.& Valenstein, E. (1972) Sexual receptivity: Facilitation by medial preoptic lesions in female rats. *Science*, **175** (4025), 1003-1005

Propper, C.R., Jones, R.E., & Lopez, K.H (1992) Distribution of arginine vasotocin in the brain of the lizard *Anolis carolinensis. Cell and Tissue Research,* **267**, 391-398

Rink, E. & Wullimann, M.F. (2001) The teleostean (zebrafish) dopaminergic system ascending to the subpallidum (striatum) is located in the basal diencephalon (posterior tuberculum). *Brain Research,* **889**, 316-330

Riters, L. and Ball, G. (1999) Lesions to the medial preoptic area affect singing in the male european starling (*Sturnus vulgaris*). *Hormones and Behavior,* **36**, 276-286

Rodriguiz, R.M., Chu, R., Caron, M.G. & Wetsel, W.C. (2004) Aberrant responses in social interaction of dopamine transporter knockout mice. *Behavioural Brain Research*, **148**, 185-198

Ross, M.R., Losey, G.S., & Diamond, M. (1983) Sex change in a coral reef fish: dependence of stimulation and inhibition on relative size. *Science,* **221**, 574-575

Ruskin, D., Bergstrom, D., & Walters, J. (2002) Nigrostriatal lesion and dopamine agonists affect firing patterns of rodent entopeduncular nucleus neurons. *Journal of Neurophysiology*, **88**, 487-496

Saegusa, M., Hashimura, M., Yoshida, T., & Okayasu, I. (2001) beta- Catenin mutations and aberrant nuclear expression during endometrial tumorigenesis. *British Journal of Cancer,* **84**, 209-217

Sakurada, T., Mima, K., Kurisaki, A., Sugino, H. & Yamauchi, T. (2005) Neuronal cell type-specific promoter of the *alpha CaM kinase II* gene is activated by Zic2, a Zic family zinc finger protein. *Neuroscience Research*, **53**, 323-330

Salero, E. & Hatten, M.E. (2007) Differentiation of ES cells into cerebellar neurons. *Proceedings of the National Academy of Science,* **104**(8), 2997-3002

Salero, E., Perez-Sen, R., Aruga, J., Gimenez, C., & Zafra, F. (2001) Transcription factors *Zic1* and *Zic2* bind and transactivate the *apolipoprotein E* gene promoter. *Journal of Biological Chemistry*, **276**, 1881-1888

Sanek, N.A. & Grinblat, Y. (2008) A novel role for zebrafish zic2a during forebrain development. *Developmental Biology*, **317**, 325-335

Schultz, W. (2007) Multiple dopamine functions at different time courses. *Annual Review of Neuroscience*, **30**, 359-388

Seeman, P.P. (1987) Dopamine receptors and the dopamine hypothesis of schizophrenia. *Synapse*, **1**, 133-52

Semsar, K. & Godwin, J. (2004) Multiple mechanisms of phenotype development in the bluehead wrasse. *Hormones and Behavior,* **45**, 345-53

Semsar, K., Kandel, F.L.M., & Godwin, J. (2001)Manipulations of the AVT system shift social status and related courtship and aggressive behavior in the bluehead wrasse. *Hormones and Behavior*, **40,** 21–31

Striedter, G.F. (1991) Auditory, elecctrosensory,and mechanosensory lateral line pathways through the forebrain in catfishes. *Journal of Comparative Neurology*, **312**, 311-331

Sun, Z. & Reiner, A. (2000) Localization of dopamine D1A and D1B receptor mrnas in the forebrain and midbrain of the domestic chick. *Journal of Chemical Neuroanatomy*, **19**, 211-224

Toyama, R., Gomez, D.M., Mana, M.D., & Dawid, I.B. (2004) Sequence relationships and expression patterns of zebrafish zic2 and zic5 genes. *Gene Expression Patterns*, **4**, 345–350

Tetsu, O. & McCormick, F. (1999) ß-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature*, **398**, 422-428.

Thorpe, C.J., Weidinger, G., and Moon, R.T. (2005) Wnt/ß-catenin regulation of the Sp1-related transcription factor sp5l promotes tail development in zebrafish *Development,* **132**, 1763-1772

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C. D'Amico, M., Pestell, R., & Ben-Ze'ev, A. (1999) The cyclin D1 gene is a target of the ß-catenin/LEF-1 pathway. *Proceedings of the National Academy of Science*, **96**, 5522-5527

Wang, J.J. & Bannon, M.J.M.J. (2005) Sp1 and sp3 activate transcription of the human dopamine transporter gene. *Journal of Neurochemistry*, **93**, 474-482

Wang, R.Y. and Aghajanian, G.K. (1977) Physiological evidence for habenula as major link between forebrain and midbrain raphe. *Science*, **197**(4298), 89-91

Warner, R.R. (1984) Mating behavior and hermaphroditism in coral reef fishes. *American Scientist,* **72**, 128-136

Warner, R.R. and Swearer, S.E. (1991) Social control of sex change in the bluehead wrasse, *Thalassoma bifasciatum* (Pisces: Labridae). *Biological Bulletin*, **181**, 199-204

Wilczynski, W., Yang, W.J., & Simmons, D. Sex differences and hormone influences on tyrosine hydroxylase immunoreactive cells in the leopard frog. *Journal of Neurobiology,* **56***,* 54-65

Williams, I.M., Carletti, B., Leto, K., Magrassi, L., & Rossi, F. (2008) Cerebellar granule cells transplanted *in vivo* can follow physiologicaland unusual migratory routes to integrate into the recipient cortex. *Neurobiology of Disease*,**30**, 139-149

Wong, D.F., Broussolle, E.P., Wand, G., Villemagne, V., Danals, R.F., Links, J.M., Zacur, H.A., Harris, J., Naidu, S., Braestrup, C., Wagner Jr., W.N., & Gjedde, A. (1987) In vivo measurement of dopamine receptors in human brain by positron emission tomography, age and sex differences. *Annuals of the New York Academy of Sciences*, **515**, 203-214

Wullimann, M.F., Hofmann, M.H., & Meyer, D.L. (1991) The valvula cerebelli of the spiny eel, *Macrognathus aculeatus,* receives primary lateral-line afferents from the rostrum of the upper jaw. *Cell and Tissue Research*, **266**, 285-293

Yanez, J. & Anadon, R. (1996) Afferent and efferetnt connections of the habenula in the rainbow trout (*Oncorhynchus mykiss*): an indocarbocyanine dye (DiI) study. *The Journal of Comparative Neurology*, **372**, 529-543

Yang, Y., Hwang, C.K., Junn, E., Lee, G., & Mouradian, M.M. (2000) Zic2 and sp3 repress sp1-induced activation of the human D_{1A} dopamine receptor gene. *Journal of Biological Chemistry*, **275**, 38863-38869

Yang, C.C., Kim, H.H.S., Seo, H.H. & Kim, K.K.S. (1998) Identification and characterization of potential cis-regulatory elements governing transcriptional activation of the rat tyrosine hydroxylase gene. *Journal of Neurochemistry*, **71**, 1358-1368

Yokota, N., Aruga, J., Takai, S., Yamada, K., Hamazaki, M., Iwase, T., Sugimura, H., & Mikoshiba, K. (1996) Predominant expression of human zic in cerebellar granule cell lineage and medulloblastoma. *Cancer Research*, **56**, 377-383

Young, L.J., Lopreato, G.F., Horan, K., & Crews, D. (1994) Cloning and in situ hybridization analysis of estrogen receptor, progesterone receptor, and androgen receptor expression in the brain of whiptail lizards *(Cnemidophorus uniparens* and *C. inornatus). The Journal of Comparative Neurology*, **347**, 288-300