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THE EXPRESSION OF p68 PROTEIN IN THE AUSTRALIAN ZEBRA FINCH BRAIN ACROSS DEVELOPMENT

by

CHUKWUEMEKA OKEKE

Under the Direction of Laura Carruth

ABSTRACT

Steroid hormones and receptors play a role in regulating biological events underlying brain development and sexual differentiation. Current evidence indicates that circulating sex steroid hormones are not entirely responsible for development of neural sex differences in song birds such as the zebra finch. p68, as a coactivator specific for estrogen receptor alpha ($ER\alpha$) and an essential factor in early tissue development and maturation might play a role in sexual differentiation. Zebra finches have a sexually dimorphic song control nuclei in the brain, males have larger song nuclei than females, and are ideal model for investigating the mechanisms controlling sexual differentiation of the brain and behavior. Western blot analysis showed a significant sex difference at post hatch day 10 (P10). Immunohistochemistry showed localization of p68 immunoreactive cells in the ZF brain including nuclei that compose the avian song system. p68 is probably developmentally regulated and may be modulated by endogenous estrogen and estrogen receptors suggesting a role for p68 in sexual differentiation.

INDEX WORDS: p68, coactivator, RNA helicase, steroid receptor, song control nuclei,
zebra finch (ZF)

THE EXPRESSION OF p68 PROTEIN IN THE AUSTRALIAN ZEBRA FINCH
BRAIN ACROSS DEVELOPMENT

by

CHUKWUEMEKA OKEKE

A Thesis Submitted in Partial Fullfillment of the Requirement for the Degree of

Master of Science

in the College of Arts and Sciences

Georgia State University

2007

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CHUKWUEMEKA OKEKE
2007

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BRAIN ACROSS DEVELOPMENT

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CHUKWUEMEKA OKEKE

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Georgia State University
May 2007

ACKNOWLEDGEMENTS

I would like to thank my advisor and mentor Dr. Laura Carruth, for making this thesis possible. I would also like to thank my committee members, Dr. Matthew Grober and Dr. Andrew Clancy for being supportive throughout my project. Furthermore, my gratitude goes to Dr. Rebecca Herman, Kelli Duncan, and Mahin Shahbazi all of whom provided their support. My sincere gratitude to our collaborator Dr. Zhi-Ren Liu who kindly provided the p68 rgg-1 antibody, and Ms. Candice Long and Ms. Latesha Warren in the Biology Department for making this project possible. Finally, I would like to thank my fiancé Ogochukwu Okafor and my family for their immense love and support throughout this project.

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LIST OF ABBREVIATIONS

α – alpha

ANOVA – one-way analysis of variance

β – beta

bp – base pair

ER- α – estrogen receptor alpha

♀ - female

IHC – immunohistochemistry

ir – immunoreactive

♂ - male

ZF – zebra finch

1

Introduction

1.1 The Australian zebra finch

The Australian zebra finch (ZF; *Taeniopygia guttata*) is the most common and familiar passerine songbird found in Central Australia inhabiting arid regions. Despite their restricted distribution in the wild, zebra finches are one of the most popular cage birds and are highly adaptable to domestic environments. In the wild, ZFs live in large social flocks (Zann, 1996). This allows for successful housing of several birds in one large cage before identifying breeding pairs. Both male and female ZFs participate in egg incubation after hatch but only the female develops a brood patch. Incubation period ranges from 11 – 15 days in the domestic pairs. As with all songbirds, songs are used for attracting and bonding with mates. Song production in the ZF is highly sexually dimorphic, and only the males can sing a courtship song (Zann, 1996).

The organizational hypothesis of vertebrate brain sexual differentiation states that the presence of androgens during a “sensitive period” in development permanently alters the developing brain, which in turn causes it to function and respond to steroids in a masculine manner in adulthood. The aromatization hypothesis states that early estrogen treatment can also masculinize the central nervous system through the aromatization of testosterone into estrogen via the action of the enzyme aromatase (Nelson, 2005). Most research on the role of steroid hormones in sexual differentiation of the avian brain has

been conducted in ZFs and has focused on the structures important for the learning and production of songs (Breedlove and Hampson, 2002). This thesis will establish the presence of a separate entity intrinsic to the brain development which demonstrates that the traditional organizational role of hormones in development is not the central mechanism for sexual differentiation of the avian song system.

1.2 Development of the Zebra finch song nuclei

Song learning and production are controlled by a series of interconnected regions or nuclei located within the telencephalon of the brain. The terminology for all identified telencephalic nuclei will follow the revised nomenclature guidelines proposed by Reiner et al. (2004a; 2004b). HVC (previously described as the caudal nucleus of hyperstriatum ventrale, HVc, or the high vocal center; Reiner et al., 2004a; 2004b), robust nucleus of the archistriatum (RA) and hypoglossal nucleus (nXIIts; previously described as nucleus intermedius of the medulla or IM; Reiner et al., 2004a; 2004b) form the motor pathway for song production (Nottebohm et al., 1976; Simpson and Vicario, 1990). Neuronal projections originating at the HVC and through the Area X or X, the nucleus dorsolateralis anterior thalami (DLM) and the lateral portions of the magnocellular nucleus of the anterior neostriatum (LMAN) ending at the RA are involved in song learning and perception (Bottjer et al., 1984). Together, these nuclei form the song learning and song production pathways in the songbird brain (Fig. 1). HVC projects to RA, which in turn projects to the tracheosyringeal portion of the hypoglossal nucleus

(nXIIIts). This nucleus contains motor neurons that innervate the vocal organ, also known as the syrinx (Nottebohm et al., 1976).

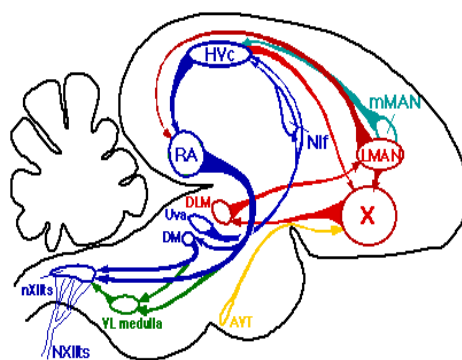


Fig 1: Schematic illustration of the adult male zebra finch brain and the song system (Diagram from Heather Williams, http://www.npa.uiuc.edu/courses/physl490b/models/birdsong_learning/bird_song.html)

Sexual dimorphisms exist at a number of levels (including behavioral, cellular, and morphological) within the avian song system (Arnold, 1992; Wade et al., 1999). Behaviorally, only males sing a courtship song and morphologically, the volumes of HVC and RA as well as the soma size and number of neurons within these nuclei are greater in males than in females. The projection from HVC to RA is more robust in males than in females. Area X, which is easily identified in males, is not easily visible in females using standard NISSL staining. These neural dimorphisms which are assumed to permit song production in males and inhibit it in females develop between P1 (post hatch day 1) and 2 months of age (Fig. 2). The most striking rate of differentiation occurs between days 15 and 35 posthatching (Bottjer et al., 1985; Kirn and DeVogd, 1989; Nixdorf-Bergweiler, 1996), but the song nuclei develop by P10, and sex differences in p68 mRNA expression, with females expressing more than males at P1 (Carruth et al., 2001; 2002), eight days earlier than the earliest previously reported sexually dimorphic

phenotype in androgen receptor (AR) expression in the ZF brain (Gahr and Metzdorf, 1999).

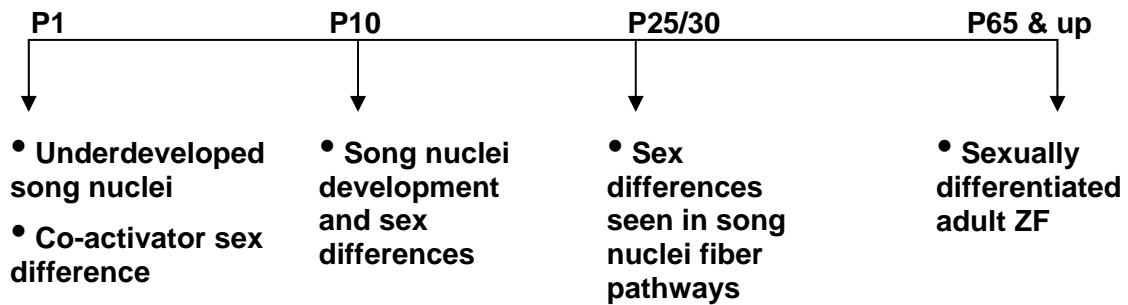


Fig. 2: Schematic representation of development of the zebra finch song nuclei.

1.3 Song Learning and Production in the Zebra finch

Song learning in the ZF consists of three distinct phases; the auditory or sensory phase, the sensory-motor phase, and crystallization or adult (see Fig. 3). During the early auditory phase from P1 through P35, the fledglings listen to and internalize features of songs produced by an adult male tutor, usually the father. Immediately following the auditory phase is the sensory-motor phase which consists of practicing the subsongs and plastic song formation and starts from about day 25 through adulthood. During the subsong stage the young zebra finch produces unorganized sounds and as they develop into the plastic stage production of clusters of stereotypical song is observed. Subsequently, the birds practice vocalization of memorized songs with specific song characteristics (De Voogd, 1990) until crystallized or stereotypical song (adult song) with normal variations in volume, duration, syllabic structure, and phrase order is produced.

ZFs are age-limited learners the crystallized song attained at adulthood remains the same and never changes (De Voogd, 1990).

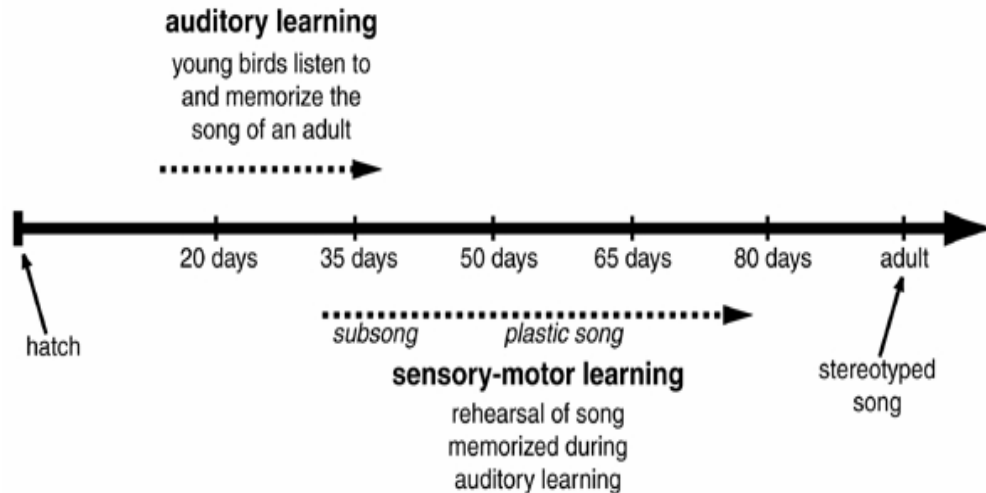


Fig. 3: Schematic representation of song learning and production timeline (Johnson, F. www.psy.fsu.edu/~johnson/johnsonlab/johnson.htm)

1.4 Role of Steroid Hormones and Steroid Hormone Receptors in Avian Brain Development

Attempts to understand the role circulating sex steroid hormones play in the development of the sex differences in the ZF brain have had conflicting results. Previous studies have shown that early treatment of females (shortly after hatching) with estrogen partially masculinizes their singing behavior and the associated brain nuclei. Neonatal treatment of female ZFs with estrogen stimulated them to sing as adults (songs were not as complex as male typical ZF songs) and resulted in the development of larger but not completely masculinized song nuclei (HVC and RA) than that of the untreated females (Gurney and Konishi, 1980; Nordeen et al., 1987; Simpson and Vicario, 1991). Though

estrogen treatment modified the song control nuclei, partially increased the size of neurons located therein and resulted in male-like vocalizations in females, it failed to completely masculinize the female ZF brain. Subsequently, inducing testicular formation during early female development, by treating genetic females with the aromatase inhibitor fadrozole, did not initiate complete masculinization of the female song system or singing (Wade and Arnold, 1996; Wade, 2001). In addition, blocking synthesis of sex steroid hormones, such as estrogen, did not disrupt normal development of the song circuit (Mathews and Arnold, 1990; Wade and Arnold, 1994) suggesting sex steroid hormones might not be the only endogenous factors responsible for sexual differentiation of brain and behavior in ZFs. Masculinization of avian song system structure and function is most likely induced by sex-specific development regulated by factors intrinsic to the brain (Arnold and Wade, 2004). We hypothesize that p68 is one of the essential factors involved in the development of the neural song system.

1.5 Estrogen receptor α and coactivator functions

Binding of estrogen to the estrogen receptor (ER) induces a conformational change in the ligand binding domain (E/F) and dissociation of the heat shock protein 90 (*hsp 90*) (Pratt et al., 1997; DeMarzo et al., 1991). Upon activation, due to the characteristic conformational changes, ERs dimerize and bind estrogen receptor elements (EREs) on target genes subsequently stimulating gene expression. ER- α is flanked by two activation function regions, activation function 1 (AF-1) located in the N-terminal A/B domain and activation function 2 (AF-2), located in the C-terminal ligand binding

domain. AF-1 region is cell and promoter specific (Metzger et al., 1995) while AF-2 promotes ligand dependent transcriptional activity (Lopez et al., 1999). AF-1 and AF-2 have been shown to activate transcription independently and also synergistically through direct binding of coactivator p300 to ER- α (Kobayashi, 2000). Nuclear receptor coactivators such as the steroid receptor coactivator (SRC-1) have been identified as essential elements for enhancing steroid hormone receptor activity (Tetel, 2000). Each coactivator possesses an intrinsic histone acetyltransferase (HAT) activity which uncoils the condensed histone complex of DNA making it more accessible for transcription (Tetel, 2000).

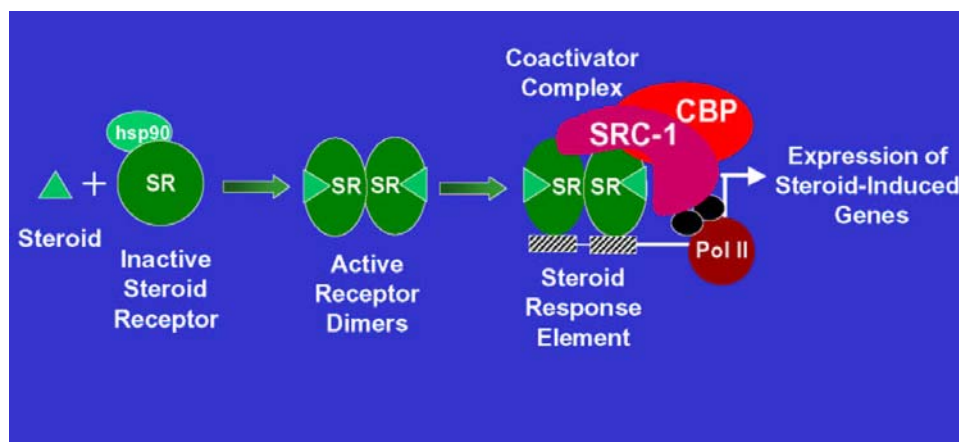


Fig. 4: Schematic representation of mechanisms of estrogen receptor mediated transactivation of steroid-induced genes (Tetel, 2000)

1.6 p68 Protein

p68 is a member of the DEAD-box protein family of putative RNA helicases and contains an ATPase, a motif that is responsible for RNA helicase activity. It is proposed to be important in diverse cellular processes, including cell growth and differentiation.

p68 expression correlates with organ differentiation and maturation of the fetus but does not always correlate to the proliferation in adult tissue (Stevenson et al., 1998). In addition, p68 is also thought to be acting as a transcriptional coactivator that is specific for the estrogen receptor alpha (ER- α) activation function 1 (AF-1) found at the N-terminal region of the ER- α .

1.6.1 p68 as an estrogen receptor alpha (ER- α) coactivator

p68 has been identified as a coactivator of AF-1 of the ER- α A/B domain (Endoh et al 1999; Watanabe et al., 2001). This transactivation interaction of p68 ER- α A/B domain is potentiated by phosphorylation of the Ser¹¹⁸ residue by mitogen-activated protein kinase (MAPK). The protein kinase activation of ER- α potentiates AF-1 activity by increasing the binding affinity for p68. Colocalization of p68 and coactivator p300 as well as interactions with the CBP/p300 complex and RNA pol II suggests the presence of p68 in the ligand-binding activation function II (AF-2) of ER- α proposing a direct role for p68 in transcriptional regulation (Rossow and Janknecht, 2003). It is postulated that the functioning of p68 as a coactivator of ER- α influences the activities of certain target genes and hormones (Endoh et al 1999). The mRNA of p68 is expressed in a sexually dimorphic manner in the P1 ZF brain with females expressing more than males (Carruth et al, 2000), indicating that additional studies of p68 at the protein level are warranted. Sexual differentiation in ZF brain may occur by this coactivator, working in conjunction with the other coactivators, to enhance the ER-alpha transcription of target genes.

1.6.2 p68 RNA helicase

RNA helicases are highly conserved and found in all organisms from bacteria to humans to viruses. RNA helicases have been suggested to play a role in unwinding of double-stranded RNA (Tanner and Linder, 2001) and RNA-protein complexes. Most RNA helicases belong to the superfamily II which includes the DEAD (Asp-Glu-Ala-Asp) and DExH (Asp-Glu-x-His) box families as identified by sequences in their conserved motifs (Wassermann and Steitz, 1991). p68 contains a helicase core between bp134-430 containing the DEAD box motif which comprises the NTP binding and hydrolysis domain and an IQ motif at bp 554-560 which includes the CaM binding and PKC phosphorylation sites (see Fig. 4; Yang et al., 2003, Kahlina, 2004) Previous studies have established a role for RNA helicases in several RNA metabolic activities including nuclear transcription, pre-mRNA splicing, nucleocytoplasmic transport and translation (de la Cruz et al., 1999). p68 RNA helicase, a prototypical member of the DEAD-box family of proteins (Ford et al., 1988, Hirling et al., 1989) was first discovered as a result of a cross reaction with an antibody against the simian virus 40 (SV40) large T antigen (Lane and Hoeffler, 1980).

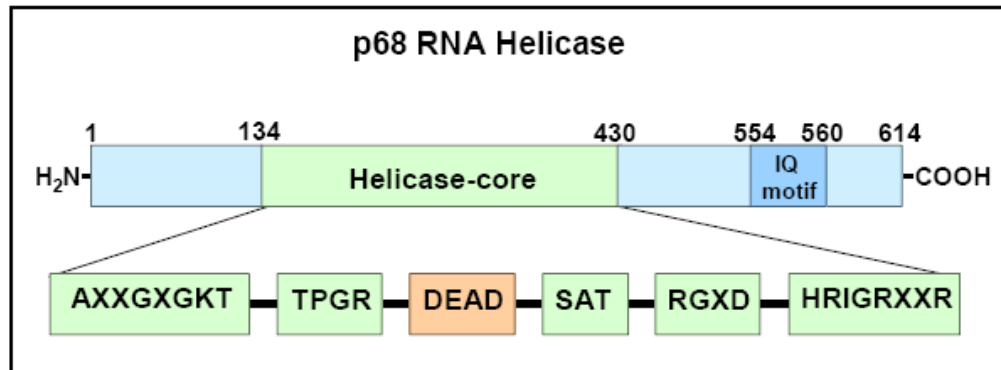


Fig. 5: Schematic representation of domain structure of p68 RNA helicase. (Kahlina, 2004)

p68 RNA helicase exhibits ATP-dependent and RNA-dependent ATPase activities *in vitro* (Iggo and Lane, 1989). ATPase assays have shown that double-stranded RNA (dsRNA) is much more effective than single-stranded RNA in stimulating ATP hydrolysis by the recombinant p68 protein (Huang and Liu, 2002). Furthermore, according to Huang and Liu (2002), p68 unwinds RNA duplexes in a bi-directional manner and this unwinding dsRNA requires energy derived from the hydrolysis of NTP or ATP. RNA helicases have also been shown to unwind RNA-protein complexes (Staley, 1998). p68 RNA helicase is highly conserved and human and murine p68 share 98% homology (Lemaire and Heinlein, 1993). The 3'-UTR of human and chicken p68 are 87% identical, 63% for the *Xenopus*-human comparisons (Seufert et al., 2000), and 70% similarity in humans and rodent (Makalowski and Boguski, 1998). The p68 homologue of *Saccharomyces cerevisiae* (yeast) is 55% identical to the human protein (Iggo et al., 1991).

2

Research Question

2.1 SA 1: Are there sex differences in p68 protein levels at critical points in the ZF brain across development?

The neural dimorphisms which are assumed to permit song production in males and inhibit it in females, develop between approximately P1 and 2 months of age, with the most striking rate of differentiation occurring between days 15 and 35 posthatching (Bottjer et al., 1985; Kirn and DeVoogd, 1989; Nixdorf-Bergweiler, 1996).

Previous studies have shown differential gene expression and protein concentrations in the male and female ZF as early as P1, but the song nuclei do not form until P10.

Therefore, we expect to see significant sex differences in p68 protein expression in male and female ZFs at three different time points, P10, P30 and adulthood (over 65 days old).

2.2 SA 2: Do the brain regions involved in song learning and production exhibit p68 protein expression?

We expect to observe expression of p68 protein in the four major telencephalic song nuclei (HVC, RA, LMAN, and Area X) and the accessory song nuclei (DLM and NCM) in brains of male P30 and adult ZFs. Also, we expect to identify p68 expression in the neurons of the subventricular zone (SVZ) which would later migrate and differentiate into specific neurons in the ZF song nuclei and brain. We hypothesize that the presence

of p68 immunoreactive neurons (p68-ir) is due to the role of estrogen in development of song nuclei and the role of p68 as an ER- α coactivator.

3

Materials and Methods

3.1 Animal care and tissue collection

All zebra finches used for the study will be housed at the Carruth lab aviary (Georgia State University) in three flight cages. Five birds of each sex (male and female) at ages P1, P10, P30 and adult were used for western analysis. Only males (n=5) were used for the immunohistochemical studies just to establish the presence of p68 immunoreactivity in the song system and other brain regions. Adult and hatchling ZFs were checked and cared for on a daily basis. All animals were kept on a 12 hour light: dark photoperiod and feed finch seed *ad libitum*. The diet was supplemented once/week with hardboiled eggs (with shell) and spinach chopped together. To stimulate breeding, adult birds were misted with water daily. Experimental animals were collected, deeply anesthetized with an overdose of isoflurane, rapidly decapitated, and examined with the dissecting microscope in order to observe the gonads to identify or verify their sex. Brain tissue for the Western blot analysis, was subdivided upon collection into the telencephalon (contains the song nuclei) and the “rest of the brain” (RB; all brain tissue excluding the telencephalon). Tissue was quickly collected and either placed in sterile

microcentrifuge tubes containing 4% paraformaldehyde for immunohistochemistry or flash frozen for protein isolation for western blot analysis.

3.2 Sex identification

All experimental animals were sexed by observing the presence of gonads, testis (male) or ovaries (female) under the dissecting microscope. To further verify sex in P1 birds, in which the gonads can be small and difficult to identify, a sexing PCR was performed (described below). The sexing PCR was conducted on genomic DNA using PCR amplification to identify the sex specific alleles (ZZ-male and ZW- female).

3.3 Preparation of ZF Genomic DNA

3.3.1 DNA digestion

Using surgical tools, a small piece of the P1 body (such as one leg or wing) was removed and placed in a clean 1.5 ml microcentrifuge tube. Tools were cleaned with distilled water and 2M NaOH between birds to prevent cross-contamination of DNA samples. 1ml DNA digestion buffer (100mM NaCl, 10mM Tris (pH 8.0), 25 mM EDTA (pH 8.0), 0.5% SDS stored at room temp) and 100ug of proteinase K was added to each tube. Tube contents were mixed gently using a P1000 pipet to avoid introducing bubbles and then incubated overnight in 50°C water bath to allow for tissue digestion. The next morning the digested tissue is stored at -20°C until the DNA isolation procedure was conducted.

3.3.2 DNA isolation

Tissue lysates were mixed gently using a pipet to ensure proper lysing of cells. Gel phase lock (GPL) tubes were used to perform solution fractionation using centrifugation (Eppendorf microcentrifuge). Before separation of lysate fractions, GPL tubes were prepared by spinning for 30 sec at 14 000 x g at 4°C in an eppendorf centrifuge 5417 R (Brinkmann Instruments, Inc., Westbury, NY). 125µl of digested tissue from each animal was added to labeled GPL tubes respectively. Subsequently, 62.5µl phenol and 62.5µl chloroform were added and mixed gently. Solution was centrifuged for 5 min at 14 000 x g in 4°C. Following addition of another 125µl of chloroform to each sample, GPL tubes were centrifuged for 5 min at 14 000 x g in 4°C and aqueous layer immediately transferred to autoclaved 0.5ml tubes and stored at -20°C.

3.3.3 DNA precipitation

To precipitate DNA from the aqueous layer containing the genomic DNA 125µl isopropanol was added to the aqueous layer and solution was mixed gently by hand to prevent shearing of genomic DNA. Samples were then incubated at room T for 3 min for precipitation. The DNA precipitate was then collected using a pipet. DNA was washed twice in 450µl of RNase free 70% EtOH and then centrifuged for 5 min at 12 000 rpm in an Eppendorf mini spin centrifuge (Brinkmann Instruments, Inc., Westbury, NY) at room T to pellet the DNA. Pellets were dried in a vacuum chamber to ensure complete removal of ethanol which could disrupt signal amplification during PCR. Finally, the DNA pellets were dissolved in 30µl of TE buffer and stored at -20°C.

3.4 Polymerase Chain Reaction (PCR)

PCR allows for *in vitro* amplification of DNA fragments using heat-resistant DNA polymerases which permits multiple denaturation of a template DNA, annealing of primers and synthesis of DNA. Each 0.2ml thin-walled PCR tubes contained 5µl Mg free PCR buffer, 6µl 25mM MgCl₂, 1µl 10mM dNTPs (1µl dATP, 1µl dGTP, 1µl dCTP, 1µl dTTP, 6µl nuclease free water), 2µl of 10mM fZF sex chromosome-specific primer (5'-YTKCCAAGRATGAGAACTG-3'), 2µl of 10mM rZF sex chromosome-specific primer (5'-TCTGCATCACTAAAKCCTTT-3'), 31µl of distilled water, 3µl of DNA template, and 0.5µl of Taq polymerase. The reaction was carried out in a thermocycler as follows; 5 min at 94°C for 1 cycle, 45 sec at 94°C, 1 min at 50°C, and 1 min at 72°C for 35 cycles, 5 min at 72°C for 1 cycle. PCR products were stored at 4°C prior to gel electrophoresis.

3.4.1 Agarose Gel Electrophoresis

PCR products were electrophoresed in 1.5% agarose gel using a 6X or 10X gel loading buffer. 100bp DNA ladder was used as marker to identify band sizes and corresponding base pairs. Double band at 353bp and 389bp indicate presence of female sex chromosomes (ZW – heterogametic sex) and single band at 351bp indicate presence of male only sex chromosomes (ZZ – homogametic sex).

3.5 Preparation of Nuclear and Cytoplasmic extracts

Brain tissue (telencephalon and rest of brain) were collected from each experimental animal (male and female) at ages P10, P30 and adult and stored at -80°C prior to cellular fractionation. The majority of the song control nuclei are located in the telencephalon and protein analysis in this region though diluted highlights the expression pattern. Tissue samples were weighed and then homogenized in Buffer A (10mM Hepes, pH 7.9, 10mM KCl, 0.1mM EDTA, 1µl of protease inhibitor, 200µl of 10% IGEPAL per 5ml of Buffer A). Lysing was carried out at 500µl of Buffer A/50mg tissue sample. Lysates were vortexed for 15 sec, placed on ice for 10 min and centrifuged at 15 000 x g for 5 min in 4°C. The supernatant (containing the cytoplasmic extract) was immediately transferred to autoclaved and pre-chilled eppendorf tubes and stored at -20°C. The residual pellets were re-suspended in 150µl of Buffer B (20mM Hepes, pH 7.9, 10mM KCl, 1mM EDTA, 10% Glycerol, added before use: 1µl of protease inhibitor per 1ml of Buffer B). Subsequently, the solution was vortexed 15 sec at 10 min intervals for 60 min. Lysate solution was centrifuged 15 000 x g for 10 min in 4°C and supernatant (containing the nuclear extract) was immediately transferred to autoclaved and pre-chilled eppendorf tubes and stored at -20°C. Tissue was dissociated via homogenization with a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY)), and centrifugation was done using an Eppendorf microcentrifuge. Analysing nuclear extracts and cytoplasmic extracts allows for better understanding of p68 expression patterns as it performs its multiple functions, as a nuclear receptor coactivators and in pre-translation activities in the cytoplasm.

3.6 Determination of protein concentration

Protein concentrations in the nuclear and cytoplasmic extracts were determined using the BCATM Protein Assay Kit (Pierce, Rockford, IL). The required volume of BCA working reagent was determined as follows;

Total Working Reagent (W.R.) = (# standards + # unknown samples x (# replicates) x (vol. of W.R per sample)

W.R. was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B (50:1; Reagent A : Reagent B). Standard solutions contained increasing protein concentrations ranging from (20-2,000µg/ml). 25µl each of 2 replicates of either the standard or unknown samples were pipetted into the microplate. Subsequently, 200µl of W.R. was added to each microplate well and the incubated at 37°C for 30 min. After incubation, optical density was measured at a wavelength of 562 nm on a BIO-TEK Synergy HT, microplate reader (Bio-Tek Instrument, Inc., Winooski, VT). Absorption values were calculated using the KC4 for Windows program, version 3.02 (Bio-Tek Instrument, Inc., Winooski, VT).

3.7 Western blotting analysis

Western blots provide a method for identifying specific polypeptide molecules found within a protein complex. The western analysis includes separation of proteins by SDS Page electrophoresis followed by the transfer of these protein fragments onto a membrane (PVDF membrane). Incubation of the membrane with an antibody cultured against a specific antigen and subsequent recognition of the bound antibody by a

secondary anti-immunoglobulin coupled to horseradish peroxidase allows for detecting the protein of interest.

3.8 SDS PAGE

Electrophoretic separation of proteins was conducted via SDS-PAGE as initially described by Laemmli (1970). 20 μ g of total protein samples were denatured by heating to 90°C in a solution of thiol compounds contained in the 6X sample loading buffer (2.5 ml of 1.25M Tris-HCl Buffer, 1.0g SDS (Sodium dodecyl sulfate), 2.5ml β -mercaptoethanol, 10ml of 50% glycerol, 5mg bromophenol blue in 50ml distilled water). The thiol compounds β -mercaptoethanol and SDS allow for proper separation of proteins during electrophoresis. 20 μ l of solution containing 20 μ g of total protein samples, 6X sample loading buffer and distilled water was heated at approximately 95°C for 5 min. After cooling to room T, the solution was centrifuged for 5 sec and loaded on the gel. Subsequently, the gel was run at 180V and 30 mA for 30 min a 4-12% Tris·HCl gel, 10 wells 30 μ l each (Bio-Rad). 10 μ l of biotinylated marker (Cell Signalling, Danvers, MA) and 6 μ l of Pre-stained (Blue) marker (N.E. Biolabs, Ipswich, MA) were used for identification of band sizes and protein weight.

3.9 Transfer to PVDF membrane

After electrophoresis, proteins were transferred to a polyvinyl fluoride (PVDF) membrane (Trans-Blot SD, Bio-Rad, Hercules, CA). The PVDF membrane was inactivated in 100% methanol and rinsed in deionized water before being placed into the transfer apparatus. The SDS gel containing separated protein samples was detached from

peroxidase-labeled goat anti-mouse antibody (Upstate Biotechnology, Lake Placid, NY), 1:2000 goat anti-rabbit antibody to bind the anti-actin antibody and 1:2000 anti-biotin to amplify the marker signal, at room T for 30 min. Immunoreactivity was detected by chemiluminescence (LumiGLO® Chemiluminescent Substrate, Upstate Biotechnology, Lake Placid, NY) and quantified on the Scion Image for Windows (Scion Corporation, Frederick, Maryland). The p68 to actin ratio was calculated and used to correct for loading and to calculate the protein expression in each protein sample.

3.11 Immunohistochemistry (IHC)

3.11.1 Tissue preparation

After 24 hours at room T, the 4% paraformaldehyde (made in PBS) was decanted and replaced with 70% ethyl alcohol (EtOH). The tissue (entire heads for P1 birds or only the brain for P30 and adults) was incubated in 70% EtOH for a minimum of 24 hrs at room T or stored in this solution until embedding. Intact heads were removed from 70% EtOH and placed in labeled paper embedding bags. Subsequently, brain tissue was tightly secured in the bags and processed through the Citadel 1000 (Shandon) which contained different concentrations of EtOH (70%, 95%, 100%), Protocol Safeclear II (a clearing agent; Fisher Diagnostic, Middletown, VA) for 23 hrs. The heads were then removed from the Citadel 1000 and embedded in paraffin wax (Paraplast, Fisher Healthcare, Houston, TX) using the Histocenter 2 (Shandon-Thermo Fisher Scientific Inc., Waltham, MA). Embedded tissue was stored at 4°C prior to sectioning on the microtome.

3.11.2 Tissue sectioning and Immunohistochemistry (IHC)

IHC was performed to localize brain regions containing p68-immunoreactive (p68-ir) neurons. 15µm sections from each paraformaldehyde fixed, and paraffin embedded whole brain tissue were sectioned using a rotary microtome. The tissue sections were mounted on chrom-alum subbed slides and deparaffinized using Protocol Safeclear II. Subsequently, tissues were rehydrated using decreasing concentrations of EtOH starting with 100% through 40% ethanol and distilled water. 0.5% Triton-X100 wash was carried out immediately to enhance accessibility of the nucleus. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide. Antigen retrieval was carried out after several buffer washes using hot 0.01M citrate buffer. Tissue sections were blocked in 5% normal goat serum (KPL, Gaithersburg, Maryland) prior to immunostaining with antibody [Carruth lab, IHC protocol, Georgia State University, Atlanta, GA). Sections were incubated for 48h at 4°C with one of two different p68 antibodies, the mouse monoclonal anti-p68, clone PAb204 (Upstate Biotechnology, Lake Placid, NY) or the mouse monoclonal anti-p68, rgg-1 (Courtesy of Dr. Zhi-Ren Liu, Georgia State University, Atlanta, GA) ; immune complexes were detected using biotin-SP-conjugated AffiniPure goat anti-mouse antibody (H+L) (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA). Streptavidin/biotin (StreptABComplex/HRP) (Vector Laboratories, Inc., Burlingame, CA) amplification system was used to amplify specific signal even though it is known to amplify unspecific signals in some cases. Enzyme mediated antibody detection was incorporated into the experiment by using

Diaminobenzidine (DAB) (KPL, Gaithersburg, Maryland) which produces a brown end product and is highly insoluble in water, as substrate for the enzyme horseradish peroxidase. Finally, tissues were dehydrated using graded series of ethanol, starting with 40% through 100% and cleared in Protocol Safeclear II clearing agent. Slides were cover slipped using a histological mounting medium, permount (Fisher Scientific, Fair Lawn, New Jersey).

4

Results

4.1 Sexing PCR

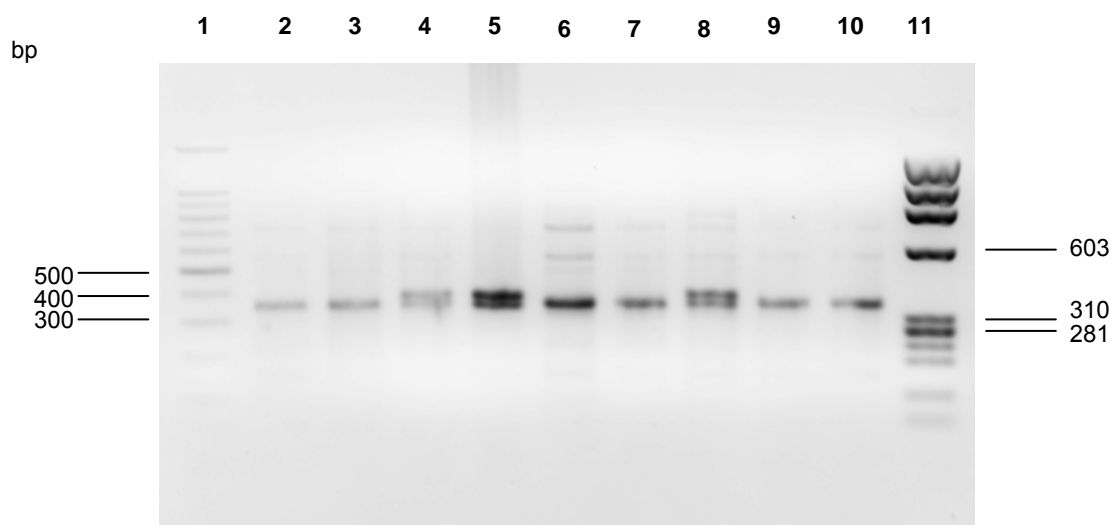


Fig. 7: Amplification and identification of the zebra finch sex chromosomes (female – ZW; male – ZZ).

The figure above shows a representation of the results from the sex identification using the ZF genomic DNA. Lanes 1 and 11 represent the markers, 100bp DNA ladder and PhiX751/HaeIII respectively used for identifying band sizes and corresponding base pairs. Lanes 2, 3, 4, 6, 7, 9 and 10 with the single bands at 351bp represent male sex chromosomes while lanes 4, 5 and 8 with double bands at 353bp and 389bp indicate presence of female sex chromosomes.

4.2 Are there sex differences in the levels of p68 protein at critical points in the development of the ZF brain?

4.2.1 Post hatchling day 10 (P10)

Nuclear extracts from the telencephalon and RB (“rest of brain”) from P10 male and female ZFs ($n = 5$) were examined for p68 protein expression. Data collected from protein expression studies on all experimental animals using Western blotting analysis were characterized by ANOVA followed by Fisher's probable least-squares difference (PLSD) test. P10 females displayed a trend toward a higher concentration of nuclear telencephalic p68 but this was not significant ($n = 5$, $p = 0.08$, Fig. 9). Subsequently, analysis of p68 expression in the RB showed an observable but not significant difference in the sexes with a pattern of expression similar to expression levels in the telencephalon, with a trend toward more expression in females compared to the males but not significant ($n = 5$, $p = 0.36$, Fig. 11). Overall analysis of the entire brain (telencephalon and RB) of both sexes showed a significant sex difference ($n = 5$, $p = 0.05$, Fig. 12) in p68 expression with higher concentration observed in females.

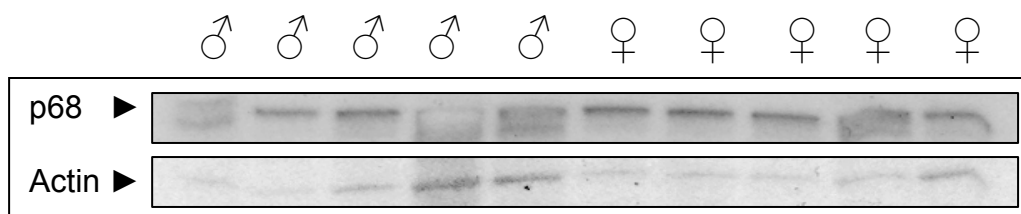


Fig. 8: Immunoblot showing detection of p68 protein expression in nuclear extracts from the telencephalic brain region of the P10 male and female zebra finches using monoclonal mouse antibody, rgg – 1 6G78A2. β -actin (1:5000) was used as loading control.

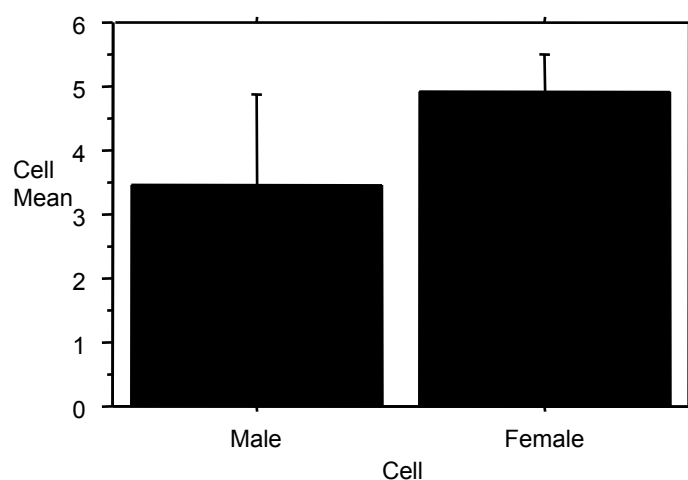


Fig. 9: Quantification of p68 protein expression in nuclear extracts from the telencephalic brain region of the P10 male and female zebra finches with error bars indicating mean \pm SD obtained from independent animals (n=5) of each sex. p68 was detected using monoclonal mouse antibody, rgg – 1 6G78A2. (p-value = 0.08).

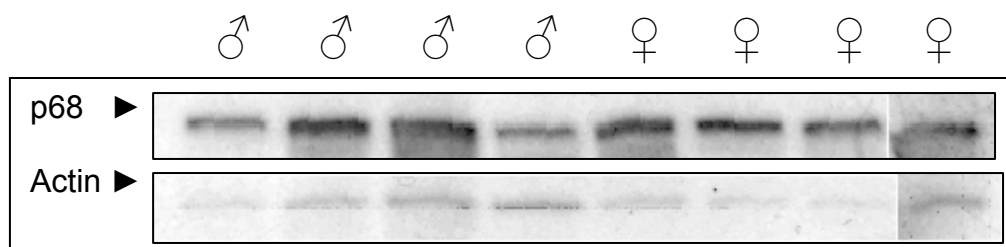


Fig. 10: Immunoblot showing detection of p68 protein expression in nuclear extracts from the rest of brain of the P10 male and female zebra finches using monoclonal mouse antibody, rgg – 1:6G78A2. β -actin (1:5000) was used as loading control.

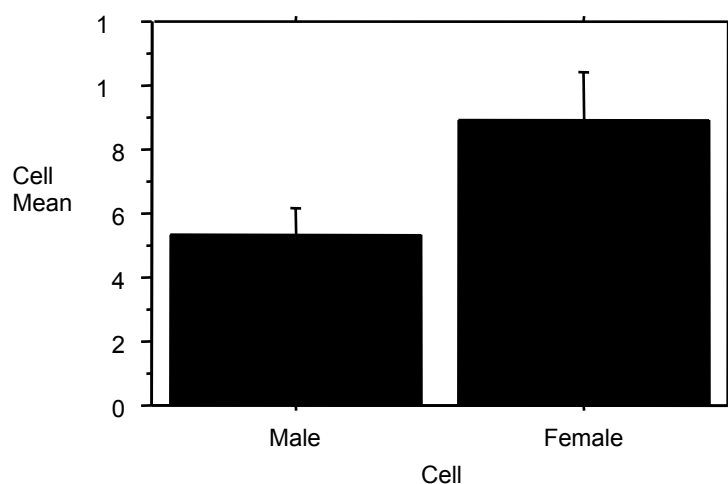


Fig. 11: Quantification of p68 protein expression in nuclear extracts from the rest of brain of the P10 male and female zebra finches with error bars indicating mean \pm SD obtained from independent animals (n=5) of each sex. p68 was detected using monoclonal mouse antibody, rgg – 1: 6G78A2. (p-value = 0.36).

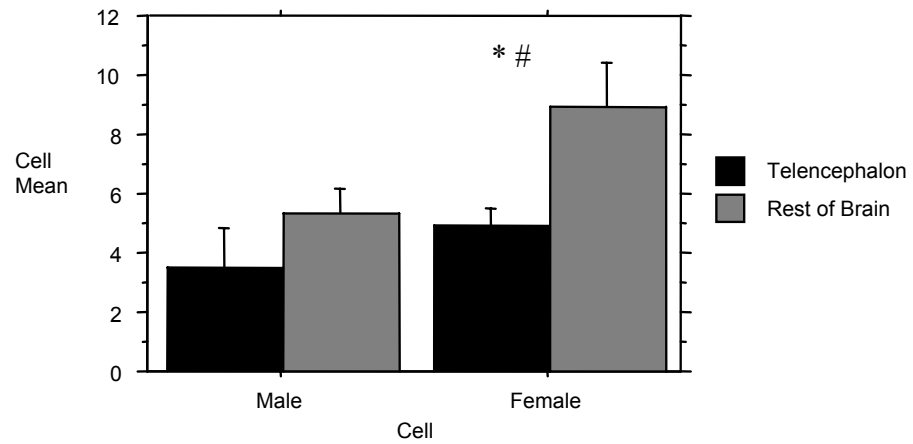


Fig. 12: Quantification of p68 protein expression in nuclear extracts from the telencephalon and rest of brain of the P10 male and female zebra finches with error bars indicating mean \pm SD obtained from independent animals (n=5) of each sex. p68 was detected using monoclonal mouse antibody, rgg – 1 6G78A2. (Comparing #telencephalon and rest of brain, p-value = 0.02; comparing *male and female, p-value = 0.05).

4.2.2 Post hatchling day 30 (P30)

Nuclear extracts from telencephalon and RB of the P30 male and female ZFs were tested for p68 protein expression. At P30, the female ZF showed a trend toward lower concentration of p68 in the nucleus of cells localized in the telencephalon compared to males but this was not significant ($n = 4$, $p = 0.07$, Fig. 14). Subsequently, analysis of p68 expression in the RB did not show an observable nor significant difference in the sexes ($n = 4$, $p = 0.81$, fig. 16). Analysis of the entire brain (telencephalon and RB) of both sexes show did not show significant sex differences (Fig. 17) in p68 expression as well.

4.2.3 Adults

Nuclear and cytoplasmic extracts from telencephalon and rest of brain of the P30 male and female ZFs were examined for p68 protein expression. Male ZFs showed a slightly higher, but not significant, level of p68 expression in the nuclear extract from the telencephalon. Although there was suggestive difference in the concentration of p68 the nuclear extracts of the telencephalon, it does not show any significance ($n = 4$, $p = 0.13$, Fig. 21). Also, the nuclear extracts from RB showed no significant difference in protein expression ($n = 4$, $p = 0.53$, Fig. 23). Subsequently, analysis of the nuclear extract from the entire brain (telencephalon and RB) of both sexes show did not show significant sex differences ($n = 4$, $p = 0.17$, Fig. 24) in p68 expression either. Analysis of the cytoplasmic extracts showed no sex differences in the telencephalon ($n = 4$, $p = 0.85$, Fig. 26) nor RB ($n = 4$, $p = 0.68$, Fig. 28). Furthermore, analysis of the cytoplasmic extracts from the entire brain (telencephalon and RB) of both sexes show did not show significant sex differences ($n = 4$, $p = 0.72$, Fig. 29) in p68 expression either.

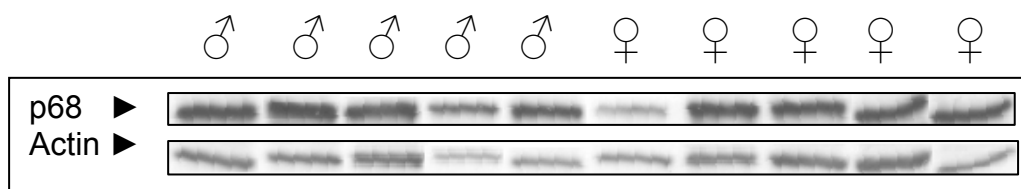


Fig. 13: Immunoblot showing detection of p68 protein expression in nuclear extracts from the telencephalon of the P30 male and female zebra finches using monoclonal mouse antibody, rgg – 1 6G78A2. β -actin (1:5000) was used as loading control.

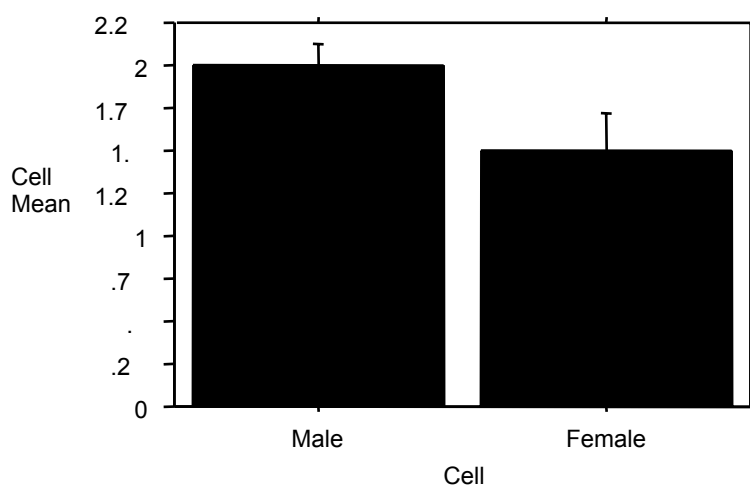


Fig. 14: Quantification of p68 protein expression in nuclear extracts from telencephalon of the P30 male and female zebra finches with error bars indicating mean \pm SD obtained from independent animals (n=5) of each sex. p68 was detected using monoclonal mouse antibody, rgg – 1 6G78A2. (p-value = 0.07).

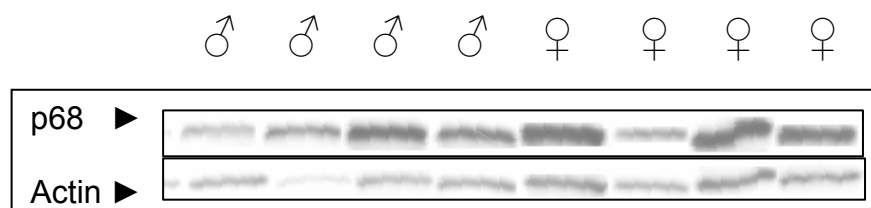


Fig. 15: Immunoblot showing detection of p68 protein expression in nuclear extracts from the rest of brain of the P30 male and female zebra finches using monoclonal mouse antibody, rgg – 1 6G78A2. β -actin (1:5000) was used as loading control.

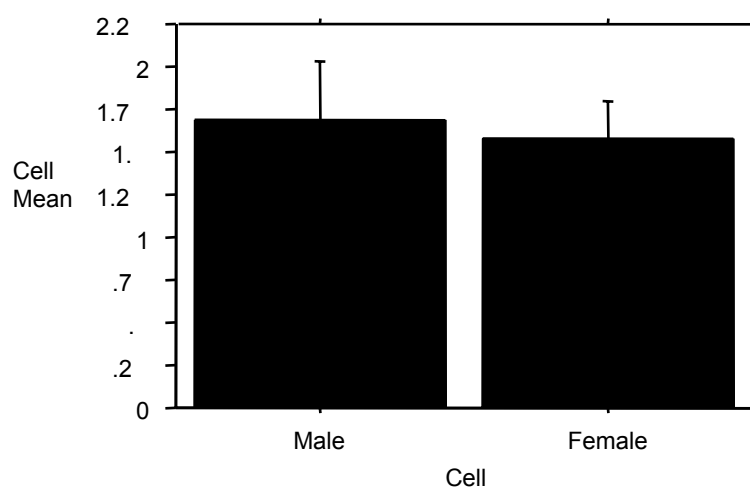


Fig. 16: Quantification of p68 protein expression in nuclear extracts from the rest of brain of the P30 male and female zebra finches with error bars indicating mean \pm SD obtained from independent animals ($n=4$) of each sex. p68 was detected using monoclonal mouse antibody, rgg – 1 6G78A2. (p -value = 0.81).

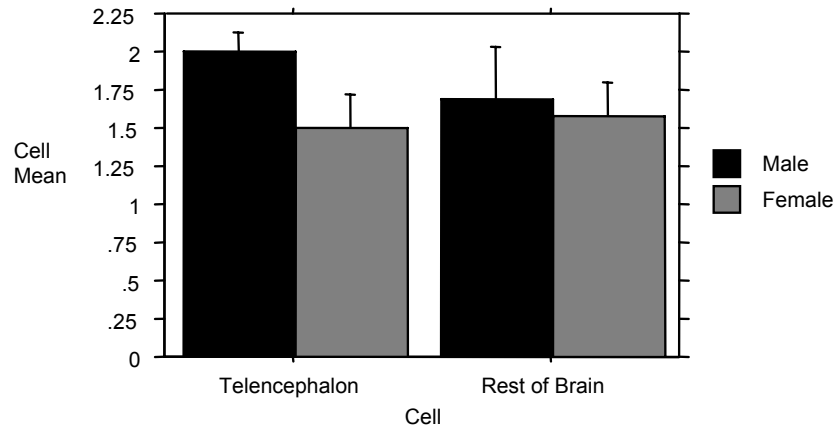


Fig. 17: Quantification of p68 protein expression in nuclear extracts from the telencephalon and rest of brain of the P30 male and female zebra finches with error bars indicating mean \pm SD obtained independent animals (n=5) of each sex. p68 was detected using monoclonal mouse antibody, rgg – 1 6G78A2. (Comparing telencephalon and rest of brain, p-value = 0.65; comparing male and female, p-value = 0.23).

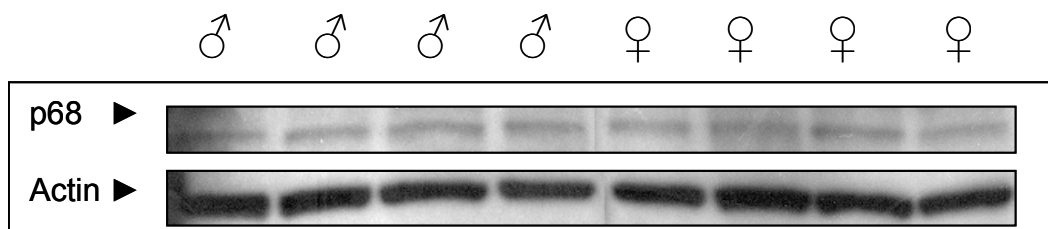


Fig. 20: Immunoblot showing detection of p68 protein expression in nuclear extracts from the telencephalon of adult male and female zebra finches using monoclonal mouse antibody, rgg – 1 6G78A2. β -actin (1:5000) was used as loading control.

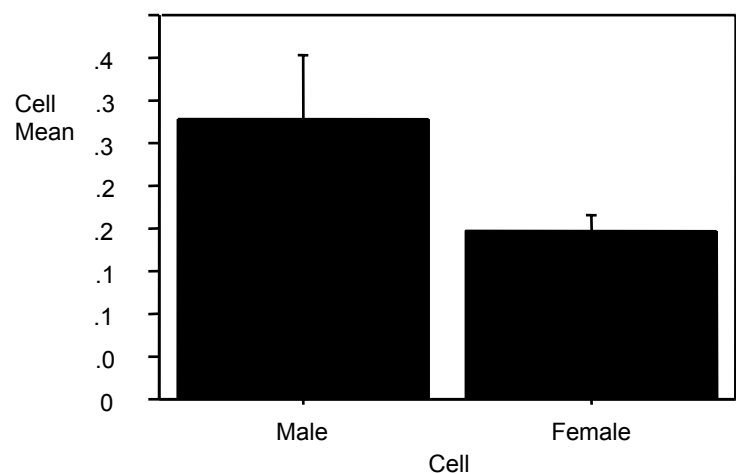


Fig. 21: Quantification of p68 protein expression in nuclear extracts from telencephalon of the adult male and female zebra finches with error bars indicating mean \pm SD obtained from independent animals ($n=4$) of each sex. p68 was detected using monoclonal mouse antibody, rgg – 1 6G78A2. (p-value = 0.13).

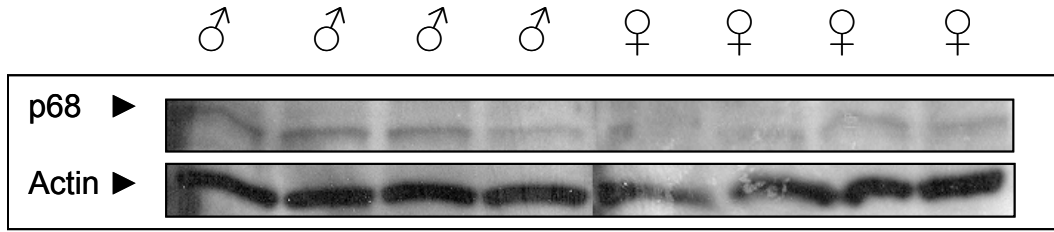


Fig. 22: Immunoblot showing detection of p68 protein expression in nuclear extracts from rest of brain of adult male and female zebra finches using monoclonal mouse antibody, rgg – 1 6G78A2. β -actin (1:5000) was used as loading control.

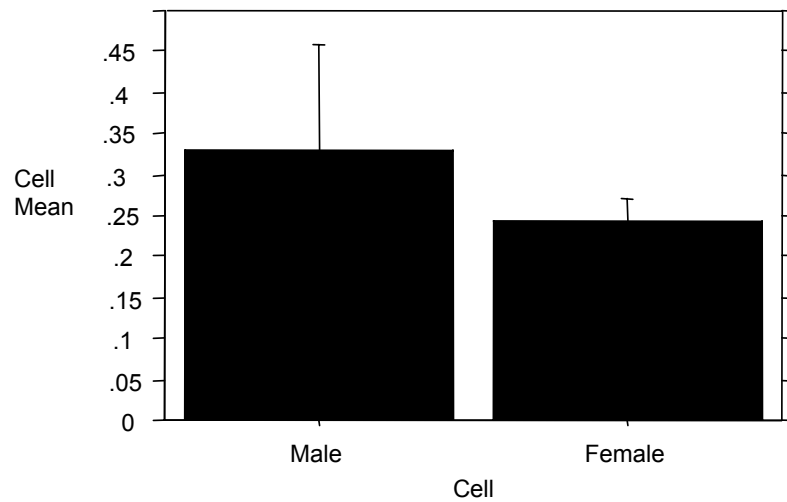


Fig. 23: Quantification of p68 protein expression in nuclear extracts from rest of brain of the adult male and female zebra finches with error bars indicating mean \pm SD obtained from independent animals ($n=4$) of each sex. p68 was detected using monoclonal mouse antibody, rgg – 1 6G78A2. (p-value = 0.53).

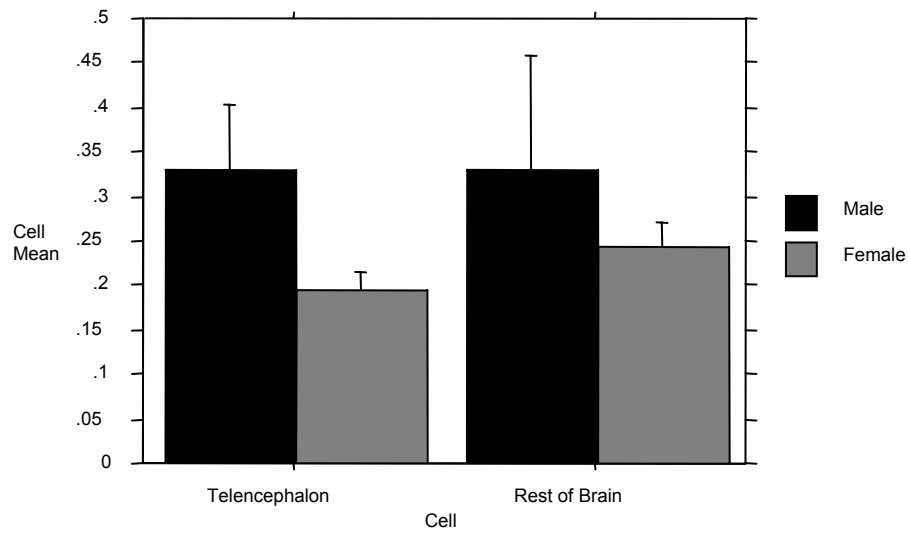


Fig. 24: Quantification of p68 protein expression in nuclear extracts from the telencephalon and rest of brain of the P30 male and female zebra finches with error bars indicating mean \pm SD obtained from independent animals (n=4) of each sex. p68 was detected using monoclonal mouse antibody, rgg – 1 6G78A2. (Comparing telencephalon and rest of brain, p-value = 0.75; comparing male and female, p-value = 0.17).

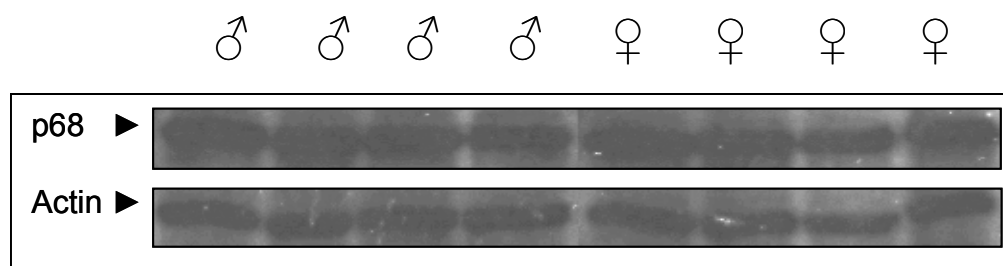


Fig. 25: Immunoblot showing detection of p68 protein expression in cytoplasmic extracts from telencephalon of adult male and female zebra finches using monoclonal mouse antibody, rgg – 1 6G78A2. β -actin (1:5000) was used as loading control.

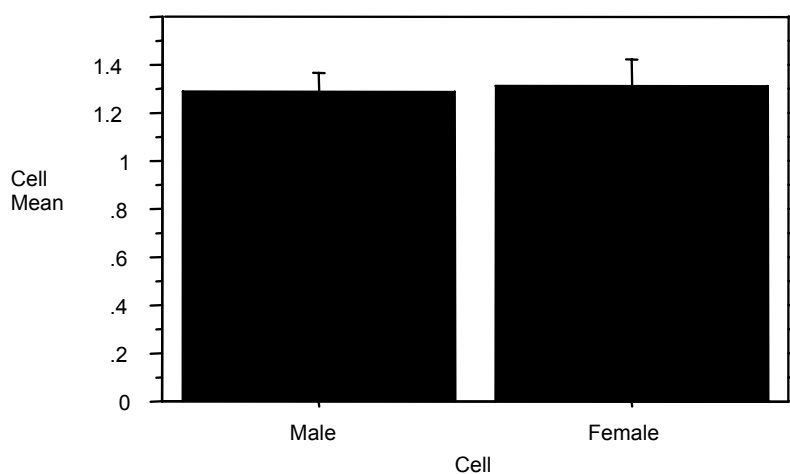


Fig. 26: Quantification of p68 protein expression in cytoplasmic extracts from telencephalon of the adult male and female zebra finches with error bars indicating mean \pm SD obtained from independent animals ($n=4$) of each sex. p68 was detected using monoclonal mouse antibody, rgg – 1 6G78A2. (p -value = 0.85).

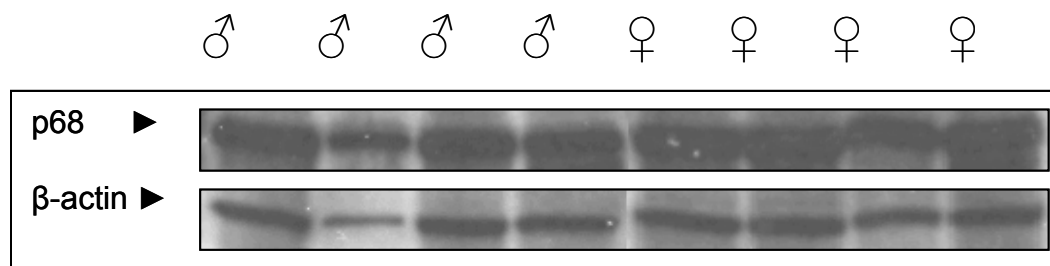


Fig. 27: Immunoblot showing detection of p68 protein expression in cytoplasmic extracts from rest of brain of adult male and female zebra finches using monoclonal mouse antibody, rgg – 1 6G78A2. β -actin (1:5000) was used as loading control.

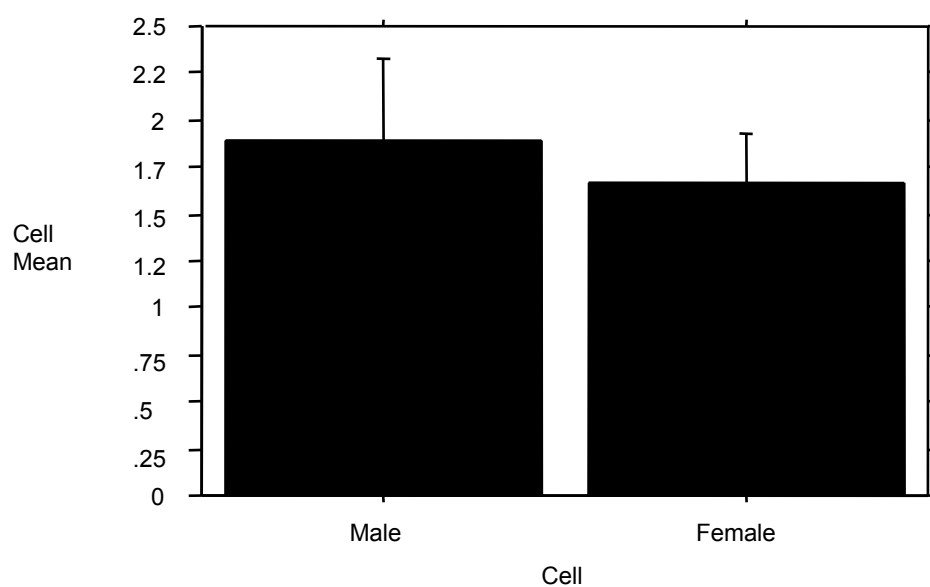


Fig. 28: Quantification of p68 protein expression in cytoplasmic extracts from rest of brain of the adult male and female zebra finches with error bars indicating mean \pm SD obtained from independent animals ($n=4$) of each sex. p68 was detected using monoclonal mouse antibody, rgg – 1 6G78A2. (p -value = 0.67).

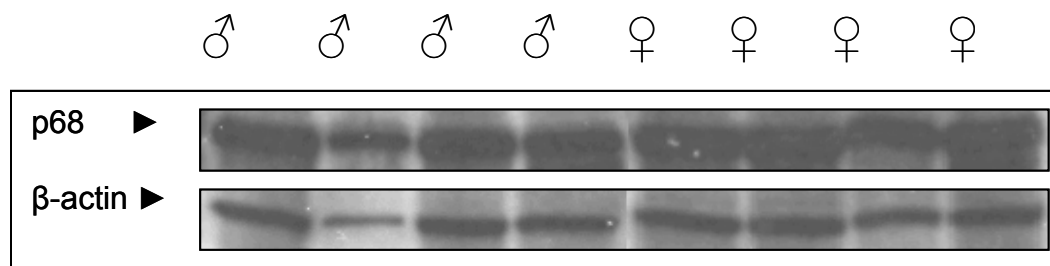


Fig. 27: Immunoblot showing detection of p68 protein expression in cytoplasmic extracts from rest of brain of adult male and female zebra finches using monoclonal mouse antibody, rgg – 1 6G78A2. β -actin (1:5000) was used as loading control.

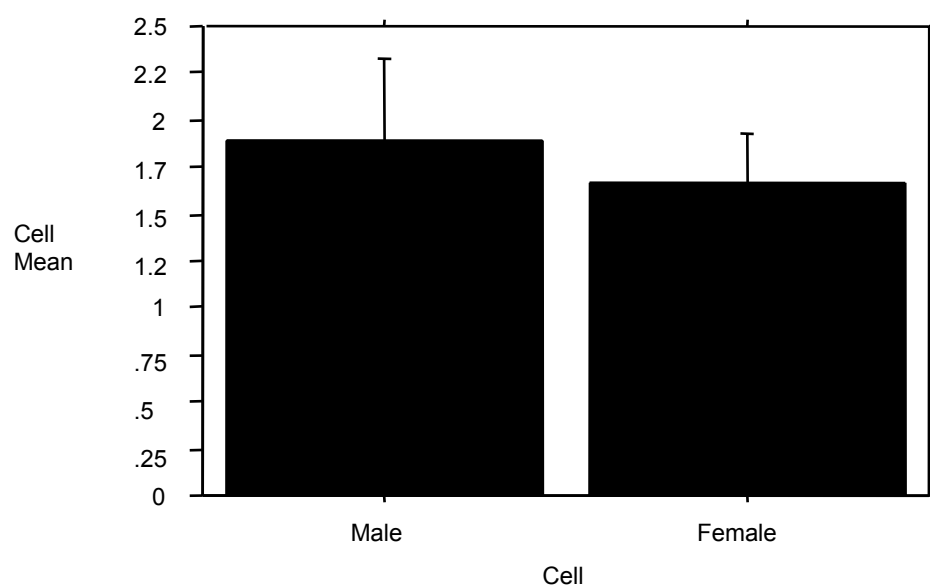


Fig. 28: Quantification of p68 protein expression in cytoplasmic extracts from rest of brain of the adult male and female zebra finches with error bars indicating mean \pm SD obtained from independent animals ($n=4$) of each sex. p68 was detected using monoclonal mouse antibody, rgg – 1 6G78A2. (p -value = 0.67).

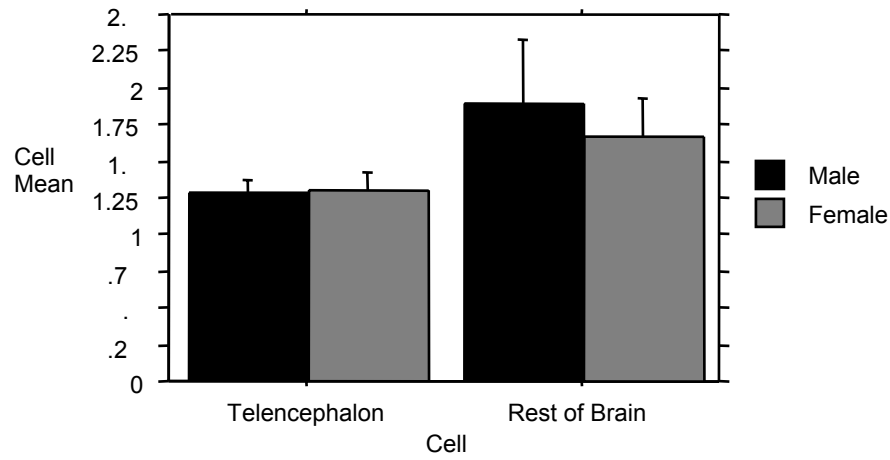


Fig. 29: Quantification of p68 protein expression in cytoplasmic extracts from the telencephalon and rest of brain of the adult male and female zebra finches with error bars indicating mean \pm SD obtained from independent animals ($n = 4$) of each sex. p68 was detected using monoclonal mouse antibody, rgg – 1 6G78A2. (Comparing telencephalon and rest of brain, p -value = 0.09; comparing male and female, p -value = 0.71).

4.3 Are there any differences in the levels of p68 in the ZF telencephalon compared to the rest of the brain?

4.3.1 Post hatchling day 10 (P10) and Post hatchling day30 (P30)

Statistical analysis of p68 expression in the telencephalon and RB showed significantly lower levels of p68 protein in the female telecephalon compared to the female RB ($n = 5$, $p = 0.02$, Fig. 12). No observable differences were identified in the distinct brain regions of the P30 ZF ($n = 4$, $p = 0.65$, fig. 17).

4.3.2 Adult

Statistical analysis of p68 expression in the nuclear extracts from telencephalon and RB showed no observable differences in the distinct brain regions ($n = 4$, $p = 0.75$, Fig. 24). Cytoplasmic extracts from telencephalon and RB showed a higher, but not significant, concentration of p68 in the RB than in the telencephalon ($n = 4$, $p = 0.09$, Fig. 29).

4.4 Are there any observable differences in p68 protein levels across developmental timeline in male ZFs?

p68 protein levels in males of different ages (P10, P30 and adult; $n = 3$) were evident with the highest concentration of p68 being observed at P30 followed by protein levels at adulthood and then P10 (Fig. 19). Though observable, these differences were nonetheless not significant.

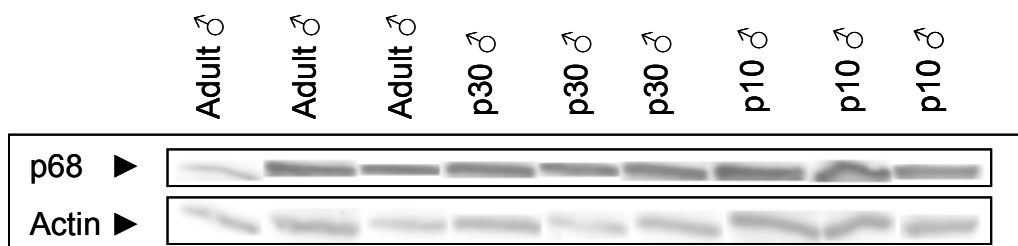


Fig. 18: Immunoblot showing detection of p68 protein expression in nuclear extracts from the telencephalon of male zebra finches across development using monoclonal mouse antibody, rgg – 1 6G78A2. β -actin (1:5000) was used as loading control.

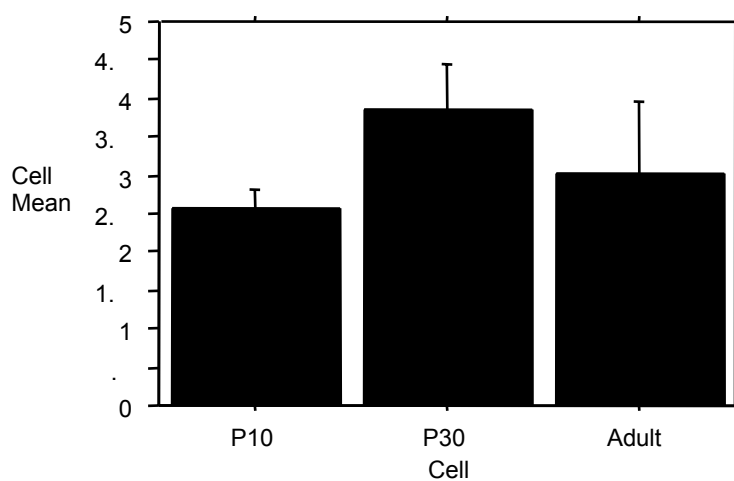


Fig. 19: Quantification of p68 protein expression in nuclear extracts from the telencephalon male zebra finches across development with error bars indicating mean \pm SD obtained from independent animals (n=3) of each sex. p68 was detected using monoclonal mouse antibody, rgg – 1 6G78A2.

4.5 Are there any observable differences in p68 protein levels in the nucleus compared the cytoplasm?

Testing for p68 expression in cytoplasmic extracts was done only in adults. Analysis of nuclear and cytoplasmic extracts from the entire brain of both sexes showed a significantly higher concentration of p68 in the cytoplasm than in the nucleus ($n = 4$, $p = <0.001$, Fig. 30). However, sex ($n = 4$, $p = 0.45$, Fig. 30) and brain region ($n = 4$, $p = 0.08$, Fig. 30) comparison failed to show significant sex differences.

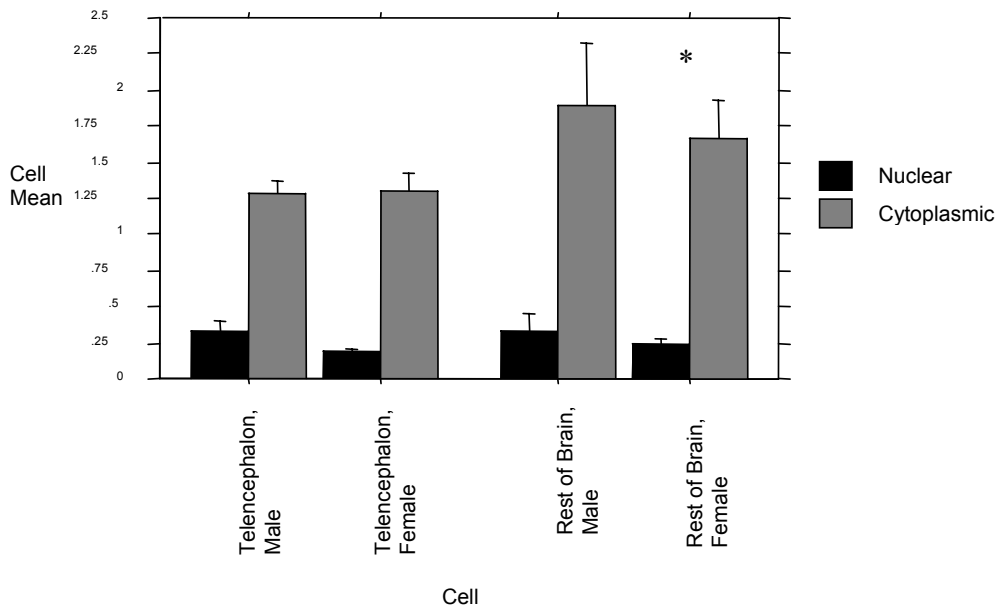


Fig. 30: Quantification of p68 protein expression in nuclear and cytoplasmic extracts from the telencephalon and rest of brain of the adult male and female zebra finches with error bars indicating mean \pm SD obtained from independent animals ($n=4$) of each sex. p68 was detected using monoclonal mouse antibody, rgg – 1 6G78A2. (Comparing telencephalon and rest of brain, p -value = 0.08; comparing male and female, p -value = 0.45; *comparing nuclear and cytoplasmic extracts, p -value = <0.001).

4.6 Distribution of p68 protein in the male zebra finches

Here we have mapped p68 immunoreactive neurons (p68-ir) across the brain in P30 and adult male zebra finches. We report varying levels of distribution in the telencephalon, diencephalon, and the brainstem (Table 1). p68-ir neurons were identified through immunostaining of cells with anti-p68 mouse monoclonal antibodies rgg-1 and PAb204.

In the telencephalon, p68 was localized throughout the rostrocaudal region of the telencephalon spanning the pallium (hyperpallium, mesopallium, nidopallium, and arcopallium) and subpallium (including the striatal and pallidal divisions). In the subpallium of the telencephalon (striatum), moderately stained cells were seen in the medial striatum (Mst; not shown) of both ages. Also, in area X within the Mst (Fig. 45), moderate staining was detected in the P30 male brain compared to the faint staining in the adult. Lightly stained p68-ir cells were seen in the lateral striatum (Lst; not shown) of the P30 but not the adult.

In the hyperpallium subdivision of the pallium, the hyperpallium apicale (HA) contained large areas of light staining as did the hyperpallium densocellulare (HD; not shown) in both P30 and adult. Moderate to light staining was observed in the mesopallium (Fig. 33) at both ages. In the nidopallium subdivision, moderate staining was observed in the nidopallium (N) (Fig. 32) compared to the diffuse line of stained cells seen in the adult. Furthermore, heavy staining was seen in the nidopallium caudale, NC (figure not shown), HVC (Fig. 35), and HVC shelf (Fig. 36) of the P30 compared to that of the adult which showed moderate staining in these areas. Moderate staining was

detected in the caudal medial nidopallium (NCM; Fig. 36) and field L (L; not shown) in both ages. The lateral magnocellular nucleus of the anterior nidopallium (LMAN), medial magnocellular nucleus of the anterior nidopallium (MMAN) and nucleus basorostralis (B or Bas) showed absence of p68-ir neurons in the adult. Some p68-ir magnocellular cells were detected in the male P30 LMAN (LMAN; not shown). In the arcopallium subdivision, heavy staining was detected in the arcopallium (A; Fig. 32) of the P30 male ZF while moderate staining was seen in the adult. Both ages showed moderate staining in the robust nucleus of the arcopallium (RA; not shown).

In the diencephalon, varying levels of protein expression was detected. Heavily stained cells were found in the P30 medial dorsolateral nucleus of the thalamus (DLM; Fig. 38), hippocampus (Hp; not shown), paraventricular nucleus (PVN) and medial preoptic nucleus (POM) compared to the adult with moderately stained cells (Adult DLM, POM; Fig. 44). Moderate expression was found in the adult nucleus septalis lateralis (Adult SL; Fig. 46 and 47), and nucleus stria terminalis (P30 NST; Fig. 37) of both ages. Lightly labeled cells were detected in the subventricular zone (SVZ) of both ages (P30 Fig. 34).

In the brainstem, heavily stained cells were found in the nucleus tuberis (P30 Tu; Fig. 39) and optic tectum (P30 TeO; Fig. 40 and 41) of the P30 compared to that of the adult. The subthalamic nuclei (STN; not shown) showed moderate expression of p68 in both ages. Moderate to lightly stained cells were detected in the ventral tegmental area (VTA), cerebellum (Ce), and purkinje cells (Pc), data not shown.

Brain Region	Abbreviation	Male P30 distribution	Male Adult distribution
Subpallium of the Telencephalon			
<i>Striatal subdivision</i>			
Medial striatum	Mst	++	++
Area X within songbird Mst	Area X or X	++	+
Lateral striatum	Lst	+	
Pallium of the Telencephalon			
<i>Hyperpallium subdivision</i>			
Hyperpallium apicle	HA	+	+
Hyperpallium densocellulare	HD	+	+
<i>Mesopallium subdivision</i>			
Mesopallium	M	++	+
<i>Nidopallium subdivision</i>			
Nidopallium	N	++	+
Nidopallium caudale	NC	+++	++
HVC	HVC	+++	++
HVC shelf	HVC shelf	+++	++
Lateral magnocellular nucleus of the anterior nidopallium	LMAN	++	
Medial magnocellular nucleus of the anterior nidopallium	MMAN		
Caudal medial nidopallium	NCM	++	++
Nucleus basorostralis	B or Bas		
Field L	L	++	++
<i>Arcopallium subdivision</i>			
Arcopallium	A	+++	++
Robust nucleus of the arcopallium	RA	++	++
Diencephalon			
Medial dorsolateral nucleus of the thalamus	DLM	+++	++
Nucleus septalis lateralis	SL	++	++
Hippocampus	HP	+++	
Subventricular zone	SVZ	+	+
Paraventricular nucleus	PVN	+++	++
Medial preoptic nucleus	POM	+++	++
Nucleus striae terminalis	NST	++	++

Brain Region (Cont.)	Abbreviation	Male P30 distribution	Male Adult distribution
Diencephalon cont.			
Nucleus tuberis	Tu	+++	++
Brainstem			
Hypoglossal nucleus-12 th cranial nerve	nXII		
Supraspinal nucleus	SSp		
Ventral tegmental area	VTA	++	+
Substantia nigra	SNc		
Subthalamic nucleus	STN	++	++
Optic tectum	TeO	+++	++
Cerebellum			
Cerebellum	Ce	+	+
Purkinje cells	Pc	++	+

Table 1: Localization levels of p68-ir cells in different brain regions of the p30 and adult male zebra finches. Staining intensities, +++ : high, ++ : medium, and + : light.

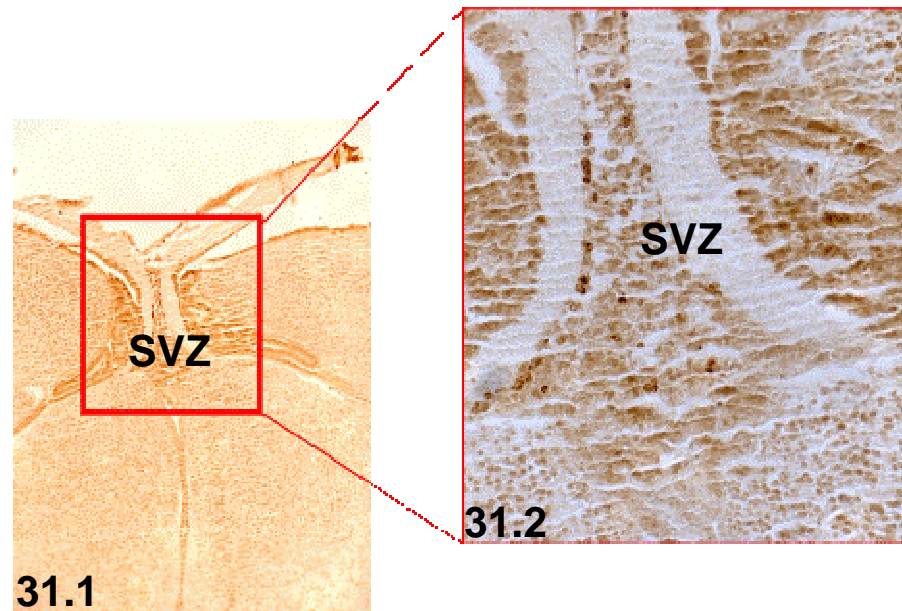


Fig. 31: Microphotograph of a male P1 ventricle and surrounding subventricular zone (SVZ) at 5X (31.1) and 10X (31.2).

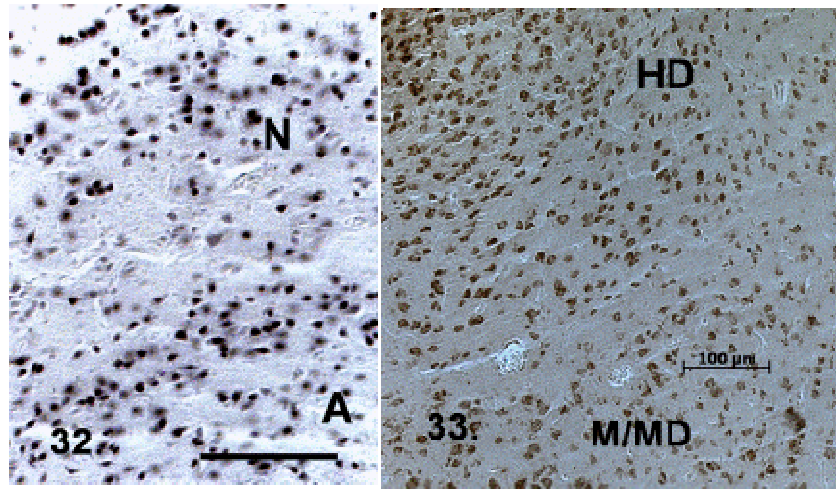


Fig. 32. Microphotograph of a P30 male Nidopallium (N) and Arcopallium (A), 40X, bar = 100µm.

Fig. 33. Microphotograph of a P30 male Hyperpallium Densocellulare (HD) and Mesopallium (M; boarder with Mesopallium dorsale). 20X.

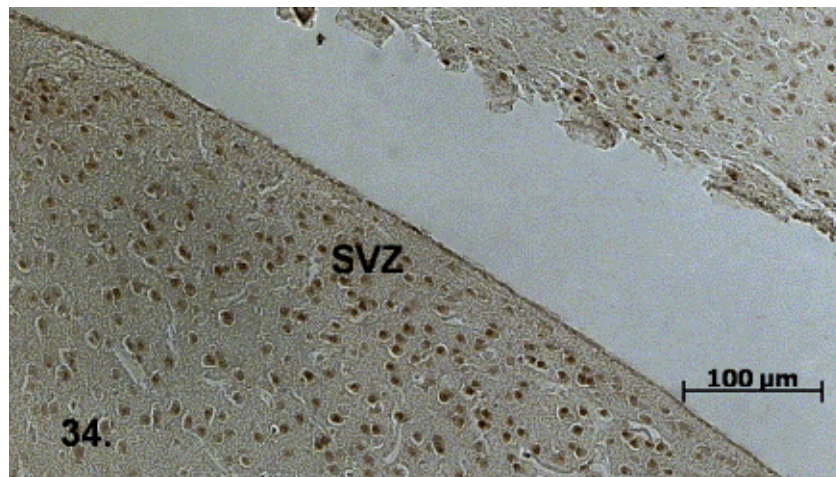


Fig. 34. Microphotograph of a male P30 ventricle and surrounding subventricular zone (SVZ), 20X

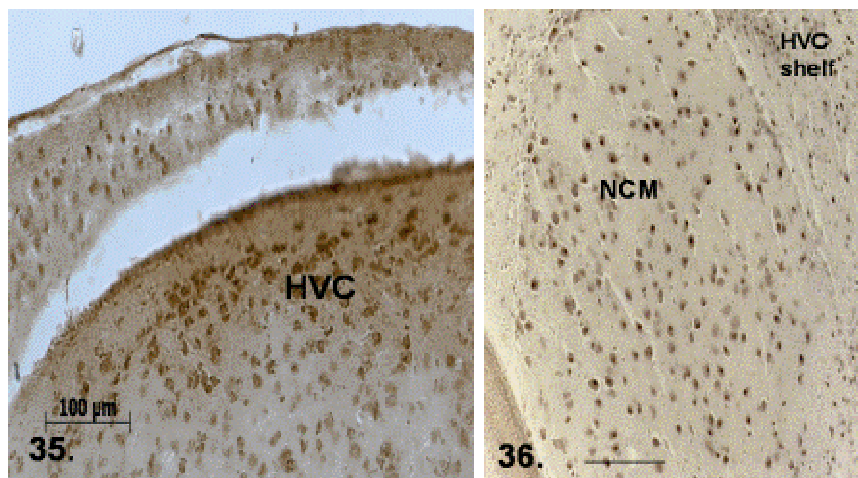


Fig. 35. Microphotograph of a male P30 HVC sitting below a lateral ventricle. 20X
Fig. 36. Microphotograph of a male P30 diencephalon containing the NCM and the HVC shelf 20X

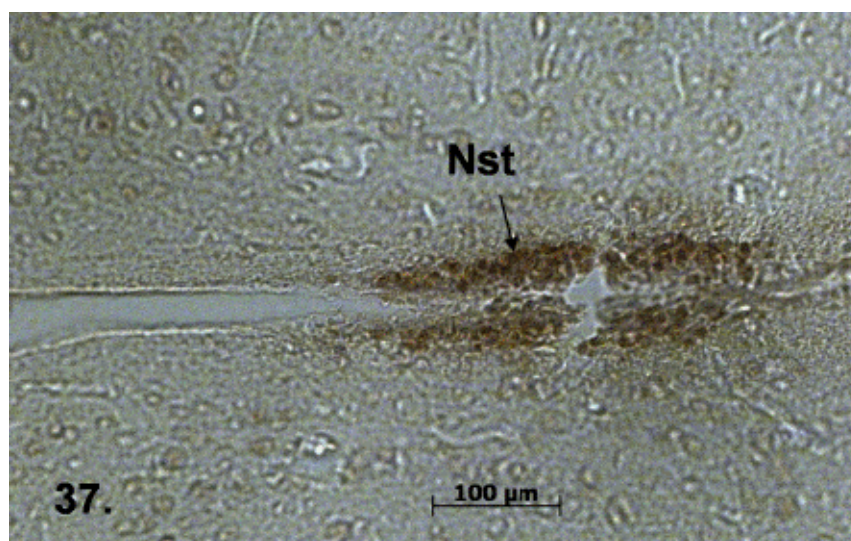


Fig. 37. Microphotograph of a male P30 Nst, 20X.

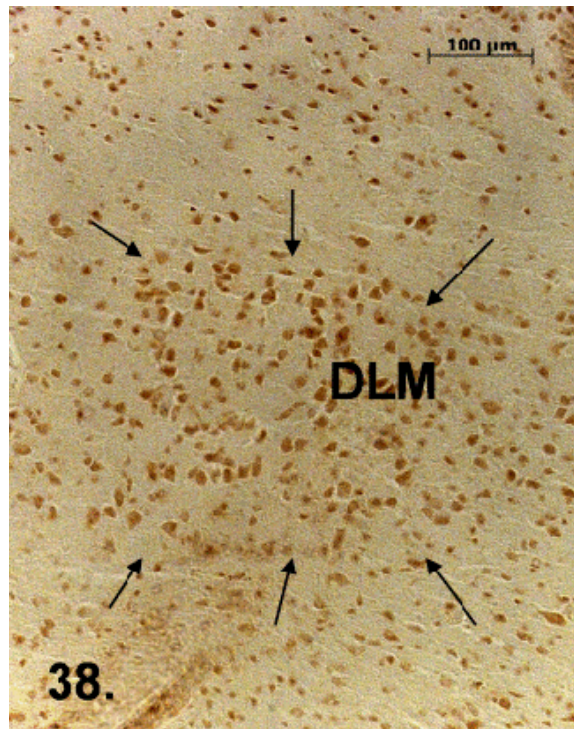


Fig. 38. Microphotograph of a male P30 DLM containing p68-ir neurons. Arrows indicate the boundaries of nucleus, 20X.

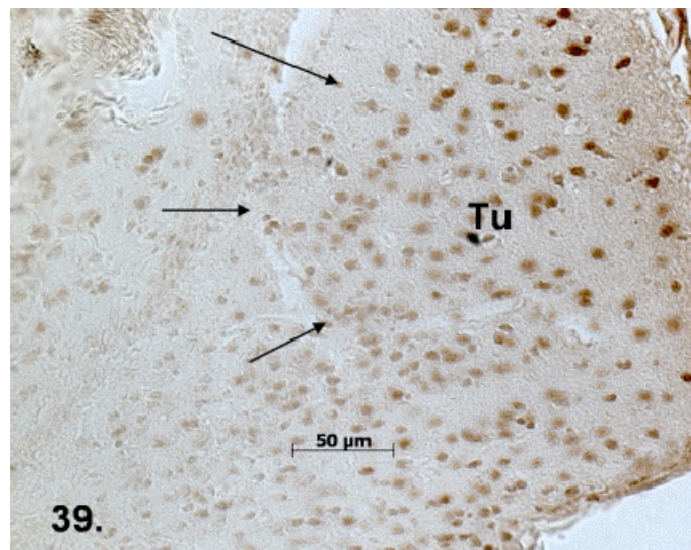


Fig. 39. Microphotograph of a male P30 Tu containing p68-ir neurons. Arrows indicate the boundaries of Tu 20X.

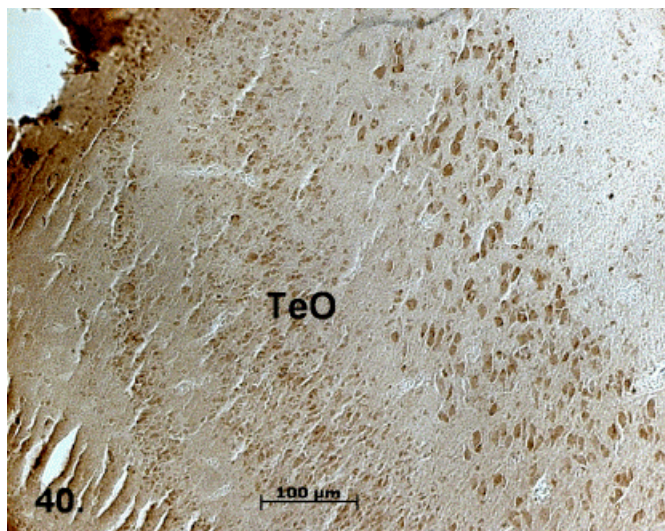


Fig. 40. Microphotograph of a male P30 TeO containing p68-ir neurons. 20X.

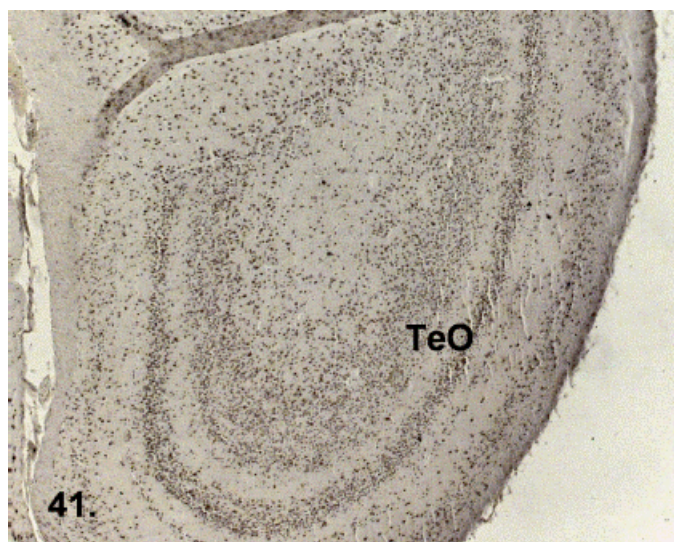
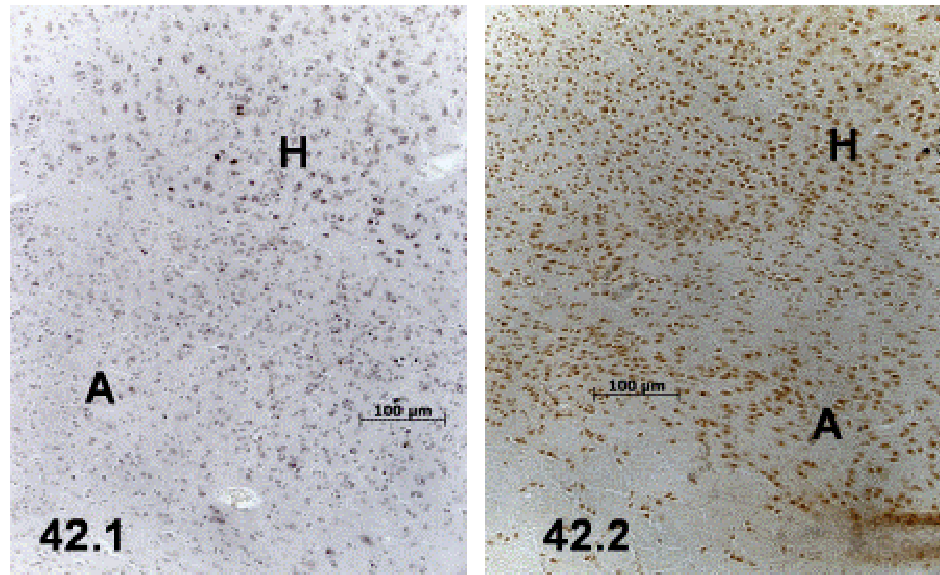


Fig. 41. Microphotograph of a male P30 TeO containing p68-ir neurons. 10X.



Figs. 42 Microphotographs of the adult male Hyperpallium (H) and Arcopallium (A) showing the border between these two regions. 20X (42.1 DAB/Ni; 42.2 DAB)

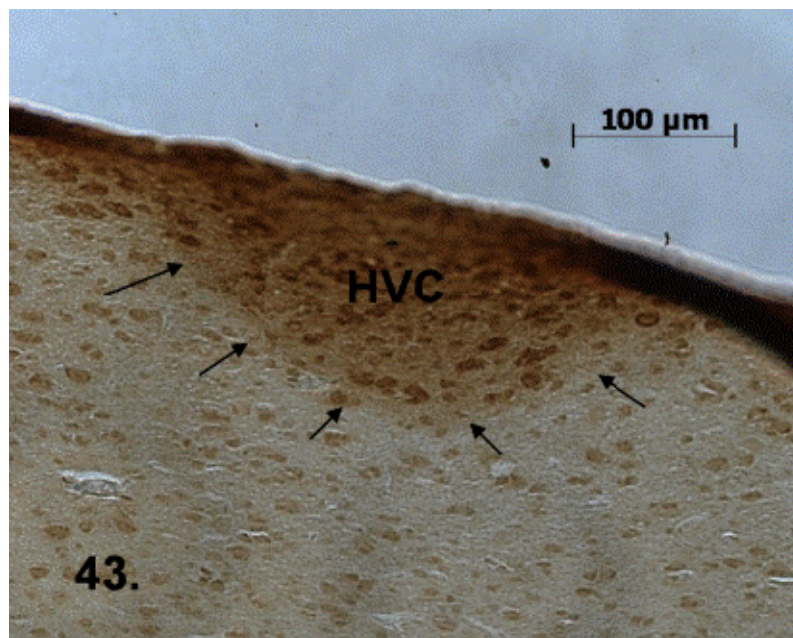


Fig. 43. Microphotograph of an adult male HVC. Arrows indicate the boundary of the nucleus. 20X

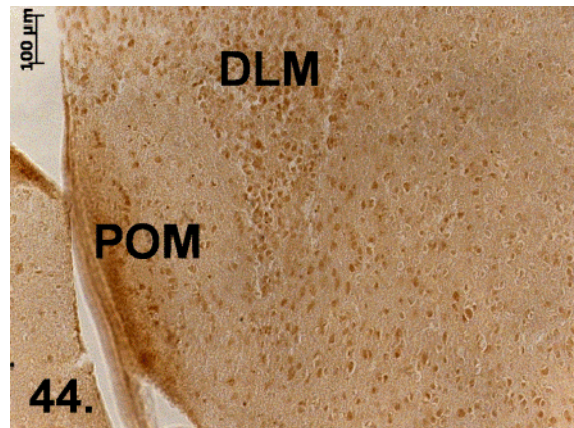


Fig. 44. Microphotograph of an adult male diencephalon containing the POM and DLM. 20X

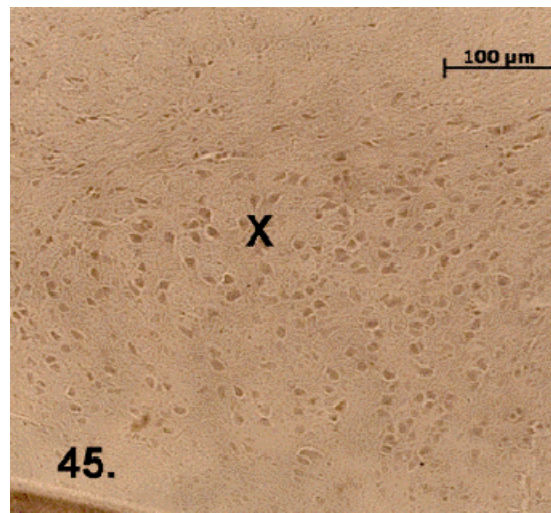


Fig. 45. Microphotograph of an adult male area X located within the medial striatum (Mst), 20X.

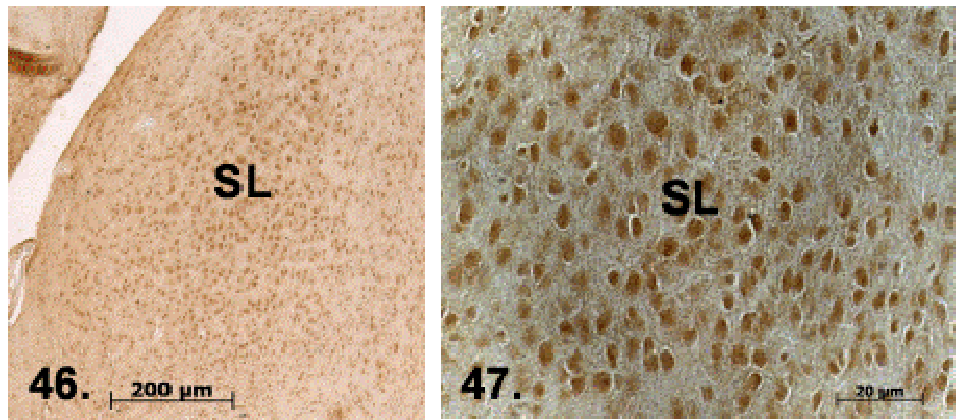


Fig. 46. Microphotograph of an adult male SL (nucleus septalis lateralis) containing p68-ir neurons. 10X.

Fig. 47. Microphotograph of an adult male SL containing p68-ir neurons. 40X.

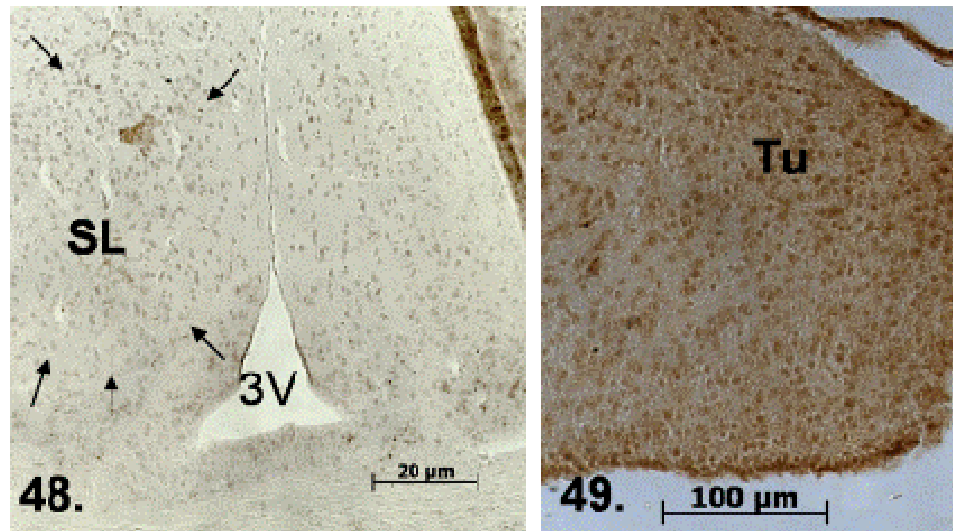


Fig. 48. Microphotograph of an adult male SL containing p68-ir neurons and 3rd ventricle (3V). Arrows indicate the boundaries of SL. 5X.

Fig. 49. Microphotograph of an adult male Tu containing p68-ir neurons. 20X

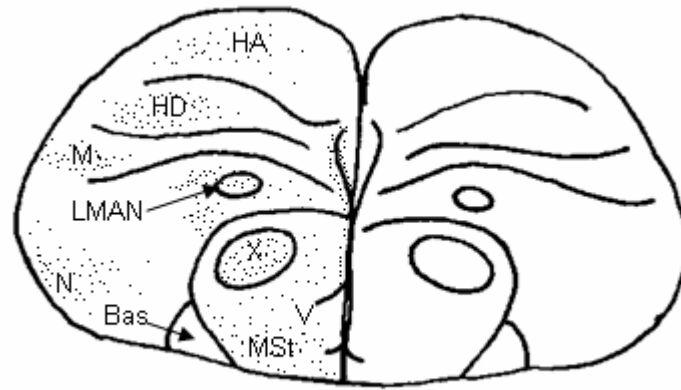


Fig. 50: Schematic representation of the distribution of p68 protein in the mature ZF brain. Abbreviations: HA: hyperpallium apicale; HD: hyperpallium densocellulare; M: mesopallium; LMAN: lateral magnocellular nucleus of anterior neostriatum; X: area X; N: neostriatum; Bas: nucleus basorostralis; MSt: medial striatum; V: ventricle.

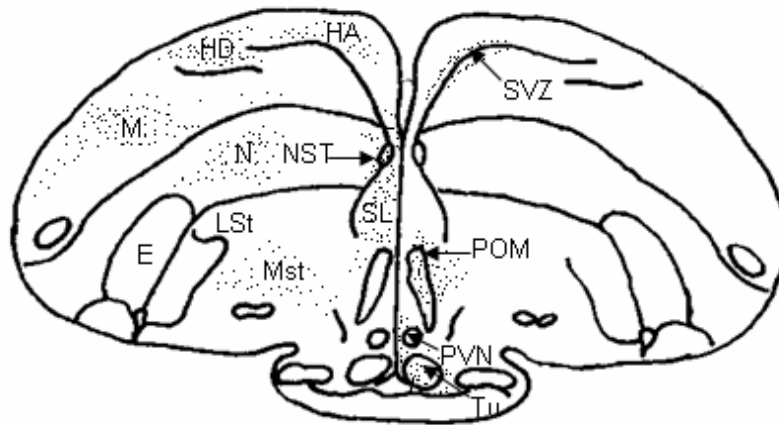


Fig. 51: Schematic representation of the distribution of p68 protein in the mature ZF brain. Abbreviation: E: entopallium; LSt: lateral striatum; NST: nucleus striae terminalis; SL: nucleus septalis lateralis; SVZ: subventricular zone; POM: medial preoptic nucleus; PVN: paraventricular nucleus; Tu: nucleus tuberis.

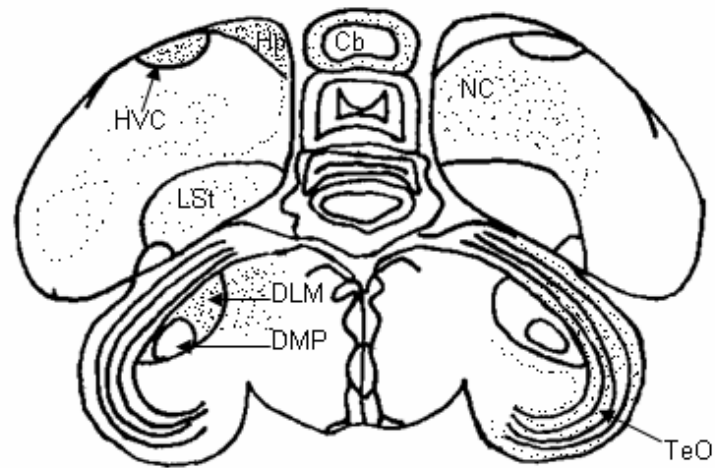


Fig. 52: Schematic representation of the distribution of p68 protein in the mature ZF brain. NC: nidopallium caudale; TeO: optic tectum; Hp: hippocampus, DLM: medial dorsolateral nucleus of the thalamus; DMP: nucleus dorsomedialis posterior thalamus.

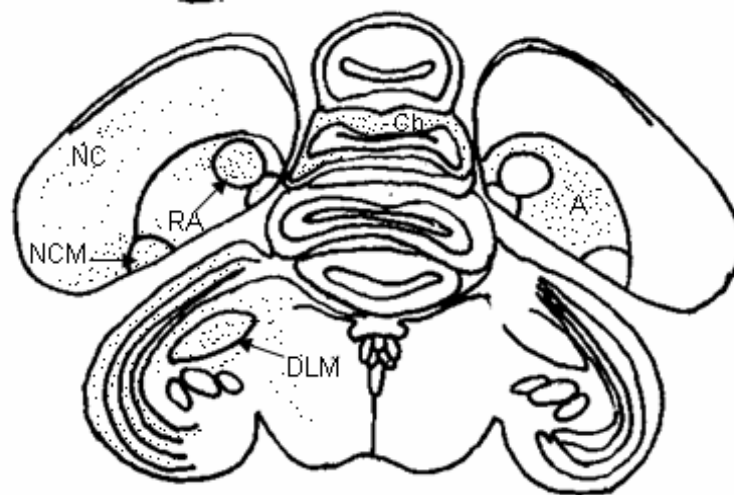


Fig. 53: Schematic representation of the distribution of p68 protein in the mature ZF brain. Abbreviation: RA: robust nucleus of the arcopallium; Cb: cerebellum; A: archistriatum, NCM: caudal medial nidopallium.

5

Discussion

Previous studies on the role of steroid hormones in zebra finch neural development demonstrated that sex steroid hormones only play a partial role in masculinizing the male ZF brain (Jacobs et al, 1999; Gahr and Metzdorf, 1999) and blocking steroid action, via receptor antagonists or synthesis inhibitors, did not disrupt the normal development of the male song circuit (Mathews and Arnold, 1990; Wade and Arnold, 1994). Furthermore, inducing testicular formation in genetic females early in development did not completely masculinize the female song system (Wade and Arnold, 1996; Wade, 2001). In addition, in a ZF that had both genetically male and female brain tissue and was exposed to the same gonadal hormones it had song control nuclei that were sexually dimorphic (Agate et al., 2003). These studies support the idea that circulating steroid hormones are not entirely responsible for the development of neural sex differences in the ZF and that other factors intrinsic to the brain may play a role in the ZF neural development.

We investigated the role of a protein, p68, which has a dual role as both an RNA helicase and an ER- α coactivator, in the development of the zebra finch song system. The mRNA of p68 is expressed in a sexually dimorphic manner in the telencephalon and “rest of brain” of P1 ZFs with females expressing more of the protein than males (Carruth et al., 2000). We have examined the expression of p68 male and female ZFs at three critical time points during development, P10, P30 and adulthood. We have also mapped the

distribution and localization of p68-ir cells in the male P30 and adult ZF brain and identified various brain regions, including the song nuclei that contain p68-ir neurons.

p68 protein is known to function in several RNA metabolic activities including nuclear transcription, pre-mRNA splicing, nucleocytoplasmic transport and translation (de la Cruz et al., 1999). It is involved in diverse cellular processes such as cell growth and differentiation (Stevenson et al., 1998). We hypothesized that p68 is involved in the mechanisms underlying ZF sexually dimorphic brain development. This study examined possible differences in both sex as well as age, at critical time points in song system development. Also, because of the diversity of functions associated with p68, which can be found in either the cell cytoplasm or nucleus, we compared p68 protein levels in nuclear and cytoplasmic extracts from the adult ZF brain.

Developmental time points critical to the formation of the ZF song system were identified with reference to previous anatomical studies. At about five days after hatching the RA can be identified followed by HVC at 10 days post hatching and LMAN at approximately two weeks after hatching. At about three weeks post hatch, the axons from the HVC have migrated into the dorsal portion of the RA (Konishi and Akutagawa, 1985). By three weeks post hatch, LMAN, unlike HVC and RA, has become sexually dimorphic with the lateral portion of the MAN being considerably larger in males than in females.

Both neurogenesis and cell death have been suggested to play roles in the distinctive sex differences found in the ZF song system morphology (DeVoogd, 1991). In ZF females, neuronal cell death has been determined to occur between 20 and 30 days

after hatching in the HVC and 30 through 45 day after hatching in the RA. Furthermore, increased axonal projections from the juvenile male P25 ZF HVC into the RA and the additional appearance of myelinated axons in the RA (Holloway and Clayton, 2001) support studying factors and mechanism underlying the sexual dimorphism found in the ZF song system. Studying the p68 expression during the juvenile period is essential to further investigate if p68 is involved in differential development of ZF dimorphic brain morphology.

Male ZFs have been shown to produce slightly larger pulses of estradiol (E2) than females at about 12 and 14 days after hatching (Adkins-Regan et al., 1990). This sex difference in E2 production may explain the sex difference in p68 expression in the nuclear extract from female P10 ZFs compared to that of males (Fig. 11). In addition, p68 may be involved in the accumulation of ER in the LMAN and HVC of young zebra finches (Gahr & Konishi, 1988) explaining the significance in protein expression in telencephalon as compared to the rest of brain at age 10 (Fig. 11). ER in LMAN and HVC are present in females until about P40 when they drastically decrease in concentration (Gahr and Konishi, 1988). A larger sample size might help clarify the trend toward significance seen p68 protein expression in the nuclear extract from the telencephalon from female ZFs as compared to males with lower protein levels ($n = 5$, p -value = 0.08, Fig. 10). The previous studies that demonstrated a significant sex difference in p68 mRNA expression has sample sizes that were at least twice as large as the sample sizes used in this study.

The lack of strong sex differences in p68 expression may have resulted from the small sample size ($n = 5$) used in these experiments. A larger sample size as well as larger protein yields will be needed to investigate the suggestive trends in p68 levels found in the nuclear fraction of telencephalic cells of male and female zebra finches at age P30 and adulthood. While the mRNA Northern blot expression data demonstrated a sex difference in p68 greater in females than males (Carruth et al, 2000) the data presented here suggests lower p68 levels in female telencephalon at age 30 ($p = 0.07$) and adulthood ($p = 0.13$). These lower protein levels could be as a result of increased neuronal cell death and increasing number of pyknotic (dying) cells seen in female zebra finches shortly after third week post-hatching (Krin and DeVoogd, 1989). This could also be supported by neuronal differentiation and divergence at about day 30 which has been shown to augment the large sexual dimorphism seen in the ZF song system (DeVoogd, 1991).

Contrary to previous reports of describing p68 localization exclusively in the nucleus, p68 also has been shown to partially reside in the cytoplasm (Rossow and Janknecht, 2003). Intracellular localization studies by Rossow (et al., 2003) revealed that full length human p68 protein is solely localized in the nucleus whereas truncated variants were found in both the nucleus and the cytoplasm. According to Goh (et al. 2004), overexpression of NS5B, the viral RNA-dependent RNA polymerase (RdRp) of the Hepatitis C virus (HCV) induces redistribution of p68 from the nucleus into the cytoplasm. This relocation has been speculated to play a role in viral replication processes. Here we have shown, using Western blot protein expression studies that p68

protein is found in both the nucleus and cytoplasm of neuronal cells in the zebra finch brain. In adult zebra finches we have demonstrated a significantly higher level of p68 protein in the cytoplasm than in the nucleus of neuronal cells. So far the diverse biological functions associated with p68 which include modulating of RNA structures for RNA splicing, transcription and functioning as an ER- α coactivator primarily indicate nuclear localization of p68. Functions of the cytoplasmic p68 protein is not well understood, its function as a RNA helicase in unwinding duplex RNA occurs in the cytoplasm. Also, Dbp2p, the yeast homologue of human p68, has been shown to affect both nonsense-mediated mRNA decay and rRNA processing by altering rRNA structure (Bond, 2001).

Immunohistochemical studies using monoclonal anti-p68 mouse antibodies (PAb204 and rgg-1) identified p68 localization in the subventricular zone (SVZ) of P1, P30 and adult male zebra finches. A concentration of neural stem cells has been identified in the SVZ of the cerebral cortex in adult mammalian brain (Gage, 2000). These cells undergo proliferation and subsequently differentiation to become integral part of the neural circuitry (Garcia-Vergudo et al., 1998). Localization of p68 protein in the SVZ indicates that p68 may play a role in cell proliferation, migration and possibly differentiation.

While androgens and estrogens play an important role in the development of the ZF song system they are not completely responsible for sexual differentiation of the avian song control system (Wade and Arnold, 2004). We have observed p68-ir neurons in the four major song control nuclei, HVC, Area X, LMAN and RA in both P30 and adult male

zebra finches. These regions of the ZF brain have been shown to be steroid sensitive and contain androgen receptors (AR) and estrogen receptors (ER) (Nordeen et al., 1987; Holloway and Clayton, 2001; Kim et al., 2004; Fusani and Gahr, 2006). The earliest expression of AR mRNA in the song system have been shown at post-hatch day 11 (P11) and also treatment with estrogen subsequently increases AR mRNA expression in these song nuclei (Kim et al. 2004). This increase in AR mRNA is a masculine pattern characteristic of the dimorphism found in the zebra finch brain. Localization of p68-ir cells in the steroid sensitive song system further suggests a role for p68 in the development of the sexually dimorphic zebra finch song system. While the studies indicating if p68 can also act as a coactivator of AR have not been conducted this may be likely because many coactivators (such as SRC-1, SRC-2, SRC-3, and L7/SPA) function as coactivators for more than one steroid receptor. If p68 plays a role in zebra finch song nuclei development it is likely due to actions of estrogen in development of song nuclei and the role of p68 as an ER- α coactivator. In the diencephalon, extensive AR mRNA has been identified in the nucleus tuberis (Tu; Kim et al., 2004) and correlates with the heavy localization of p68-ir cells in this nucleus.

p68 is known to play a role in cell growth and its expression correlates with fetal organ differentiation and maturation thus functions as an important element in cellular and tissue development. Steroids such as androgens and estrogens regulate biological events underlying brain development and sexual differentiation. As an ER- α coactivator, p68 may act to enhance the expression of estrogen induced genes and this function may be further highlighted by its interaction with other coactivators such as the CBP/p300

complex and transcription factors as well as RNA polymerase II. The localization of p68-ir cells in the song control nuclei and other steroid sensitive brain regions suggests a possible role for p68 protein in the development and functioning of those regions.

Further investigation is required to elucidate the proposed role for p68 in the development of the ZF song system. With p68 protein acting as an ER- α coactivator, an overlap in the expression of both proteins the brain regions specific for song learning and production will strongly support a role for p68 in the development of the avian song system. In addition, a change in the level of endogenous p68 in the ZF brain due to exogenous estrogen treatment or blocking estrogen activity using fadrozole, an aromatase inhibitor would strengthen the postulated role of p68 in brain development. This theory is supported by previous studies which show that p68 interacts with the sex steroid receptor, ER- α to induce ligand dependent transactivation through AF-1 of hER- α (Endoh et al., 1999). Also, investigating the expression and localization of phosphorylated p68 will shed more light on the functional role of p68 in developmental regulation of the ZF brain. The role p68 in sexual differentiation and song development is not clear and requires further investigation.

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