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Running title: Molecular correlates of brain development in ontogeny

Abbreviations (alphabetical order):

(r)qPCR: (relative) quantitative polymerase chain reaction

AR: androgen receptor

cMYC: MYC Proto-Oncogene

Dio2: Type II iodothyronine deiodinase

Dio3: Type III iodothyronine deiodinase

DLM: dorsolateral nucleus of the thalamus

ERα: Estrogen receptor alpha

ERβ: estrogen receptor beta

EYA3: EYA Transcriptional Coactivator And Phosphatase 3

GADD45γ: growth arrest and DNA-damage-inducible protein gamma

HVC: High Vocal Center

LAT1: L-type amino acid transporter 1

LMAN: lateral magnocellular nucleus of the anterior nidopallium

MCT8: Monocarboxylate transporter 8

OTAP: Organic Anion Transporting Polypeptides

OTX2: Orthodenticle Homeobox 2

PC3: NGF-Inducible Anti-Proliferative Protein

pCNA: Proliferating Cell Nuclear Antigen

POA: Preoptic area

RA: robust nucleus of the arcopallium

SOX2: Sex determining region Y-box 2

THRα: Thyroid hormone receptor alpha

THRβ: Thyroid hormone receptor beta

TLX: Nuclear Receptor Subfamily 2 Group E Member 1

TrSM: tractus septopallio-mesencephalicus

TSH-R: Thyroid stimulating hormone receptor

Abstract

Development of the songbird brain provides an excellent experimental model for understanding the regulation of sex differences in ontogeny. Considering the regulatory role of the hypothalamus in endocrine, in particular reproductive, physiology, we measured the structural (volume) and molecular correlates of hypothalamic development during ontogeny of male and female zebra finches. We quantified by rqPCR the expression of 14 genes related to thyroid and steroid hormones actions as well as 12 genes related to brain plasticity at 4 specific time points during ontogeny and compared these expression patterns with the expression of the same genes as detected by transcriptomics in the telencephalon. These two different methodological approaches detected specific changes with age and demonstrated that in a substantial number of cases changes observed in both brain regions are nearly identical. Other genes however had a tissue-specific developmental pattern. Sex differences or interactions of sex by age were detected in the expression of a subset of genes, more in hypothalamus than telencephalon. These results correlate with multiple known aspects of the developmental and reproductive physiology but also raise a number of new functional questions.

Key words: Hypothalamus, sexual dimorphism, zebra finch, telencephalon, hormones, ontogeny

1. Introduction

The hypothalamus is a key brain region regulating fundamental aspects of behavior and physiology. The anatomy of this area and the mechanisms underlying its development are highly conserved across vertebrate species (1). The hypothalamus is located in ventral position in the forebrain at the rostral pole of the midbrain on either side of the third ventricle and has an intricate functional and developmental relationship with the pituitary gland through which it controls a variety of the endocrine functions (2). The hypothalamus is anatomically complex and composed of different nuclei arranged in a patchwork manner (2-5). Functionally, the hypothalamus orchestrates via releasing factors the activity of the anterior pituitary and thus the functioning of major endocrine glands including the gonads, the thyroid, and adrenals glands (via the anterior pituitary) and physiological processes such as growth or lactation. It also releases, via the posterior pituitary, peptide hormones such as oxytocin and vasopressin (vasotocin in birds) that control a variety of processes such as uterine contractions, milk ejection, or water reabsorption in the kidneys. Hypothalamic nuclei also project to multiple other brain areas where they release diverse signaling molecules (6, 7). Through these diverse mechanisms, the hypothalamus modulates a vast array of functions including namely sleep-wake cycles, metabolic/electrolyte balance, body temperature, food intake, fear, stress, reproduction and reproductive behaviors, aggression and territoriality, and many more (for general overviews see: (8, 9)). The hypothalamus regulates these complex processes by integrating inputs from the various brain areas it is connected to, diverse sensory inputs and feedback from numerous circulating hormones.

These complex functions of the hypothalamus only develop progressively during ontogeny and some of them, in particular those related to reproduction, also become sexually differentiated (9). The anatomical and functional development of the hypothalamus during ontogeny has been investigated in a number of mammalian species including rats, monkeys and humans (5) and many studies have also been devoted to this question in birds, mostly in chicken (*Gallus domesticus*) and Japanese quail (*Coturnix japonica*) (10). For example, multiple papers on the changes during development of testosterone metabolism, aromatase expression and neurogenesis in the quail hypothalamus have been published by our group (11). The amount of information available on the anatomy, neuroendocrinology, neurochemistry and sexual differentiation of the avian hypothalamus is by far too important to be even superficially summarized here but multiple reviews on these topics are currently available namely in the two collection of book chapters already cited (8, 9). To our knowledge, there is however only limited information on the ontogeny of the hypothalamus and its neuroendocrine functions in zebra finches (*Taeniopygia guttata*) even if this experimental model has been extensively used to analyze in detail the production of sex steroids by the brain (see (12, 13)). It has in particular been demonstrated that all enzymes needed to produce sex steroids from cholesterol are expressed in the zebra finch brain and that the production of estradiol by aromatization of testosterone significantly contributes to the

circulating concentrations of estrogens and plays a key role in the development of connections within the song control system (see (12, 13), reviewed in (10)). The brain as well as the gonads and the adrenal glands thus all contribute to the hormonal milieu in this species.

In birds, functional studies indirectly suggest that hypothalamic function in relation to the control of male sexual behavior differentiates during the early post-natal life in response to estradiol secreted by the female ovary (10). In quail, adult females do not display male-typical copulatory behavior even when treated with exogenous testosterone, however blocking estrogen production or action during embryonic development produces adult females that will show the behavior in response to testosterone. Conversely, this capacity to show male-typical copulatory behavior is lost in males if they are treated during their first weeks of life with exogenous estradiol or testosterone that will act via aromatization (14). Copulatory behavior seems to differentiate by the same mechanisms in zebra finches since, like in quail, adult females do not display male-typical copulatory behavior even when treated with exogenous testosterone and this capacity is lost in males if they are treated during their first weeks of life with exogenous testosterone (14-17) (reviewed in (10)). This suggests that in both species male-like copulatory behavior differentiates under the influence of estrogens: they demasculinize the females, and the male phenotype, as far as copulatory behavior is concerned, develops in the absence of hormonal influences (see for review: (10)). Based on research in other avian species and in vertebrates in general, male-typical copulatory behavior is activated by the action of testosterone and its metabolites in the preoptic area-hypothalamus (18, 19). The sex difference affecting this behavior should therefore be paralleled by neuroanatomical or neurochemical differences in this brain area (20) but the detail of these differences has never been investigated in zebra finches.

Zebra finches have been extensively used in biological research, namely in the context of the control of singing behavior by steroids and of the development of sex differences affecting this behavior and the underlying brain structures (21-24). This work has actually identified a number of quite unique features. Like in many other songbird species (25), singing behavior is sexually differentiated in zebra finches, but this sex difference is here more prominent than in other species in that adult females never sing, even after being treated with exogenous testosterone (26-28). This exacerbated behavioral sex difference corresponds to extreme sex differences in the brain circuits controlling the behavior (29).

Song production is controlled by a set of interconnected nuclei comprising a motor pathway connecting HVC (formerly High Vocal Center, now used as a proper name) to the robust nucleus of the arcopallium (RA) and to the motor-neurons innervating the syrinx. There is in addition an anterior forebrain pathway that controls sensorimotor song learning and plasticity and indirectly connects HVC to RA via Area X of the striatum, the medial part of the dorsolateral nucleus of the thalamus (DLM) and lateral magnocellular nucleus of the anterior nidopallium (lMAN) (30, 31). Most of these nuclei are larger in males than in females in the majority of songbird species (25) but this difference is extreme in zebra finches. HVC and RA are 4 to 6 times larger in

males than females and Areas X is even not detectable at all in females by standard neuroanatomical methods (29) (but see (32) for more recent data indicating existence of a residual Area X in females).

The ontogenetic development of these neuroanatomical sex differences is also peculiar in zebra finches. Whereas estrogens demasculinize all male-typical characteristics that have been investigated in birds (10), an early postnatal treatment of females with estrogens masculinizes singing behavior and the volume of the song control nuclei (14, 26-28). However, blocking estrogens production (with an aromatase inhibitor) or estrogen action (with an estrogen receptor blocker) in males failed to produce the expected results (33, 34), therefore leaving the mechanism through which sex steroids regulate the sexual differentiation largely unexplained (10, 35).

The study of a gynandromorphic zebra finch i.e., a bird male one side and female on the other side, indicated that genetic information directly contributes to the sexual differentiation of these structures, although the specific genes that are involved have not to this date been identified (36). However, the female side of the gynandromorph was more male-like than in a normal female suggesting that hormonal stimuli, either coming from the gonads or produced in the brain and diffusing to the contra-lateral hemisphere, also contribute to the sexual differentiation (10). The mechanisms mediating the sexual differentiation of singing and of the song system are thus largely unexplained but they are clearly different from the mechanisms controlling the sexual differentiation of the hypothalamus (see for review (10)).

In the present study we investigated some molecular correlates of the hypothalamic development of male and female zebra finches by quantifying via relative quantitative polymerase chain reaction (rqPCR) a variety of messenger RNA related to reproduction, to the activity of steroid and thyroid hormones. We also measured an array of mRNAs involved in brain development, plasticity and stem cell activity to test if there are sexual differences in molecular correlates of development as such. The mRNAs were assayed from hypothalami that had been collected at key points in development, namely 1-3 days post-hatch (dph), the fledging period at the very beginning of the sensory period of song learning (20 dph), the period of gonadal maturation or puberty at the middle of the period of sensorimotor song learning (65 dph) and adulthood (120 dph) (31, 37). Given that we also had developed during the activity of the "Plastocine research network" funded by the Belgian Science Policy (project Plastocine P7/17) a full transcriptome of the male and female zebra finch telencephalon at the same ages, we additionally compared the changes with age of the expression of these mRNA in these two brain areas that based on the present results seem to develop and become sexually differentiated based on divergent control mechanisms.

2. Material and methods

2.1 Animals

Male and female zebra finches (*Taeniopygia guttata*; n=20 for each sex) originating from a breeding colony at the University of Liège were used for the gene expression study. The body and gonadal weights reported here were also obtained from these birds. These birds had been raised in a large indoor aviary containing nesting material, along with their parents within a large group of conspecifics allowing complex social interactions and exposure to tutor song. Throughout their life, birds were held on a 12L:12D dark/light cycle and they received food and water ad libitum. Egg food, perches, cuttlebones and sand were provided as enrichments. The experiment was performed according to the Belgian law on animal experimentation and approved by the Ethical Committee for Animal Experimentation at the University of Liège (protocol 1396). The zebra finches used for MRI based measurements were raised in the indoor colony of the University of Antwerp. Birds were raised in similar conditions as in University of Liege. The Committee on Animal Care and Use at the University of Antwerp (Belgium) approved all experimental procedures (permit number 2012–43 and 2016–05).

2.2 Experiment

2.2.1 In-vivo Brain volume measurement

We used a sub-set (n=13 males, n=16 females) of the recently published volumetric brain scans and the population-based brain template (38) to estimate the volume of the hypothalamus at 20, 65 and 120 dph in the same subjects. Briefly, zebra finches were anesthetized with isoflurane (IsoFlo®, Abbott, Illinois, USA; induction: 2.0–2.5%; maintenance: 1.4–1.6%). A primary T2-weighted Turbo RARE pilot scan was done to enable uniform slice positioning across imaging sessions. Then a T2-weighted 3D RARE scan was obtained with settings of TE 11 ms (TEeff 55 ms), TR 2500 ms, RARE factor 8, FOV ($18 \times 16 \times 10$) mm^3, acquisition matrix ($256 \times 92 \times 64$) zero-filled to ($256 \times 228 \times 142$) for reconstruction yielding an isotropic voxel dimension ($0.07\ mm^3$). The FOV of the 3D RARE scan captured the entire zebra finch brain. Maximum accuracy of the age of acquisition was ensured (relative errors of age at acquisition are: 1.39% at 20; 0.97% at 65, 0.78% at 120,).

For volume measurements, we first delineated the hypothalamus on the population-based template according to the spatial definitions described in (39, 40) and used in (41) for other molecular studies. The structure considered extends in rostro-caudal direction from the tractus septopallio-mesencephalicus (rostral edge of the POA) to the oculomotor nerves (caudal edge of tuberal hypothalamus). Second, the delineation was warped back to the native space of each individual scan using the spatial transformation parameters estimated in the deformation-based morphometry procedure described in (38) using the 'Deformations' utility of SPM12

(Statistical Parametric Mapping, r6225, Wellcome Trust Center for Neuroimaging, London, UK, http://www.fil.ion.ucl.ac.uk/spm/). Third, we computed the volume of the back transformed (warped to native space) hypothalamus delineation by counting the number of voxels in the latter and multiplying this result with the voxel volume. In addition, we back transformed the whole brain mask to extract the whole brain volume for each anatomical scan. We also acquired MRI scans of 1-3 day old chicks (N=5; males and females) ex vivo with brains intact in skull. For this, the heads of the chicks after decapitation were stored in Fluorinert™ (3M™, FC-770) and scanned directly within 48 hours.

2.2.2 Body weight and gonadal development

Birds (N=5 for both males and females at each age sampled) were individually weighed to the nearest of 0.01 g just before being killed for gene expression studies. Brain from birds of both sexes were collected at 4 different developmental stages: 1-3, 20, 65 and 120 dph. Gonads were immediately removed and weighed. The data of left testis for male and ovaries for female is reported here. Body weight, gonadal volume measurements and gene expression were measured in birds than were different from birds used for brain volume measurements by MRI.

2.2.3 Gene expression:

Birds were killed by quick decapitation and heads kept on ice. Brains were quickly removed. The telencephalon was separated from the rest of the brain first. It should be noted that these birds used for gene expression studies are not the same subjects that were used for measuring by in vivo MRI brain volume changes during ontogeny.

a) Quantitative PCR: gene expression in hypothalamus

The preoptic area—hypothalamic block was dissected first by two coronal cuts at the level of the tractus septopallio-mesencephalicus (rostral edge of the POA) and of the oculomotor nerves (caudal edge of tuberal hypothalamus), two parasagittal cuts placed approximately 2 mm lateral to the brain midline and one horizontal cut approximately 2 mm above the floor of the brain. Anatomically, the tractus septopallio-mesencephalicus and the caudal edge of hypothalamus are clearly visible even in the brains of 1-3 dph birds. This aspect of the dissection was therefore probably very accurate. The horizontal cut located 2 mm above the brain floor could have been slightly more variable but inspection of the variance in results in figures 2 and 3 suggests that dissections were quite reproducible since there are only limited differences in mRNA expression between subjects.

Each sample was homogenized in TRIreagent (Ambion Inc., Cat no. AM9738) by bead beater, and total RNA was extracted by chloroform precipitation and quantified, as per the manufacturer's protocol. 1µg RNA was DNase treated (Ambion Inc., Cat no. AM1906) to remove genomic DNA, if any, and single strand cDNA was

prepared by MAXIMA single strand cDNA prep kit (Fermentas, Cat no. K1641). Gene specific primers were designed using PRIMER quest (http://www.idtdna.com/Primerquest) software (supplementary Table 1), with stringent conditions of Tm: 60^0C, amplicon length: 75-95 bp, G+C: 50%. qPCR was performed on the Rotor Gene Q, Qiagen. The PCR reaction for each gene was standardized with primer concentration and total reaction volume to achieve a standard slope of -3.2 to -3.4. Melting curve analysis showing a single peak confirmed the primer specificity. Each sample was run in duplicate along with negative RT and non-template controls in each plate. A 15 µl reaction volume consisted of 1 µl of cDNA (10ng/µl), 1 µl each of forward and reverse gene specific primer (500 – 1000nM), 7.5 µl of SYBR Green I master(1x) and 4.5 µl nuclease free water. GAPDH, β-actin and cyclophilin were tested as reference gene candidates and the ct values of all the three housekeeping genes obtained from 5 samples per age group were tested with NormFinder (https:// moma.dk/ normfinder-software). GAPDH with a stability value of 0.031 (as compared to 0.043 for beta actin and 0.041 for cyclophilin) was found to be the most stable gene. The combined stability values of GAPDH and beta-actin was 0.035 which is a little lower than for GAPDH alone. There were random individual variations in CT of GAPDH between samples but no significant difference was found between the CT values of different groups of sex/age. The present results are thus based on GAPDH as a reference gene. A fold change in the relative mRNA level of a gene was calculated using the formula $2^{-(\Delta\Delta Ct)}$ (42). Briefly, the fluorescence exceeding background levels gave the cycle threshold (Ct), which was used to calculate ΔCt (Ct [target gene] – Ct[reference gene]). Then, these Ct values were normalized against the Ct value of a sample (calibrator) consisting of cDNA mix of all the samples. Finally, the negative value of this powered to 2 ($2^{-\Delta\Delta ct}$) was plotted and used for all analyses. The final values thus represent relative qPCR and are referred to as rqPCR in the rest of this paper.

b) Transcriptome analysis: gene expression in telencephalon

Within the framework of the "Plastoscine" initiative consortium, we performed a full transcriptome analysis of the finch telencephalon sampled at age 1-3, 20, 65 and 120 dph (data GEO "to be submitted"). Three biological replicates (N=3) were analyzed and sequenced for each sex and age and these biological replicates each consisted of a pool of RNA from the telencephalon of 3 different birds in equal proportions (thus, 9 subjects were used at each age). This approach allowed to balance individual differences between samples and thus to increase the power of the study. In contrast, the hypothalamic tissue used for the gene expression studies by qPCR came from 5 of these 9 birds collected at each age in both males and females. Thus, although it is not possible to relate for each individual subject the mRNA expression in the hypothalamic and telencephalic samples, all data are derived from the same subjects but only a subset of them was used for the hypothalamic studies.

Briefly, total RNA was isolated for RNA sequencing. Samples were lysed in QiazolTM lysis reagent (Qiagen 79306). Total RNA was extracted using the RNeasy mini kit (Qiagen 74106,) according to the manufacturer's instructions. The RNA sequencing was performed in the NXTGNT sequencing facility of Ghent University (Ghent, Belgium). RNA samples were quantified and quality controlled using the Quant-iTTM Ribogreen® RNA Assay (Invitrogen R11491) kit and Agilent RNA 6000 Nano (Agilent 5067-1511; on Agilent Bioanalyzer). Upon DNase treatment, library preparation (Truseq stranded mRNA, Illumina RS-122-2101 and RS-122-2102) was performed on 1µg of each sample with fragmentation performed at 94^{o} C for 8 min. Each sample was sequenced on the Illumina NextSeq500 (over four lanes). The Zebra Finch reference genome (Ensembl; assembly taeGut3.2.4, release 87) was used for mapping of both RNA-Seq data. FastQC (v0.10.1) was used to assess RNA-seq data quality and indicated no major problems. Alignment and mapping of reads to the reference genome was done by STAR (v2.5.2b). Samtools (v0.1.18) was used for downstream analysis and htseq-count (v0.6.0) (based on GTF file, Ensembl, assembly 3.2.4, release 87 was applied for data summary. Finally, normalization and differential gene expression analysis was performed by the R-package EdgeR with its GLM functionality (v3.8.6). Full details of the results from transcriptomics and other methodologies will be reported elsewhere (Diddens et al. 2020, in preparation).

2.3 Statistics

Statistical analyses and graph preparations were done with the GraphPad Prism software version 8.0 (San Diego, CA, USA). $P < 0.05$ was considered as a statistically significant difference. Two way-ANOVA was used to compare body weight, gonadal volume and gene expression between male and female zebra finches at different ages. The total brain volume, hypothalamus volume and their ratios were compared using repeated measures (RM) 2 way-ANOVA.

Specific post hoc tests were carried out based on the results of 2 way-ANOVA following recommendations of the GraphPad Prism 8 software. If a significant age by sex interaction was detected, two sets of post hoc tests were performed with the Tukey multiple comparisons test to compare changes during ontogeny in males and females separately and with the Sidak multiple comparisons test to compare male and female expression at specific time-points. If there was a significant effect of age but no significant effect of sex and no interaction, the Tukey multiple comparisons test was used to compare gene expression at different time points in ontogeny in both sexes together. If there was a significant effect of age and sex but no significant interaction, Tukey multiple comparisons post hoc tests were used to compare gene expression in ontogeny in males and females separately. Finally, if there was a sex effect and no interaction, no post hoc test was needed since there are only 2 sexes.

We also calculated the correlations between gene expression in the hypothalamus and gonadal weight in each sex separately with the use of the Pearson product-moment correlation coefficient (r). The correlation between genes was tested for significance and the p values were adjusted for multiple comparisons by the Benjamini–Hochberg false discovery rate (https://tools.carbocation.com/FDR) correction to give a corrected p value (= q value) that was used to decide whether a correlation was significant or not.

3. Results

3.1 Weight and gonadal changes in ontogeny

The 2-way ANOVA of body weight identified a significant effect of age during ontogeny ($F_{3, 32}$ = 149.4, P<0.0001) but no sex difference ($F_{1, 32}$ = 2.965, P=0.0947) and no sex by age interaction ($F_{3, 32}$ = 0.634, P=0.5985; Fig. 1a). Post hoc tests revealed that at 20 dph, the body weight of males and females were already significantly higher than at 1-3 dph and birds attained their highest body weight at 65 dph, only a marginal increase occurred afterwards (see results of Tukey's post hoc tests in Fig. 1a).

Gonadal weight (testis in males, ovary in females) similarly showed a significant effect of age ($F_{3, 32}$ = 56.16, P<0.0001, Fig. 1a) but no effect of sex ($F_{1, 32}$ = 0.105, P=0.7478) and no interaction between these factors ($F_{3, 32}$ = 0.6138, P=0.6110). Tukey's post hoc tests revealed that 65 dph birds showed a significantly larger gonadal volume than 1-3 and 20 dph old birds and a significant additional increase was observed at 120 dph in both males and females (Fig. 1a).

3.2 Hypothalamus volume changes during ontogeny

As delineation of the hypothalamus, based on location of TrSM and infundibular area, was not reliable enough in MR images of 1-3 dph chicks, these data are not presented here and we only included the volume of the whole brain for 1-3 dph old birds (Fig. 1b) but these data were not included in statistical analyses.

Whole brain volumes of both male and female zebra finches repeatedly measured in the same subjects from 20 dph to 120 dph were significantly affected by age ($F_{7, 54}$ = 79.41, P<0.0001) and by an interaction between age and sex ($F_{2, 54}$ = 3.173, P=0.049), but there was no overall effect of sex ($F_{1, 27}$ = 0.808, P=0.376, 2-way RM ANOVA; Fig 1b). In males, brain volume was significantly different at all ages examined with the largest volume being present at 65 dph and the smallest at 120 dph (Tukey's post hoc test, Fig 1b). In females also the whole brain volume was numerically larger at 65 dph but it did not differ significantly from volumes measured at 20 and 120 dph (Tukey's post hoc test). No difference between male and female volumes were detected at any of the ages tested (Sidak's multiple comparison test).

The hypothalamic volumes in male and female finches were significantly affected by age ($F_{27, 54}$ = 51.07, P<0.0001), sex ($F_{1, 27}$ = 6.799, P=0.0147) and their interaction ($F_{2, 54}$ = 6.321, P=0.0034, 2-way RM ANOVA, Fig. 1b). In both sexes the hypothalamus volume progressively decreased with age but in males all ages were significantly different while in females 120 dph only was lower than both 20 and 65 dph (Tukey's post hoc test). The hypothalamic volume was also larger in males than in females at 20 dph and although a numerical difference persisted it became non significant in older birds (Sidak's multiple comparison test).

The hypothalamus to brain ratio was significantly affected by age ($F_{27, 54}$ = 12.03, P<0.0001) and sex ($F_{1, 27}$ = 8.66, P=0.006) but not by their interaction ($F_{2, 54}$ = 1.06, P=0.352, 2-way RM ANOVA; see figure for results of post-hoc tests)

3.3 Expression of hormone related genes

We measured by rqPCR the expression of a total of 14 hormone related/hormone responsive genes in the finch hypothalamus categorizing them into thyroid related/responsive genes, steroid related/responsive genes and genes related to the gonadotropin releasing hormone (GnRH-1) and its receptors (GnRH-R1). TSHβ gene expression was also measured but excluded from further analysis due to too large Ct values (>34). The details of the functions of the genes under analysis are described in supplementary Table 1. We also analyzed the transcriptome (ages 1-3 dph, 20 dph, 65 dph and 120 dph) of the zebra finch telencephalon and compared these results with the mRNA of all genes measured for hypothalamus except for THRα that was not detected in the telencephalon. All data were analyzed by 2-way ANOVA and detail of statistical results for of all genes in both regions are provided in the Table 1 and 2.

i) In hypothalamus

Expression of the thyroid hormones receptors, TSH-R and THRβ was significantly affected by age, sex and their interaction (Fig.2). TSH-R and THRβ expression increased monotonously with age in males but reached a peak at 20 dph in females and decreased afterwards. THRα expression showed a significant effect of age and of the interaction between age and sex but no significant effect of sex; the highest expression was present in both the sexes at 20 dph. The mRNA of all thyroid hormones transporter genes viz. MCT8, LAT1 and OTAP significantly changed with age but showed no overall sex difference and no interaction between sex and age. MCT8 and LAT1 expression progressively decreased with age with MCT8 expression showing a earlier and sharper decrease than LAT1, in contrast OTAP expression increased with age and reached its maximum at 65 dph (see Fig. 2 and Table 1 for details of post-hoc tests).

Similarly, expression of the deiodinases, Dio2 and Dio3 showed a significant main effect of age only without any effect of sex or their interaction. While Dio3 expression decreased with age (significantly higher at 1-3dph than at all subsequent ages), Dio2 showed a significantly maximal expression at 20 dph only and then decreased to its the lowest level at 120 dph (Fig.2).

The two way-ANOVA of the expression of steroid related genes and gonadotropins demonstrated that all these genes i.e. ERα, ERβ, AR, aromatase and GnRH-1 and GnRH-R1 were significantly affected by age and by the interaction of age and sex (except for ERβ) but there was no overall significant effect of sex. ERα, ERβ and AR similarly increased from 1-3 dph to 20 dph and after that remained essentially stable, except for a

small but significant decrease of AR in females. In contrast aromatase expression continuously increased with age and was significantly higher at 65 and 120 dph than earlier in both males and females. Males additionally showed a significantly higher expression than females at 120 dph (Fig.2). GnRH-1 expression also increased with age in both sexes but this increase only started at 65 dph in males and the major numerical increase was even delayed to 120 dph in females. Thus, GnRH-1 was significantly higher in males than in females at 65 dph (Fig.2). GnRH-R1 was also significantly affected of age and the interaction of sex by age but there was here in addition an overall effect of sex. Interestingly the expression of this receptor decreased with age in males but not in females. The expression was significantly higher at 1-3 dph than later in males but no difference in expression was found in female as a function of age. As a consequence of these patterns, expression of GnRH-R1 was higher in males than in females at 20 dph.

ii) In telencephalon

With few exceptions, the patterns of gene expression detected by transcriptomics in the telencephalon were very similar to the patterns detected by rqPCR in the hypothalamus. This was especially the case for THRβ, MCT8, Dio2, Dio3, and aromatase and to a lesser extent for LAT-1 and TSH-R (in males at least, Fig.2). The changes with age of the expression of the three sex steroid receptors (ERα, ERβ and AR) was however more different in the two brain regions. ERα increased monotonously in the telencephalon but reached a plateau already at 20 dph in the hypothalamus, ERβ decreased continuously in the telencephalon (more in females than males) but increased and reached a plateau also at 20 dph in the hypothalamus and in contrast AR peaked in telencephalon at 20 dph but increased very progressively in the hypothalamus (Fig.2).

All thyroid related genes (TSH-R, THRβ, MCT8, LAT1 and OTAP) examined in the telencephalon were significantly affected by age but were not affected by sex nor by the interaction of sex by age except for Dio3 that was affected by sex while both deiodinases were affected by the interaction of sex by age (Fig.2). The patterns of changes were in general similar to what had been observed in the hypothalamus except for OTAP which has an inverse pattern for both sexes.

TSH-R and THRβ expression continuously increased with age and the highest expression was observed at 120 dph, whereas LAT1 and MCT8 monotonously decreased with age so that the lowest expression was present at 120 dph. OTAP expression also decreased from hatching to 65 dph but then remained at the same level until 120 dph (a slight non-significant increase was even observed, Fig.2). Like in the hypothalamus, Dio2 and Dio3 showed divergent changes with age with Dio3 decreasing continuously with age while Dio2 peeked at 20 dph before falling until adulthood (Fig.2). Expression of Dio3 was higher in females than in males at 1-3 dph.

The steroid related genes expression all showed a significant effect of age only, except for ERβ that was in addition significantly affected by sex and by the interaction a sex with age. ERα continuously increased with age in the telencephalon while in the hypothalamus the maximal level of expression was already reached at 20 dph. ERβ expression decreased with age while it was increasing in the hypothalamus. This decrease was more prominent in females than in males and a sex difference was thus observed at the two earliest ages.

AR expression in telencephalon peaked at 20 dph in both sexes and then came back in older birds to the low levels observed at 1-3 dph. Changes in aromatase expression were very similar to what was observed in the hypothalamus (Fig.2) at least for males. It increased continuously with age although no sex difference was observed here contrary to what had been detected in the hypothalamus. Finally, GnRH-1 was present in the telencephalon but did not change as a function of sex, age or their interaction (Fig.2).

3.4 Expression of neuronal plasticity markers in hypothalamus and telencephalon

We measured the expression of 12 genes known to have a role in neuroplasticity by regulating either stem cell activity, or neurogenesis or axonal plasticity in hypothalamus. We also compared the hypothalamic expression of these genes with their expression in the telencephalon during ontogeny.

i) In hypothalamus

All 12 genes under study were significantly affected by age. The patterns of changes were however quite different from one gene to another. The expression of some genes decreased continuously with age in both sexes. In some cases, the decrease was similar in both sexes (OTX2, DCX, Neuropilin, Tubulin) sometimes it was more prominent in one sex than in the other (cMYC, EYA3, Nestin; Fig.3). Expression of a couple of other genes increased with age, sometimes in a clear manner (TLX), sometimes in a less pronounced way (SOX2). Finally, three genes displayed a peak in expression sometimes during development at least in one sex (GADD45γ, pCNA, PC3; see Fig. 3 and Table 2 for the details of post-hoc tests).

Overall sex differences were detected for 5 genes (TLX, EYA3, pCNA, PC3 and Nestin) but in 3 of these 5 cases there was also a significant interaction of sex by age (pCNA, PC3 and Nestin). mRNAs of TLX and EYA3 were expressed in higher amounts in males than in females throughout development (Fig.3). The interactions were observed because either males were higher than females at specific ages (pCNA at 65 dph) or the reverse was true (PC3 at 20 dph, Nestin at 1-3 dph). The interaction associated with cMYC expression (with no overall sex difference) resulted from the fact that expression in females was slightly higher than in males at some time points but the overall changes in both sexes were still fairly similar (Fig.3).

ii) In telencephalon

In the telencephalon also, expression of all 12 genes was affected by age but only one overall effect of sex (GADD45γ) and two interactions of sex with age (GADD45γ, PC3) were detected. The patterns of changes with age were sometimes very similar to what had been detected in the hypothalamus (OTX2, DCX, Neuropilin, Tubulin, and to some extent cMYC, nestin) but for the 6 other genes they were extremely different indicating a differential regulation of plasticity during ontogeny (Fig.3).

Expression of GADD45γ regularly decreased with age in males but was essentially stable at extremely low level is females which induced an overall sex difference and an interaction of age by sex. The pattern of changes in PC3 expression that generated a significant sex by age interaction was complex with both sexes showing a decrease with age but major changes were not observed at the same time points so that males displayed a higher expression at 1-3 dph and the reverse was true at 65 dph (Fig.3).

3.5. Correlation of hormone related gene expression in hypothalamus to gonadal weight

We also investigated the potential correlations between all hormone-related genes and the gonadal development index during ontogeny in zebra finches. We included in this analysis all genes of both the hypothalamo-pituitary-thyroid (HPT) and hypothalamo-pituitary-gonadal (HPG) axes since hormones modulating these two axes interact in the photoperiodic control of reproduction (43) and possibly also in non-photoperiodic species (44,45).

In males, a significantly strong positive correlation was found between testis weight and hypothalamic aromatase ($r = 0.846$, $q < 0.0001$) and GnRH-1 ($r = 0.743$, $q = 0.001$, Fig. 4) expression. Inversely, a significant negative correlation was found between testis weight and Dio2 expression ($r = -0.67$, $q = 0.005$, Fig. 4). In females, a significantly strong positive correlation was found between ovary weight and hypothalamic OTAP ($r = 0.631$, $q = 0.013$) and GnRH-1 ($r = 0.87$, $q < 0.0001$) expression. A significant negative correlation was found between ovary weight and Dio2 ($r = -0.585$ $q = 0.023$), MCT8 ($r = -0.54$, $q = 0.038$) and LAT1 ($r = -0.72$, $q = 0.0018$, Fig. 4). No other genes where significantly correlated either positively or negatively with gonadal weight.

4. Discussion

We show here that the zebra finch hypothalamus undergoes major volumetric changes during development and that concomitantly, the expression of multiple genes implicated in the function of the HPT and HPG axes as well as genes regulating brain plasticity change with age in a gene- and sex-specific manner. By comparing the results of the rqPCR assays on hypothalamic samples with a previous transcriptomics study of the telencephalon, we also demonstrate that expression of some of these genes changes in parallel in the telencephalon but that other genes have a tissue-specific developmental pattern. These results raise a number of functional issues that deserve additional discussion.

General physiology and hypothalamic volume

By measuring repeatedly in vivo with MRI the brain and hypothalamus volume of the same animals we demonstrated that these two volumes undergo specific changes during ontogeny in both male and female zebra finches. The brain volume peaked at 65 dph but the volume of the hypothalamus decreased continuously from 20 to 120 dph. Interestingly a previous *in-vivo* MRI study from our lab found that most of the volume changes in the zebra finch brain occur between 20 to 65 dph (38). It has been previously reported that the volume of the song control nuclei, HVC, RA and area X increase the most during the first 50 days of post-hatching life and become stable thereafter at least in males (46, 47). Similar results were also found in a recent study of our laboratories (48). It was therefore intriguing to observe that the hypothalamic volume continuously decreased in both sexes from 20 to 120 dph. This decrease was observed during a period when the gonads of both males and females are growing and become functionally active as found here in agreement with all previous data. These volume changes could be reflecting the death of the abundant neurons that were generated in earlier ages, the progressive pruning of connections of these neurons or any volume change affecting specific cellular or extracellular compartments.

Methodological aspects

One fascinating aspect of the present work relates to the finding that by using different methods for quantifying gene expression (quantitative PCR in hypothalamus and transcriptomic analysis in the telencephalon) we were able to observe changes with age in the expression of 26 genes and demonstrate that in a substantial number of cases, the changes observed in both brain regions are nearly identical. This was particularly striking for a few genes related to the HPT axis (e.g., MCT8, Dio2, Dio3) and some of the genes related to brain plasticity (e.g., OTX2, DCX, Neuropilin, Tubulin).

This similarity indicates that the transcriptomic approach is able to identify variations in gene expression in the same manner as quantitative PCR, which indirectly validates the accuracy and specificity of both

techniques. It is then interesting to note that the changes with age or sex of some genes was quite different in the two brain regions. Since it is unlikely that these differences reflect differences in detection procedures, they very probably reflect true tissue specificity. This is not too surprising since it is well established that different parts of the brain develop and differentiate sexually at different paces as shown here again by the differential growth with age of the hypothalamus and whole brain volumes. The sexual differentiation of the song control system and of the hypothalamus also seem to proceed under the influence of different mechanisms (see introduction and (10)). This should obviously be reflected in gene expression.

It must also be noted that relatively few sex differences or interactions of sex by age were detected in the present study. This was somewhat surprising especially for the genes related to the HPG axis, such as the AR and ER that might have been expected to be differentially expressed in males and females, at least in mature birds. It must however be considered that the present study focused on large brain areas that both contain a variety of specialized nuclei. By pooling all these nuclei, we were possibly averaging small sex differences making them undetectable. Studies by rqPCR of nuclei microdissected by the Palkovits punch method (49) would allow testing the present suggestion and would likely reveal many more localized sex differences.

The hypothalamo-pituitary-thyroid (HPT) axis

Thyroid hormones are one of the most studied class of molecules in the context of brain development in both mammals and birds. In precocial birds like chickens and quail, the thyroid gland develops in early embryonic stages and the thyrotropin releasing hormone is already detected in the embryonic hypothalamus by incubation day 4.5 (for detail see, (50)). Quantification of thyroid transporters in both male and female hypothalami identified very different expression profiles in ontogeny for each transporter. MCT8 was high at the earliest ages and decreased afterwards while an opposite trend was shown by OTAP. Another transporter, LAT1, although present at all ages, generally tended to decrease with age. In general, a very similar expression pattern of the transporters was recorded in the telencephalon with the exception that OTAP that showed here a U-shaped pattern while it was increasing constantly in the hypothalamus until 65 dph. These patterns imply that there is an age-dependent importance of each thyroid transporters that may relate to the requirements of specific substrates (T3/T4, (51)).

The thyroid receptor THRα displayed its highest expression at 20 dph and decreased at later ages in both male and female hypothalamus. THRβ peaked at the same age in females (20 dph) but later (65 dph) in males. TSH-R expression was even more different in males and females showing a peak at 20 dph in females but increasing until 65 dph in males. This might suggest that thyroid action in the hypothalamus reaches its maximal importance earlier in females than in males.

THRα was not detected by transcriptomic analysis in the telencephalon but THRβ and TSH-R expression was fairly similar to what was observed in the hypothalamus in both males and females. The failure to detect THRα does probably not reflect absence of this receptor in the telencephalon but rather a technical limitation of transcriptomics, a technique that attempts to read all transcripts present in a tissue but is, as a consequence, exposed to an increased risk of false negative. In chicken, it has been shown that these receptors have unique expression patterns during embryonic development (52) with THRα being expressed in the early development and mediating thyroid hormones actions while Dio3 is also abundantly expressed at these early stages and protects the brain from premature thyroid hormone signaling. The present study shows an overall high expression of thyroid receptors in the hypothalamus/telencephalon at later ages until adulthood indicating that thyroid hormones presumably play a persistently important role in the brain.

Interestingly the developmental patterns of the two deiodinase enzymes, Dio2 and Dio3 were similar in both sexes and both tissues, but were quite specific to each enzyme. In both male and female hypothalamus and telencephalon Dio3 decreased continuously from 1-3 dph to adulthood while Dio2 expression peaked at 20 dph and only decreased thereafter, a pattern of results expected based on previous research (41, 43). The high expression of Dio2 at 20 dph may be implicated in stimulating neural growth by locally increasing in the availability of T3. Dio2 indeed regulates the conversion of the inactive thyroid hormone (T4) into its active form (T3), which induces functional changes in the target tissue. T3 interacts with nuclear receptors, regulates gene expression (about 5% of expressed genes are modulated by T3) and acts in specific time windows to influence brain development through changes in neurogenesis, neuronal migration, neuronal and glial cell differentiation, myelination, and synaptogenesis (for details see, (53)). Thus, the high expression of Dio2 in hypothalamus and in telencephalon at 20 dph may be a crucial milestone in the developmental timeline.

An *in situ* hybridization study providing a more detailed anatomical localization of Dio2 mRNA in zebra finches showed that Dio2 expression is high in the endothelial cells lining the brain capillaries in the entire telencephalon at early ages (10 and 20 dph). At 30 dph, when song control nuclei are still growing but the rest of the brain has attained its mature size, Dio2 expression was low everywhere except in HVC, RA and Area X of male birds specifically. At 90 and 120 dph, Dio2 expression became nearly undetectable throughout the brain. In females, Dio2 expression inside the song control nuclei was already low like in other brain regions at 20-30 dph (54). Such a sex difference was not detected here but as already mentioned this might be due to dilution of a difference in a large mass of undifferentiated tissue. Dio3 in contrast was expressed at high levels only at 1-3 dph both in hypothalamus and in telencephalon suggesting that the brain needs to be protected from T3 action at this early age. Similar results have been found in mice (55).

In conclusion, it is clear that expression of thyroid hormone-related genes drastically changes with age during development of zebra finches in a sex- and tissue-specific manner. It is however difficult based on the patterns detected here to determine precisely the function of T3 and T4 at the different ages in different tissues even if all markers except TSH-R, THRβ and OTAP suggest a decreased function in older subjects.

Sex steroids and the control of reproduction

In contrast the expression in the hypothalamus of genes related to sex steroids tended to be denser in older subjects with the exception of ERβ expression in the female telencephalon that was maximal at 1-3 dph. A few expression patterns also peaked at 20 dph and remained high afterwards, namely ERα and ERβ in the hypothalamus and AR in the telencephalon of both sexes. This clearly precedes the sexual maturation of the birds, as no increase in gonadal weight was detected before 65 dph. The hypothalamus is thus ready to respond to sex steroids even before they are produced in large amounts by the gonads**.**

Previous immunohistochemical or *in situ* hybridization studies have localized sex steroid receptors in the telencephalon of zebra finches and demonstrated a specific temporal and spatial expression pattern (56-59). Quantification in the whole telencephalon provides a global view of these expression patterns and identifies interesting differences in comparison with their expression in hypothalamus, suggesting area-specific area functions of the receptors. Most of these expression patterns were affected by a significant interaction of sex by age in the hypothalamus (except ERβ) but not in the telencephalon (ERβ is here also the exception). This might relate to the fact that the hypothalamus supports a large number of sexually differentiated functions related to reproduction, which is less the case in the telencephalon, if we except the song control nuclei but they only occupy a small amount of the space. The most prominent sex difference observed here concerned expression of ERβ in the telencephalon that was much higher in females than in males especially at the early ages.

Interestingly, no sex difference in baseline E2 concentrations in the caudal forebrain of juvenile zebra finches was found by *in vivo* dialysis during the early critical window of brain masculinization (60). The sex difference in baseline E2 concentrations were only detected in subadult finches (after 46 dph). A differential expression of ERβ in females could thus trigger a sexually differentiated response even in the absence of difference in the ligand for this receptor. The function of this high receptor expression and its specific localization are however currently unknown and would clearly deserve additional work.

Aromatase expression increased progressively with age in both hypothalamus and telencephalon and was expressed in a sexually differentiated manner (males >females) only in the adult hypothalamus. This corresponds well with the developmental patterns of activity that have been previously reported (61, 62). These studies and studies analyzing expression of the aromatase protein or mRNA indeed identified increases with age

and a sex difference in the hypothalamus but not the telencephalon (63-65), unless activity was measured specifically in the synaptosomal compartment (66). The early increases in aromatase expression (already significant at 20 dph) might suggest that the enzyme is already able to supply estrogens to the brain at these early stages. This implies however that the protein should be synthetized from the mRNA and a suitable substrate (testosterone or androstenedione) is present. These conditions might not be fulfilled since aromatase activity, as measured *in vitro* by a radioenzymatic assay was shown to be very low in many nuclei of 20 day-old zebra finches (61). The *in vivo* situation thus remains unclear.

GnRH-1 expression in the hypothalamus increased with age starting at 65 dph onwards, which corresponds well with the gonadal development observed in these birds. Surprisingly GnRH-1 and GnRH-R1 expression changed with age in opposite directions especially in the male hypothalamus (increase in GnRH-1 but decrease of its receptor). This has been observed in male wistar rats where GnRH-R1 mRNA was found to be significantly decreased in old subjects in comparison to young ones. Further, fewer cells containing GnRH-R1 were also found in older subjects (67). In the telencephalon, we detected some expression of GnRH-1 though its expression was highly variable between subjects and ages. GnRH-1 protein containing fibers have been shown to be present telencephalon in birds (for details see (68)) but, to our knowledge, the perikarya at the origin of these fibers have not been mapped carefully. Most cells synthesizing GnRH-1 are classically located in the preoptic and septal areas. It is therefore possible that the GnRH-1 mRNA we detected here in the telencephalic samples was coming from the positive cells located in the septum. Since this structure was at the edge of the dissected sample, it could have been included in the dissection in some subjects but not others thus explaining the high variability. Alternatively, some mRNA have been detected in axons (e.g., (69) and the GnRH-1 mRNA detected here in the telencephalon might not be located in perikarya but rather in cell processes.

Gonadal development is obviously controlled by the hypothalamic secretion of GnRH-1 and the correlation observed here in both sexes between gonadal weight and GnRH-1 expression in hypothalamus clearly supports this notion. It is quite interesting to note that both gonadal weight and GnRH-1 expression did not increase before 65 dph whereas the sex steroid receptors were already at their maximal level of expression at 20 dph. This suggests that the hypothalamus of young birds is super-sensitive to the steroid feedback exerted by androgens and estrogens on GnRH-1 secretion and this might delay sexual maturation (puberty) (70, 71).

Molecular changes in the hypothalamus, including regulation of the HPT axis, have been directly implicated in the regulation of the gonadal cycle in both seasonal passerine (41) and galliform birds (43). The present data allowed us to study the role of similar genes in the regulation of gonadal development during ontogeny in a non-seasonal, opportunistic breeding species. Interestingly, Dio2 was negatively correlated with gonadal maturation in both male and female zebra finches. However, a complete absence of relationship of

Dio2/Dio3 with seasonal changes in gonadal status has been recently found in a non-photoperiodic but seasonal subtropical avian species, *Lonchura punctulata* (45). Dio2/Dio3 expression was also found to be unimportant in the regulation of gonadotropin activity in a German population of great tits, *Parus major* (40). Together these studies indicate that the deiodinase-based mechanism of the regulation of reproductive physiology may not be conserved during ontogeny and during seasonal cycles between different species that express different reproductive life history strategies. Alternatively, it may also be possible that initiation by Dio2 of GnRH-1 release (for details see, (72)) occurred as early as day 20 post hatching (the only age when Dio2 was high) in zebra finch and the downstream effect were only detected later in ontogeny.

Brain plasticity and development

To gain insight in the molecular basis of hypothalamic development, we measured the expression of 12 genes involved in the control of neurogenesis, neural differentiation and stem cell activation. In both males and females, OTX2, DCX, cMYC, neuropilin and tubulin were expressed at their highest level at 1-3 dph. OTX2 has been implicated in region patterning and morphogenesis (73), cMYC is a proto-ontogene associated to cell proliferation, DCX is a marker of new neurons, neuropilin is responsible among other things of axons guidance during development and tubulin is a component of the cytoskeleton implicated in cell mobility. The high expression of all these genes clearly indicates the existence of a very active neurogenesis and neural cell migration as can be expected in a young brain.

Interestingly the expression of GADD45γ was specifically high at 20dph in the hypothalamus of both males and females, and in the male telencephalon at 1-3dph. The GADD45 family genes have been shown to have an effect on neuronal function through GADD45 mediated demethylation leading to increased expression of specific genes important for neuroplasticity (74). Of special note, GADD45 has been associated with priming sex determination (75). Different expression levels of GADD45 at 1-3 dph in telencephalon or 20dph in the hypothalamus maybe further translated into sex and neurodevelopmental specific epigenetic changes in DNA methylation.

Recently, a genome wide gene expression study analyzed the transcriptional signatures induced by vocal practice in the song control nucleus RA. This study demonstrated that GADD45 family genes are induced by the activity of singing in zebra finches (76). These gene expression changes were followed by neuroanatomical changes in the song control system.

In contrast, other genes were expressed at similar levels throughout life or even increased in older animals. This was for example the case for TLX and pCNA (proliferating cell nuclear antigen) in the hypothalamus of both males and females. This is supported by other findings in murine models where TLX was

found to be low at birth but again increasing to detectable levels in adult brain (for details see, (77)). TLX has been considered as the master regulator of neurogenesis and stem cell maintenance while pCNA is playing an important role in the replication of the genome. This could imply that avian hypothalamus must display some adult neurogenesis. The hypothalamus is not classically considered as an active neurogenic niche but during the last decade, a few studies have demonstrated the presence of neurogenesis in this structure in several mammalian species (78-80) and in birds (81-84). Interestingly, DCX expression drops drastically with age in the hypothalamus, which probably suggests that the new cells that are formed in the adult hypothalamus are mostly not neurons but rather glia or other cell types. It should also be noted that in contrast to the hypothalamus, TLX and pCNA expression decreased drastically with age in the telencephalon, thus suggesting completely different cell dynamics.

Very few sex differences in these plasticity genes could be detected in the telencephalon while multiple effects of sex and interactions of sex by age were present in the hypothalamus. Sex differences are known to affect the telencephalic song control nuclei but other parts of the telencephalon are not clearly dimorphic so that the limited sex differences affecting the song control nuclei might be diluted in mass of undifferentiated tissue. In contrast the physiology of many hypothalamic nuclei might be different in males and females.

5. Conclusions

In conclusion, we present here evidence that there is significant hypothalamic structural plasticity in zebra finches during development. We identified some of the molecular correlates of hypothalamic development and compared them with the molecular correlates of telencephalon development. Considering the high expression of Dio2 in a particular age in both hypothalamus and telencephalon, we would like to speculate that a pulse of active thyroid hormone mediated by the increase of Dio2 and decrease of Dio3 expression sets up a chain of events influencing different aspects of the physiology in the developing brain. We also showed that many HPT related genes (Dio2, Dio3, MCT8, THRβ) and HPG related genes (AR and aromatase in both sexes and ERα and ERβ in males) have the same pattern of expression in both hypothalamus and telencephalon across most important milestones in both the development of the song control system and in more general aspects of the physiology, suggesting the existence of similar underlying regulatory mechanisms. The presence of a larger number of sex differences or interactions of sex by age in the expression of genes in the hypothalamus compared to the telencephalon additionally suggest that the hypothalamus is more closely related to the process of sexual differentiation.

Declaration of Interests

The authors have nothing to declare

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Author contributions

GM and GY collected the rqPCR data, analyzed all the data and prepared all graphs and figures. JH collected the data of hypothalamic volumes. MV helped with the statistical analysis. JB provided the animals and helped with analysis. LC, TDM and WVB provided the transcriptome data. GM, AVDL and JB wrote the manuscript.

Figure legends:

Fig 1: Comparison of A) body and gonadal weight in male and female zebra finch in ontogeny (1-3, 20, 65 and 120 dph). B) Changes in hypothalamic volume of male and female zebra finches in ontogeny. Absolute volume of hypothalamus, absolute volume of whole brain and their ratio are presented. The brain images represent the *in vivo* 3D images of average zebra finch brain with the red area representing the delineated hypothalamus. Whole brain volume of 1-3 dph is shown (bars) but is not included in statistical comparisons. Letters (a, b, c..) depict differences between time points in males and Greek letters (α, β, γ…) represent differences between time points in females. * indicates a significant difference between males and females at specific time points. Letters {(a), (b), (c)} represent differences for males and females where there is only an age effect but no interaction or sex effect in post hoc tests. Data points sharing a same letter are not different; points with different letters are statistically different.

Fig 2: Mean (±SE; N=5) of relative mRNA levels of genes related to the HPT or HPG axes in male and female zebra finch hypothalamus ($2^{-\Delta\Delta ct}$ value) and telencephalon (RNA counts) in ontogeny. See figure 1 for additional explanations on how results of post-hoc tests are presented.

Fig3. Mean (±SE; N=5) relative mRNA levels of neuroplasticity related genes in male and female zebra finch hypothalamus ($2^{-\Delta\Delta ct}$ value) and telencephalon (RNA counts) in ontogeny. See figure 1 for additional explanations on how results of post-hoc tests are presented.

Fig4. Scatter plots showing the significant correlations, as determined by r and q (FDR corrected p value), that were detected between gonadal weight and genes expression in the hypothalamus in males (A, B) and females (C, D) Panels A and C represent genes positively correlated to gonadal weights, panels B and D represent negative correlations. Dotted lines in the scatter plots represent the linear regression.

Table 1: Detailed Two-way ANOVA values showing the effect of age and sex on hormone related gene expression in zebra finch hypothalamus and telencephalon.

Table 2: Detailed Two-way ANOVA values comparing the effect of age and sex on neuroplasticity related gene expression in zebra finch hypothalamus and telencephalon.

Supplementary Table 1: List of gene names and the primers used for rqPCR based measurement of relative mRNA levels of zebra finch hypothalamus at different ages in development.

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Figure 1

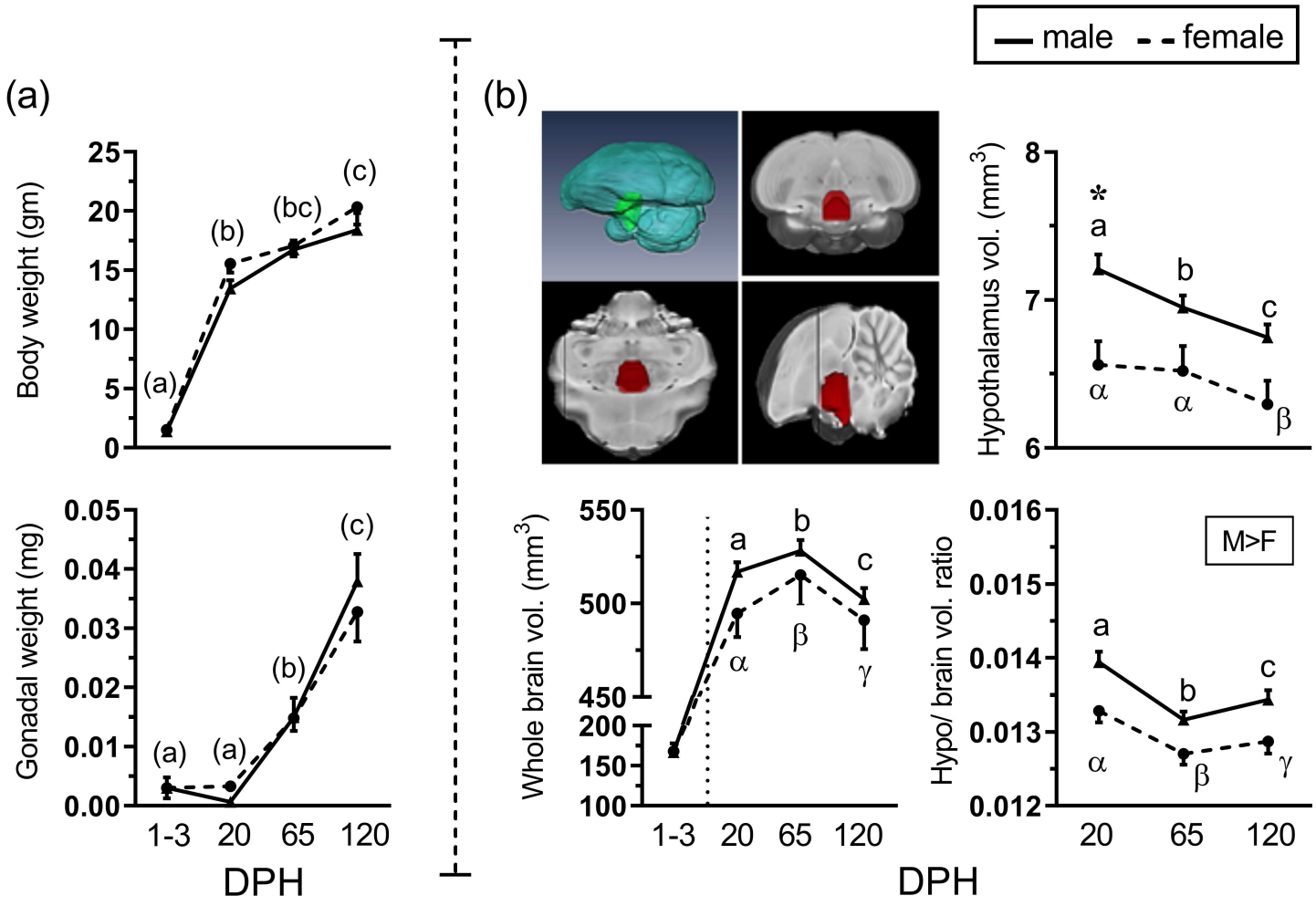
male
female
(a)
(b)
Body weight (gm)
25
20
15
10
5
0
(a)
(b)
(bc)
(c)
Gonadal weight (mg)
0.05
0.04
0.03
0.02
0.01
0.00
(a)
(a)
(b)
(c)
1-3
20
65
120
DPH
Hypothalamus vol. (mm3)
8
7
6
*
a
b
c
α
α
β
Whole brain vol. (mm3)
550
500
450
200
150
100
a
b
c
α
β
γ
1-3
20
65
120
DPH
Hypo/ brain vol. ratio
0.016
0.015
0.014
0.013
0.012
M>F
a
b
c
α
β
γ
20
65
120

Figure 2

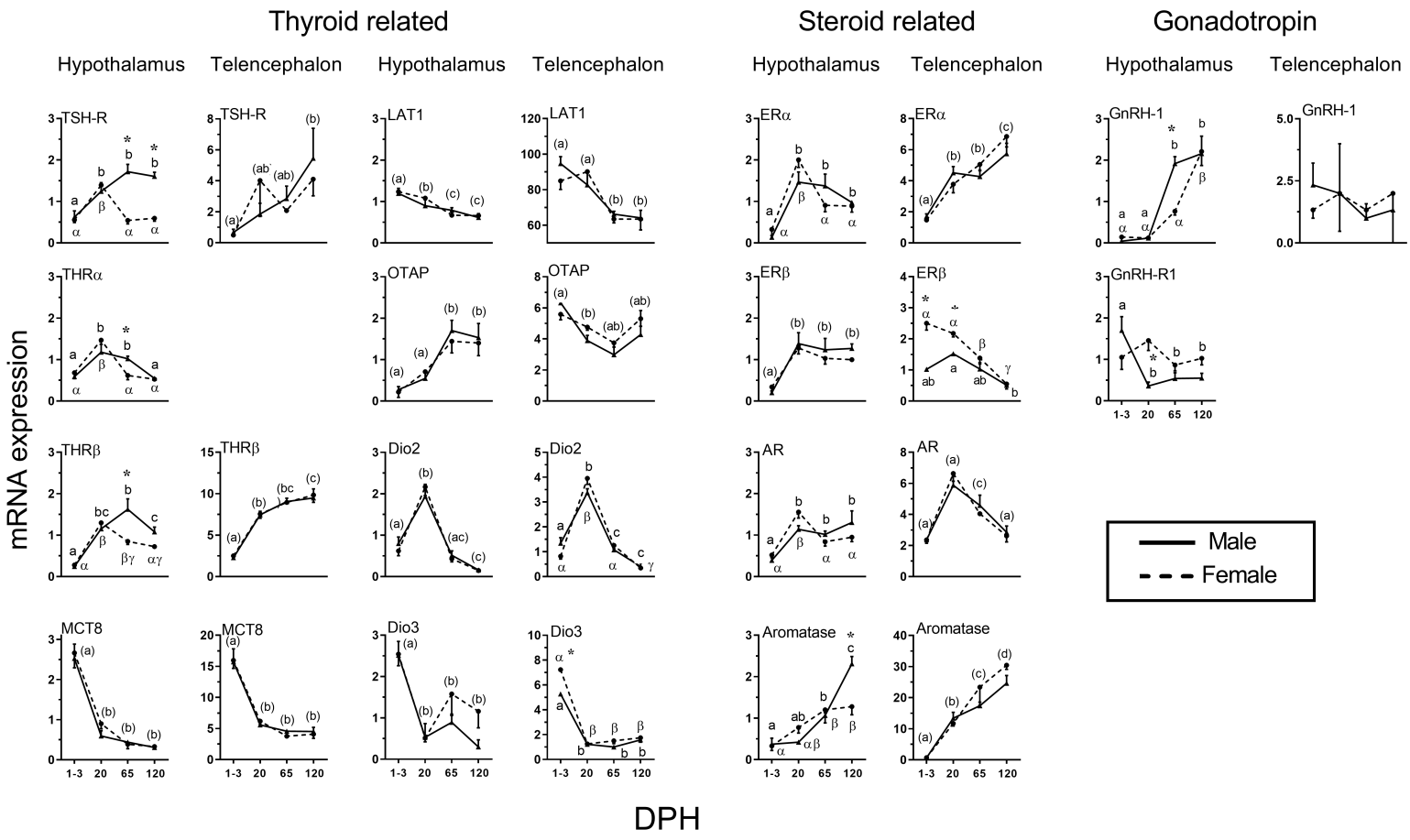
Thyroid related
Steroid related
Gonadotropin
Hypothalamus
Telencephalon
Hypothalamus
Telencephalon
Hypothalamus
Telencephalon
Hypothalamus
Telencephalon
TSH-R
TSH-R
LAT1
LAT1
ERα
ERα
GnRH-1
GnRH-1
THRα
OTAP
OTAP
ERβ
ERβ
GnRH-R1
THRβ
THRβ
Dio2
Dio2
AR
AR
MCT8
MCT8
Dio3
Dio3
Aromatase
Aromatase
mRNA expression
DPH
1-3
20
65
120
Male
Female

Figure 3

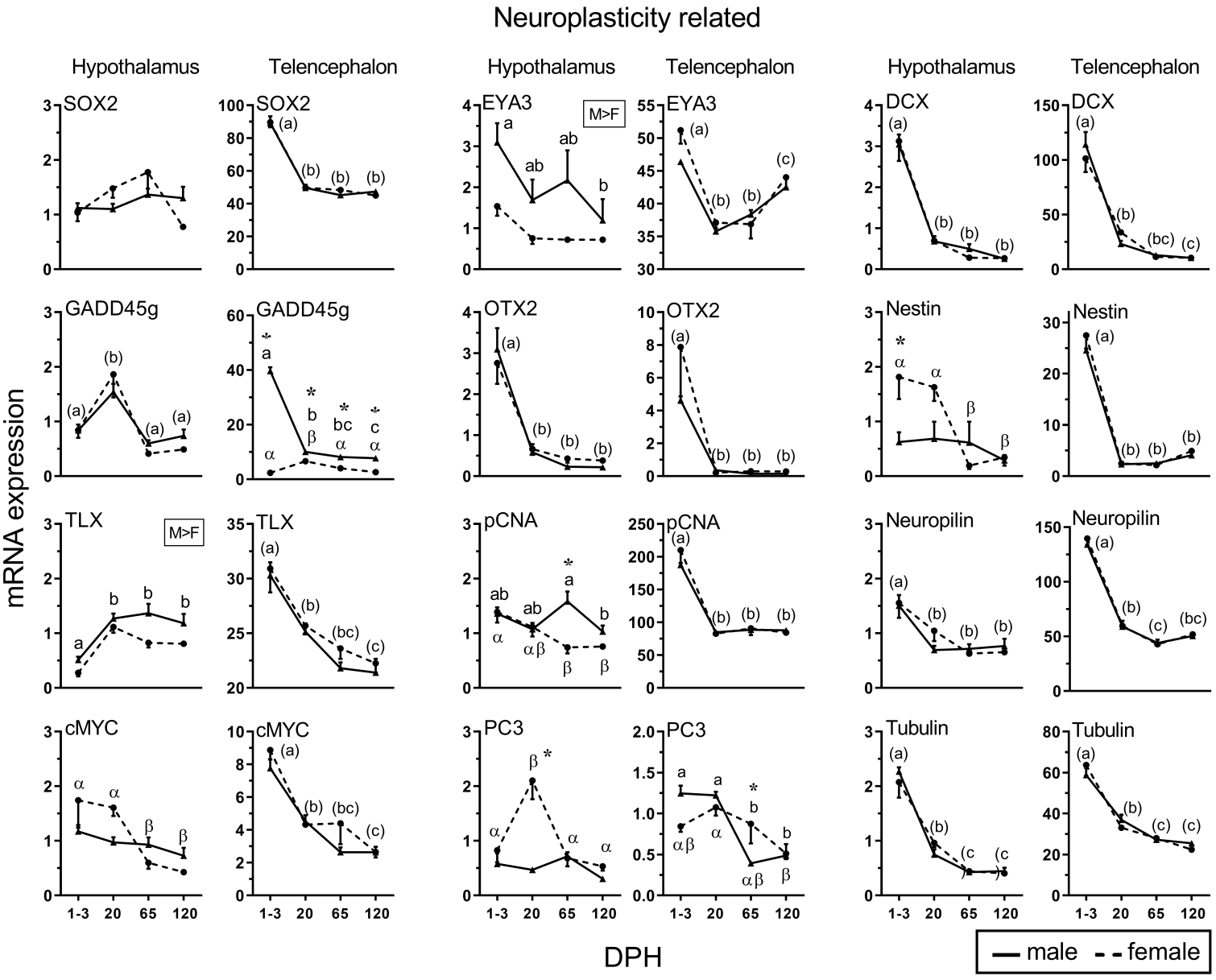
Neuroplasticity related
Hypothalamus
Telencephalon
Hypothalamus
Telencephalon
Hypothalamus
Telencephalon
SOX2
SOX2
EYA3
M>F
EYA3
DCX
DCX
GADD45g
GADD45g
OTX2
OTX2
Nestin
Nestin
TLX
M>F
TLX
pCNA
pCNA
Neuropilin
Neuropilin
cMYC
cMYC
PC3
PC3
Tubulin
Tubulin
mRNA expression
1-3
20
65
120
DPH
male
female

Figure 4

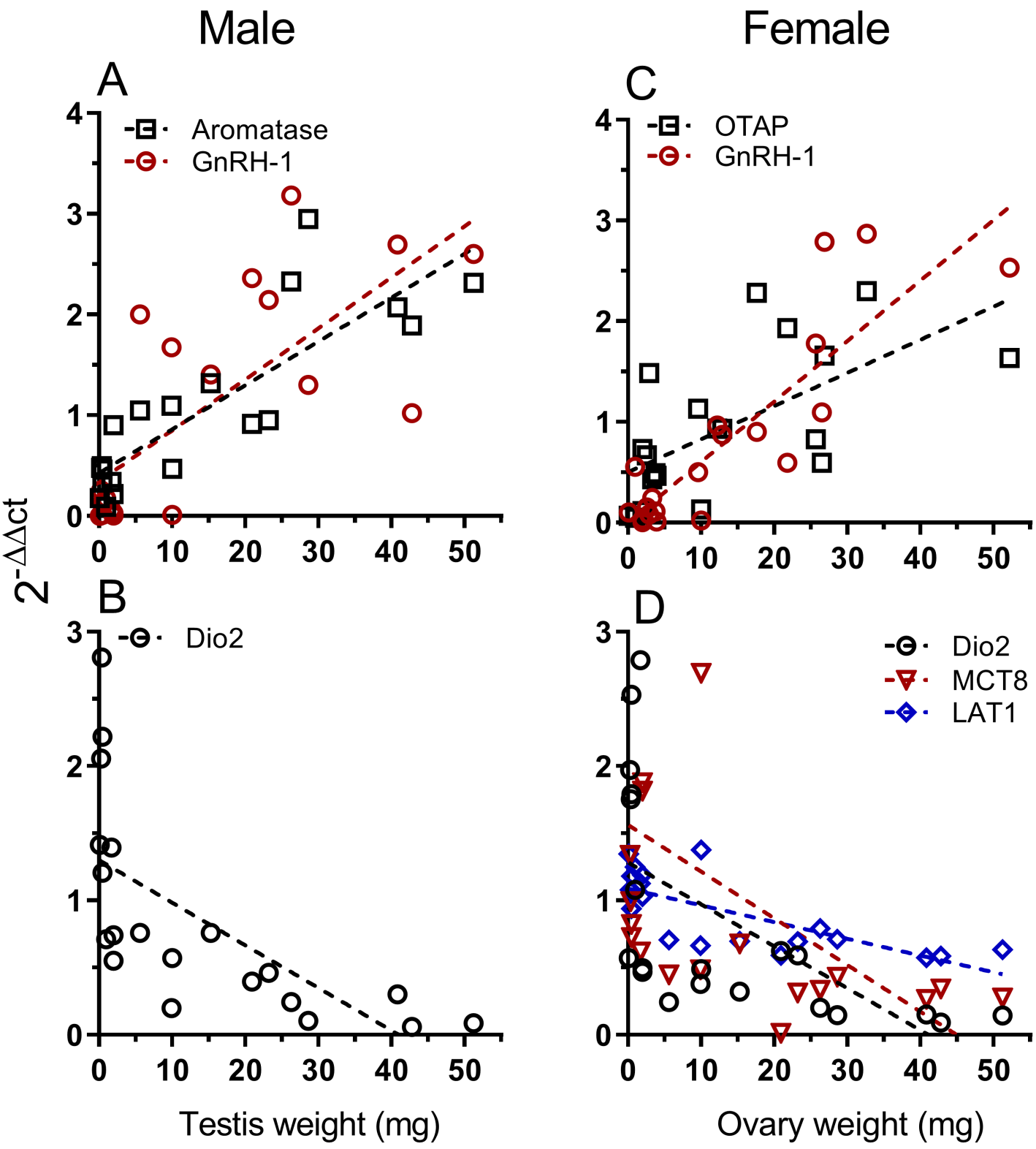
Male
Female
A
B
C
D
Aromatase
GnRH-1
Dio2
OTAP
GnRH-1
Dio2
MCT8
LAT1
2-ΔΔct
Testis weight (mg)
Ovary weight (mg)
0
1
2
3
4
0
10
20
30
40
50

Table 1: Effect of age and sex on hormone related gene expression in zebra finch hypothalamus and telencephalon: Two-Way ANOVA

Two way ANOVA results								
	Gene Name	Effect of Age		Effect of Sex		Interaction		Posthoc type
		F value	P value	F value	P value	F value	P value	
Hypothalamus	TSH-R	F3,32=10.19	<0.0001	F1,32=28.67	<0.0001	F3,32=11.54	<0.0001	1
	THR-A	F3,32=23.04	<0.0001	F1,32=0.0164	0.8989	F3,32=4.017	0.0156	1
	THR-B	F3,32=26.54	<0.0001	F1,32=7.156	0.0117	F3,32=5.556	0.0035	1
	MCT-8	F3,32=58.21	<0.0001	F1,32=0.53	0.470	F3,32=0.31	0.820	2
	LAT1	F3,32=19.99	<0.0001	F1,32=0.40	0.529	F3,32=1.08	0.369	2
	OTAP	F3,32=15.42	<0.0001	F1,32=0.18	0.670	F3,32=0.29	0.833	2
	Dio2	F3,32=59.93	<0.0001	F1,32=0.008	0.926	F3,32=0.649	0.589	2
	Dio3	F3,32=17.00	<0.0001	F1,32=3.19	0.081	F3,32=1.06	0.381	2
	ER-A	F3,32=24.80	<0.0001	F1,32=0.12	0.726	F3,32=2.94	0.048	1
	ER-B	F3,32=17.07	<0.0001	F1,32=0.92	0.344	F3,32=0.61	0.611	2
	AR	F3,32=16.32	<0.0001	F1,32=0.0005	0.981	F3,32=3.269	0.033	1
	Aromatase	F3,32=28.63	<0.0001	F1,32=1.49	0.230	F3,32=6.57	0.001	1
	GnRH-1	F3,32=47.91	<0.0001	F1,32=2.86	0.100	F3,32=4.20	0.0130	1
	GnRH-R1	F3,32=4.29	0.011	F1,32=4.62	0.039	F3,32=6.18	0.013	1
Telencephalon	TSH-R	F3,16=4.138	P=0.024	F1,16=0.0015	P=0.969	F3,16=0.83	P=0.499	2
	THR-B	F3,16=64.01	P<0.0001	F1,16=0.126	P=0.727	F3,16=0.053	P=0.9829	2
	MCT-8	F3,16=62.53	P<0.0001	F1,16=0.016	P=0.901	F3,16=0.20	P=0.8934	2
	LAT1	F3,16=14.52	P<0.0001	F1,16=0.158	P=0.696	F3,16=0.947	P=0.4411	2
	OTAP	F3,16=5.711	P=0.0075	F1,16=1.10	P=0.304	F3,16=0.827	P=0.4978	2
	Dio2	F3,16=146.9	P<0.0001	F1,16=0.1348	P=0.718	F3,16=3.71	P=0.0337	1
	Dio3	F3,16=282.9	P<0.0001	F1,16=20.83	P=0.003	F3,16=9.22	P=0.0009	1
	ER-A	F3,16=35.96	P<0.0001	F1,16=0.5089	P=0.485	F3,16=1.75	P=0.1972	2
	ER-B	F3,16=34.21	P<0.0001	F1,16=36.87	P<0.0001	F3,16=8.88	P=0.0011	1
	AR	F3,16=35.27	P<0.0001	F1,16<0.0001	P=0.999	F3,16=0.854	P=0.4846	2
	Aromatase	F3,16=44.15	P<0.0001	F1,16=2.155	P=0.1615	F3,16)=1.29	P=0.3101	2
	GnRH-1	F3,16=0.171	P=0.9142	F1,16=0	P>0.999	F 3,16=0.17	P=0.9142	No post hoc

#Shaded: not significant

Post hoc type1: Two post hoc's test done. Tukey's multiple comparison for comparing male and female gene expression in ontogeny separately and Sidak's multiple comparison to compare male and female expression at specific time-points.

Post hoc type2: One post hoc test done. Tukey's multiple comparison for comparing male and female gene expression in ontogeny together

Post hoc type3. One post hoc test done. Tukey's multiple comparison for comparing male and female gene expression in ontogeny separately.

Table 2: Effect of age and sex on plasticity related gene expression in zebra finch hypothalamus and telencephalon: Two-Way ANOVA

Two way ANOVA results								
	Gene Name	Effect of Age		Effect of Sex		Interaction		Post hoc type
		F value	P value	F value	P value	F value	P value	
Hypothalamus	SOX2	F3,32=2.98	0.0458	F1,32=0.10	0.754	F3,32=2.54	0.081	2
	GADD45g	F3,32=18.54	<0.0001	F1,32=0.04	0.834	F3,32=1.04	0.386	2
	TLX	F3,32=21.54	<0.0001	F1,32=17.88	0.0002	F3,32=1.19	0.330	3
	cMYC	F3,32=8.09	0.0004	F1,32=0.94	0.340	F3,32=3.25	0.034	1
	EYA3	F3,32=4.13	0.013	F1,32=14.46	0.0006	F3,32=0.75	0.531	3
	OTX2	F3,32=42.27	<0.0001	F1,32=0.02	0.896	F3,32=0.40	0.754	2
	pCNA	F3,32=4.15	0.0136	F1,32=7.20	0.013	F3,32=4.52	0.009	1
	PC3	F3,32=8.69	0.0002	F1,32=17.64	0.0002	F3,32=9.28	0.0001	1
	DCX	F3,32=85.30	<0.0001	F1,32=0.04	0.847	F3,32=0.20	0.897	2
	Nestin	F3,32=6.91	0.0010	F1,32=5.86	0.021	F3,32=4.28	0.011	1
	Neuropilin	F3,32=14.10	<0.0001	F1,32=0.23	0.638	F3,32=1.00	0.403	2
	Tubulin	F3,32=99.94	<0.0001	F1,32=0.0003	0.986	F3,32=1.03	0.390	2
Telencephalon	SOX2	F3,16=184.3	P<0.0001	F1,16=0.054	P=0.8195	F3,16=0.505	P=0.6842	2
	GADD45g	F3,16=347.2	P<0.0001	F1,16=997.6	P<0.0001	F3,16=439.8	P<0.0001	1
	TLX	F3,16=23.13	P<0.0001	F1,16= 1.34	P=0.2640	F3,16=0.125	P=0.9439	2
	cMYC	F3,16=37.24	P<0.0001	F1,16=2.654	P=0.1228	F3,16=1.272	P=0.3176	2
	EYA3	F3,16=32.6	P<0.0001	F1,16=2.384	P=0.1422	F3,16=1.668	P=0.2137	2
	OTX2	F3,16=12.35	P=0.0002	F1,16= 0.979	P=0.3370	F3,16=0.882	P=0.4713	2
	pCNA	F3,16=93.21	P<0.0001	F1,16=0.641	P=0.4352	F3,16=1.092	P=0.3810	2
	PC3	F3,16=14.64	P<0.0001	F1,16=0.018	P=0.8945	F3,16=5.139	P=0.0112	1
	DCX	F3,16=111.8	P<0.0001	F1,16=0.0397	P=0.8444	F3,16=1.204	P=0.3402	2
	Nestin	F3,16=257.6	P<0.0001	F 1,16=1.55	P=0.2310	F3,16=1.011	P=0.4136	2
	Neuropilin	F3,16=259.3	P<0.0001	F1,16= 0.334	P=0.5713	F3,16=0.269	P=0.8464	2
	Tubulin	F3,16=90.58	P<0.0001	F1,16=0.031	P=0.8637	F3,16=1.314	P=0.3044	2

#Shaded: not significant

Post hoc type1: Two post hoc's test done. Tukey's multiple comparison for comparing male and female gene expression in ontogeny separately and Sidak's multiple comparison to compare male and female expression at specific time-points.

Post hoc type2: One post hoc test done. Tukey's multiple comparison for comparing male and female gene expression in ontogeny together

Post hoc type3. One post hoc test done. Tukey's multiple comparison for comparing male and female gene expression in ontogeny separately.

Supplementary Table 1: Genes, primers and function

Gene name	Primers (5'-3')	Amplicon length (bp)	Melting temp (c^0)	Function/Role
GAPDH	F: TCCCATGTTCGTGATGGGTG R: GATGGCATGGACAGTGGTCA	160	84.5	Housekeeping gene; *Kozera and Rapacz, 2013*
β-actin	F: TGGATTTCGAGCAGGAGATG R:TCCATACCCAGGAAAGATGG	120	85	Housekeeping gene; *Kozera and Rapacz, 2013*
Cyclophilin A	F: CAACCCTGTCGTCTTCTTC R:TGTCTTCGGGACCTTGT	91	85.2	Housekeeping gene; *Kozera and Rapacz, 2013*
Tshβ	F: TCACTCTGTGCTCCTTCT R: GCAGATGGTGGTGTTGAT	84	83.2	Thyroid stimulating hormone, Central regulator of gonadal development, Nakao et al., 2009
Tsh-R	F: GTCTTATGGGCAGAGAATCAG R:GTCACAGCAGGAAGGTAATC	81	82	Thyroid stimulating hormone receptor, receptor for TSH; Nakao et al., 2009
THRα	F: CCACCTACTCCTGCAAATAC R:ACGGAGATGCACTTCTTG	94	85.5	Thyroid hormone receptor alpha subunit; *Decuypere et al., 2005*
THRβ	F: GCCACCGGATATCATTATCG R:GTTGGATGGAGGTTCTTCTG	86	80.5	Thyroid hormone receptor beta subunit; *Decuypere et al., 2005*
MCT8	F: GGAGAAAGACCTATCGGATTTG R:TTTGACCAGGTTCATGTAAGG	81	82.7	Thyroid hormone transporter; *Raymaekers et al., 2017*
OTAP	F: GAAAGATTCCCTTCCACCATC R:AGAGTGAAGTTTGGCCTTTATC	76	80.8	Thyroid hormone transporter; *Raymaekers et al., 2017*
LAT1	F: ATCGGCTTTGCCATCATC R:GAGAAGATGCCTTGGAGAAC	95	83.8	Thyroid hormone transporter; *Raymaekers et al., 2017*
Dio2	F: GGTTTGCTAAGTGTGGATTTG R:GTCATAGAGCGCGAGAAAG	84	80.8	Coverts T4 to active T3; *Yoshimura et al., 2003*
Dio3	F: CTACGGTGCTTACTTCGAGA R:CGAGATCTTGTAGCCCTCTG	91	83	Produces inactive T3; *Yoshimura et al., 2003*
ERα	F: CCTCCTCATCCTCTCTCATATC GAGCGGAACTACATTCTTACAC	88	79	Estrogen hormone receptor subunit; *Balthazart et al., 2009*
ERβ	F: ATCTCTCCAGTCCCTTACAA R:CCATGAAGGATGAGGGAATG	82	82.5	Estrogen hormone receptor subunit; *Balthazart et al., 2009*
AR	F: GGATGCTGTACTTCGCTCCA R: ACTCCTGGGGTGTGATCTGA	85	85.2	Androgen receptor; *Balthazart et al., 2009*
CYP19/	F: GTGTCTCGATTTGGGAGTAAG	81	79.5	Aromatase gene, influence sexual differentiation; *Balthazart et al.,*

Aromatase	R:TGCTGGGTTGTTGTTAAATATG			*2009*
GnRH-1	F: CACCAGAATTCCAGGAGTG R: GTTCTTTCTTCTGCCTTGTTC	75	83.2	Regulates release of gonadotrops from pituitary; *Ubuka et al., 2013*
GnRH-R1	F: TCTGGCACTGGTTCTGT R:GAAGAGGCCAAAGATGAAGAG	87	86.5	GnRH hormone receptor; *Ubuka et al., 2013*
SOX2	F: GTCACTTCCTCGTCTCATTC R:TACTTCAGCACCTGGTAGATA	87	83.8	Pluripotency, Stem cell activation; *Zhang and Cui, 2014*
GADD45g	F: AGCTCTGGAGAAGCTGAA R:ACTCAGGCAGGGTGATAG	80	81.8	Proliferation of progenitors, Dendritic growth; *Felling and Song, 2014*
TLX	F: CAGCAGCCAGACTTCTATTC R:CATCAGCTGGTCTTGTAAGG	86	83	Neural Stem Cell Maintenance and Neurogenesis; *Islam and Zhang, 2014*
cMYC	F: CTGATTCAGAGGACAGTGAAC GTGAGGAAACTTGACCGTAG	85	81.8	involved in cell proliferation; *Miller et al., 2001*
EYA3	F: CCAGCCCAGCACATTATT R:GACAACAGTTGAGGATGTAGAG	80	79.2	Cell division activation/ thyroid hormone activation; *Nakao et al., 2008*
OTX2	F: TCCTATCCCATGACCTACAC R:AGTCCATCCCTCCGAAAT	82	83.2	Neuroplasticity/GnRH induction; *Larder and Mellon, 2009*
pCNA	GAGGAGGAGGCTGTTACAATA GTGGCTTTGGTGAAGAAGTT	86	83.7	Proliferation and neurogenesis; *Miller et al., 2001*
PC3	ACACGACAAACGAACCAA CCTCAGCTTGAGGTATTACAG	84	80.2	Proliferation and cell cycle regulation; *Miller et al., 2001*
DCX	GAAGCCATCAAACTGGAGAC CCCAAAGAAATCGTGGAGAC	87	82.2	Neurogenesis and cell migration; *Balthazart et al., 2008*
Nestin	TTGAGGTAGGACAAGACCAT TTCCTCAGAACCTTCTCCTG	95	76	Neural cytoskeleton and plasticity; *Hendrickson et al., 2011*
Neuropilin	CAGAAATCCTCCTCCACTTTC GTCATACTTGCAGTCTCCTTC	80	81.8	Angiogenesis, axon guidance, cell survival, migration; *Schwarz and Ruhrberg, 2010*
Tubulin	GGTCTCTGAATACCAGCAATAC TATGCCTCATCCTCTTCTCC	84	82.5	Dendrite growth and neuron migration; *Aiken et al., 2017*