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Expression and activity profiling of the steroidogenic enzymes of glucocorticoid biosynthesis and the *fdx1* co-factors in zebrafish

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Abstract

The spatial and temporal expression of steroidogenic genes in zebrafish has not been fully characterized. Since zebrafish are increasingly employed in endocrine and stress research, a better characterization of steroidogenic pathways is required to target specific steps in the biosynthetic pathways in the future. Here, we have systematically defined the temporal and spatial expression of steroidogenic enzymes involved in glucocorticoid biosynthesis (*cyp21a2*, *cyp11c1*, *cyp11a1*, *cyp11a2*, *cyp17a1*, *cyp17a2*, *hsd3b1*, *hsd3b2*), as well as the mitochondrial electron-providing co-factors ferredoxin (*fdx1*, *fdx1b*) during zebrafish development. Our studies showed an early expression of all these genes during embryogenesis. In larvae, expression of *cyp11a2*, *cyp11c1*, *cyp17a2*, *cyp21a2*, *hsd3b1* and *fdx1b* can be detected in the interrenal gland, the zebrafish counterpart of the mammalian adrenal gland, whereas the *fdx1* transcript is mainly found in the digestive system. Gene expression studies using quantitative RT-PCR and whole-mount *in situ* hybridization in the adult zebrafish brain revealed a wide expression of these genes throughout the encephalon, including neurogenic regions. Using ultra-high-performance liquid chromatography tandem mass spectrometry, we were able to demonstrate the presence of the glucocorticoid cortisol in the adult zebrafish brain. Moreover, we demonstrate *de novo* biosynthesis of cortisol and the neurosteroid THDOC in the adult zebrafish brain from radiolabeled pregnenolone. Taken together, our study is a comprehensive characterization of the steroidogenic genes and the *fdx* co-factors facilitating glucocorticoid biosynthesis in zebrafish. Furthermore, we provide additional evidence of *de novo* neurosteroid biosynthesizing in the brain of adult zebrafish facilitated by enzymes involved on glucocorticoid biosynthesis. Our work provides a valuable source for establishing the zebrafish as a translational model to understand the roles of the genes of glucocorticoid biosynthesis and *fdx* co-factors during embryonic development, stress and in brain homeostasis and function.

Introduction

Glucocorticoids such as cortisol and corticosterone are steroid hormones mainly produced by the adrenal cortex in mammals and the interrenal gland in teleosts. They are key regulators of vertebrate body homeostasis including the physiological responses to stress. Several key steps of glucocorticoid biosynthesis are facilitated by cytochrome P450 enzymes (CYPs) and hydroxysteroid dehydrogenases (HSDs) (REF). (Fig. 1A). Importantly, both the enzymatic functions of CYP11A1, catalyzing the rate-limiting step of steroidogenesis, and CYP11B1, catalyzing the final step of glucocorticoid biosynthesis, crucially depend on electron transfer from NADPH via ferredoxin reductase (FDXR) and the iron sulfur protein ferredoxin (FDX1) (Fig. 1A) [reviewed in (1, 2)]. The central nervous system is a key target of steroid hormones in all vertebrates. However, the brain is also a site of *de novo* steroidogenesis(4, 5). These steroids synthesized in the central nervous system are called neurosteroids and are thought to be important for brain development and homeostasis (6), neuroprotection (7) and neurogenesis (8, 9). Glucocorticoids and the neurosteroid tetrahydrodeoxycorticosterone (THDOC) play key roles in brain homeostasis by modulating, for example, the expression of subunits of γ-aminobutyric acid (GABA) receptors (10, 11), or by regulating glutamate and GABA synapse-specific retrograde transmission in the hypothalamus (12). They also target a wide variety of cells across the central nervous system, including neurons as well as neural progenitors (13), suggesting a role in neurogenesis. Indeed, glucocorticoid injections in rodents lead to decreased proliferation of hippocampal progenitors (14, 15) and to increased apoptosis of neural progenitors as well as immature neurons in the dentate gyrus (16). Furthermore, chronic stress and changes in glucocorticoid concentrations impair neural progenitor proliferation, differentiation and cell survival in the hippocampus (15). However, little is known about the underlying mechanisms by which neurosteroids, mediate homeostatic and pathobiological processes in the brain.

Vertebrate model organisms such as the zebrafish have a significant potential to provide novel insights into such mechanisms. The zebrafish is a well-established model for studying vertebrate development and disease (18), allows the performance of *in vivo* high throughput molecule screenings (19) and the analysis of brain function and regeneration due to its high regenerative capacity compared to mammals (20, 21). In recent years, zebrafish have increasingly been used as a model for endocrine physiology and disease, as its endocrine system shares a high degree of similarities with the human endocrine system. Like humans, zebrafish are day active and their main glucocorticoid is cortisol (22). In zebrafish cortisol is produced by the interrenal gland, the zebrafish counterpart of the human adrenal gland (23, 24), and is released upon stress to induce cortisol-mediated gene transcription (25-27). Given the similarities of the zebrafish endocrine system to that of the human, the zebrafish appears to be a good model for studying the role of glucocorticoids and stress during early development and its impact in adulthood [for further in depth-reading, see (22, 28)]. However, due to a genome duplication event that have occurred in teleosts about 350 million years ago (29, 30), zebrafish can have two or more copies of a gene unique in mammals with either similar or distinct functions (31). This gene duplication event also involved the some, but not all genes involved glucocorticoid biosynthesis. Previous studies have analyzed the expression of key enzymes of steroidogenesis (Cyp11, Hsd3b, Cyp17) in the adult zebrafish brain (8, 32); however, it is not

clear from these studies which of the corresponding paralogs were analyzed. More recently, novel insights into the expression and/or role of these steroidogenic enzymes as well as their corresponding paralogs were obtained in zebrafish (33-36). This clearly emphasizes the importance of a precise characterization of the zebrafish paralogs and their role in steroidogenesis. Thus, further in-depth analysis for those genes and particularly their paralogous in the adult zebrafish brain is required to facilitate the correct targeting of steps in the biosynthetic pathways in the future.

By applying a transcriptional analysis, we herein describe the spatio-temporal expression profiles of the steroidogenic genes involved in glucocorticoid biosynthesis, namely *cyp11a1, cyp11a2, cyp17a1, cyp17a2*, *hsd3b1, hsd3b2, cyp21a2, cyp11c1,* and the ferredoxin (*fdx)* co-factors (*fdx1*, *fdx1b*) during zebrafish development. Furthermore, we report the wide distribution of these genes in the adult zebrafish brain, including their expression in many neurogenic regions. Using high-performance liquid chromatography, we furthermore demonstrate the presence of the glucocorticoid cortisol in the adult zebrafish brain and the biosynthesis of cortisol and the neurosteroid THDOC in the brain from [^{3}H]-pregnenolone, demonstrating the functional capacity of this biosynthetic pathway in the adult zebrafish brain.

Results

Spatio-temporal expression of genes of glucocorticoid biosynthesis and fdx *co-factors*

A comprehensive search for all potential zebrafish (*Danio rerio,* dr) orthologues of the human (*Homo sapiens*, hs) and mouse (*Mus musculus*, mm) glucocorticoid biosynthetic and *fdx* co-factor genes showed that zebrafish have only a single gene copy (*cyp21a2*) for 21-hydroxylase (*CYP21A2)* (Fig. 1B). Furthermore, a single *CYP11B*-like gene is present in the zebrafish genome, namely *cyp11c1* (25) (Fig. 1B). Two paralogs can be found for the human *CYP11A1* genes, *cyp11a1* and *cyp11a2* (33, 37, 38). Two paralogs can also be found for the human *CYP17A1* (*17α-hydroxylase*) genes, *cyp17a1* and *cyp17a2* (Fig. 1B). *cyp17a1* facilitates both the 17α-hydroxylation and 17,20-lyase reactions, whereas *cyp17a2* is only able to 17α-hydroxylate steroid hormones precursors (36). In the case of the *HSD3B* genes, the situation is more complex, as there is one orthologue in zebrafish, *hsd3b1*, for the duplicated human *HSD3B1* and *HSD3B2* genes. The *hsd3b2* gene appears to be a zebrafish-specific gene duplication (35). In addition, two orthologues, *fdx1* and *fdx1b* (34), for the human *FDX1* are present in the zebrafish (Fig. 1D). Phylogenetic analysis demonstrates that all zebrafish protein sequences of the examined genes group together with their corresponding human and mouse orthologues (Fig. 1B-D), suggesting conserved functions of these enzymes between the species.

To assess the gene expression profiles of steroidogenic genes involved in glucocorticoid biosynthesis during early zebrafish development , we reanalyzed a recently published data set (39) that provides a global transcriptional profiling of zebrafish development between zygote (1-cell) stage to 5 days post-fertilization (dpf). *cyp11a1* transcript levels are the highest at the zygote (1-cell) and cleavage (2-cell) stages and remain high at blastula (128-cell, 1k-cell, dome) and gastrula (50% epiboly, shield, 75% epiboly) stages (Fig. 1E). *cyp11a1* expression decreases at segmentation (4 somites, 19 somites, 25 somites) stages and remain at very low levels after primordium-5 (24 hpf) up to 120 hpf (Fig. 1E). In contrast, the *cyp11a2* transcript is not detectable until the dome stage and remains at very low levels during the gastrula (50% epiboly,

shield), segmentation stages (4 somites, 19 somites) and primordium-5 (24 hpf) (Fig. 1F). From the primordium-15 (30 hpf) stage onwards transcript levels of *cyp11a2* increase with a peak around 3-5 dpf (Fig. 1F). *cyp17a1* and *cyp17a2* transcript levels peak at 2-cell and dome stage for *cyp17a1* and *cyp17a2*, respectively (Fig. 1G, H). The expression of both genes is lower during gastrulation stages with a decrease for *cyp17a1* at 50% epiboly (Fig. 1G) and for *cyp17a2* at 75% epiboly (Fig. 1H). *cyp17a1* transcript levels increase with segmentation until the primordium stages and then slightly decrease again (Fig. 1G), whereas *cyp17a2* transcript levels are lower at segmentation stages and constantly increase after primordium-15 (30 hpf) (Fig. 1H). Transcript levels of *hsd3b1* are the highest at zygote (1-cell) stage and remain high at cleavage (2-cell) and blastula (128-cell, 1k-cell, dome) stages until they decrease at the shield stage (Fig. 1I). *hsd3b1* transcript levels remain low until primordium-5 (24 hpf). Starting at primordium-15 (30 hpf) *hsd3b1* transcript levels increase again and remain constant up to 120 hpf (Fig. 1I). In contrast, *hsd3b2* transcript levels are extremely low at the zygote (1-cell) and cleavage (2-cell) stages until the dome stage and increase at around the shield/75% epiboly stage (Fig. 1J). *hsd3b2* transcript levels decrease at the 4 somites stage and remain very low (Fig. 1J). The *hsd3b2* transcript patterns are consistent with the described role of *hsd3b2* in early embryogenesis (35). *cyp21a2* transcript is almost undetectable until primordium-5 (24 hpf) (Fig. 1K). After primordium-5 *cyp21a2* expression constantly increases and peaks around 96-120 hpf (Fig. 1K). *cyp11c1* transcript levels are low until the dome stage, increase during gastrulation (50% epiboly, shield, 75% epiboly), then again slightly decrease during segmentations stages (4 somites, 19 somites, 25 somites) (Fig. 1L). After primordium-5 (24 hpf) transcript levels of *cyp11c1* increase and reach a peak around 72-120 hpf (Fig. 1L). *fdx* transcripts can be detected at all analyzed stages (Fig. 1M, N). However, in contrast to the overall higher transcript levels of *fdx1* at early stages with a peak at the zygote (1-cell stage) and cleavage (2-cell) stages (Fig. 1M), *fdx1b* transcript levels peak at gastrulation (50% epiboly, shield, 75% epiboly) stages and after primordium-15 (30 hpf) (Fig. 1N). Thus, all analysed genes involved in glucocorticoid biosynthesis are expressed within the first days of zebrafish development. *cyp11a1*, *cyp17a1*, *hsd3b1* and *fdx1* exhibit strong maternal contribution, while their corresponding paralogs are mainly zygotically expressed.

The organ-specific expression of the steroidogenic genes involved in glucocorticoid biosynthesis and the *fdx* co-factors during zebrafish development was analyzed by whole-mount *in situ* hybridization. We focused our study on a larval stage (120 hpf), as organ-development, cortisol biosynthesis and a functional stress-axis leading to cortisol-mediated gene expression is fully established at this point of development (22, 26, 40). Our results showed no staining for the *cyp11a1*, *cyp17a1* and *hsd3b2* probes at this larval stage (Fig. 2A1, D1, H1 and Figure S1), whereas a clear staining was observed in the larval interrenal gland for *cyp11a2*, *cyp11c1, cyp17a2*, *cyp21a2*, *hsd3b1* and *fdx1b* (Fig. 2B1, C1, E1, F1, G1, J1 and Figure S1). *fdx1* transcript was detected in the liver and in the gut (Fig. 2I1 and Figure S1). The specificity of the staining was shown in parallel using the respective sense probes against the transcripts of interest (Fig. 2 A2-J2 and Figure S1).

Spatial expression of the steroidogenic genes of glucocorticoid biosynthesis and fdx *genes in zebrafish adult brain*

Knowledge about the expression of steroidogenic enzymes, involved in glucocorticoid biosynthesis, in the brain of adult zebrafish remains limited. As observed via a quantitative RT-PCR transcriptional analysis, all examined genes are expressed in the whole brain of adult zebrafish (Fig. S2), indicating a potential physiological function of those genes in the central nervous system. To define the main sites of expression of the steroidogenic genes and *fdx* co-factors of glucocorticoid biosynthesis, we next performed whole-mount *in situ* hybridization on adult zebrafish brains. We observed a wide expression for the examined genes throughout the adult zebrafish brain from the junction between the olfactory bulbs and the dorsal area of the telencephalon to the more caudal parts of the encephalon including the cerebellum (Fig. 3, 4, and 5). The specificity of the labeling was verified with either the corresponding sense probes (for *cyp11a1*, *cyp11c1*, *cyp17a2, cyp21a2*, *hsd3b1*, *hsd3b2*, *fdx1, fdx1b;* Fig. S4A) or alternatively with a second non-overlapping anti-sense probe (for *cyp11a2* and *cyp17a1*; Fig. S4B).

At the junction between the olfactory bulbs and the telencephalon, the examined steroidogenic genes are weakly expressed in the dorsal telencephalic area (Fig. 3 column 1), except *cyp11a1* which seems strongly expressed. At this section level, transcripts are mainly detected along the ventricular and periventricular layers and to a lesser extent in the brain parenchyma (Fig. 3 column 1 and Fig. 5 row A). In the telencephalon, the steroidogenic genes of glucocorticoid biosynthesis and the *fdx* co-factors are widely expressed with similar expression patterns. They are detected in most telencephalic brain nuclei, including the ventral (Vv), dorsal (Vd), and central (Vc) nuclei of the ventral telencephalic area, as well as in the medial (Dm) and lateral (Dl) zones of the dorsal telencephalon (Fig. 3 column 2 and Fig. 5 row B) [as clearly seen in higher magnification images of the Dm and Vv (Fig. 4 C1-L1 and C2-L2)]. In addition, these genes are also detected in the ventricular and periventricular layers, where neural progenitors are localized (Fig. 3 and 5), and in the posterior zone of the dorsal telencephalic area (Dp) (Fig. 3, 4 and 5 rows B and C). To summarize, the examined genes display similar distributions in the telencephalon and are detected along the ventricular/periventricular layers, in the parenchyma of the pallium and the subpallium, including the Vc, Vd, Vv, Dp, Dl, Dm, in the ventral part of the entopendoncular (ENv) and the post-commissural nucleus (Vp) (Fig. 5 rows B and C).

In the diencephalon, we observed expression of the steroidogenic genes facilitating glucocorticoid biosynthesis and the *fdx* co-factors in the anterior part of the preoptic area (PPa). They are also largely expressed in the entire posterior part of the preoptic area (PPp), in the ventromedial thalamic nucleus (VM) and in the habenula (Hav) (Fig. 3 column 3 and Fig. 5 row D). More caudally, all the examined transcripts appear to be widely expressed and display similar expression patterns (Fig. 3 columns 4 and 5; Fig. 5 rows E-G). These genes are distributed in the medial hypothalamus (Hv), the lateral hypothalamic nucleus (LH), the dorsal zone of the periventricular hypothalamus surrounding the lateral recess (LR), the periventricular nucleus of the posterior tuberculum (TPp), the anterior preglomerular nucleus (PGa) and in the periventricular grey zone of the optic tectum (TeO) (Fig. 3; Fig. 4 C3-C4 to L3-L4; Fig. 5 F-H). In the mediobasal hypothalamus, wide and similar expression patterns are observed in the Hv, the LH and the dorsal zone of the periventricular hypothalamus (Hd) (Fig. 4 C4-L4). Steroidogenic enzymes and *fdx1* gene distribution at this level highlights expression along the

diencephalic ventricle and around the lateral and posterior recess (LR and PR) (Fig. 3 columns 4 and 5; Fig. 5 rows F-H). In addition, they are detected with different staining intensities in the corpus mamillare (CM), the valvula of the cerebellum (VCe) and the torus longitudinalis (TL). In the most posterior part of the brain, the examined genes are detected in the cerebellum (CCe), notably in the cells surrounding the rhombencephalic ventricle (Fig. 5 row I).
It is noteworthy that heterogeneity in staining intensities was observed between several brain tissues hybridized with the same probes within an experiment. Thus, even though spatial expression is the same, some brains seem to be labeled faster by the very same probe than others. As we have used only male fish with similar age for our study, the heterogeneity observed cannot be attributed to age or sex differences. It remains elusive whether other inter-individual differences in, for example, the reproductive/hormonal status might explain the observed differences in expression levels of steroidogenic enzymes in the brains between fish.

Steroidogenic capacity of the adult zebrafish brain
We next investigated the question whether the expressed genes of glucocorticoid biosynthesis in the adult zebrafish brain are capable of facilitating *de novo* glucocorticoid biosynthesis. Therefore, we first carried out ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) analysis to determine the basal concentrations of steroid hormones in the adult zebrafish brain. Cortisol, the main glucocorticoid in zebrafish, was detected at a concentration of 0.09 ± 0.02 ng/mg dry mass (Fig. 6 A). We were unable to detect any precursors of cortisol, which is likely due to a number of factors including, limited sample material, the low ionization efficiency of many of the upstream steroids such as pregnenolone and the complex matrix. It is also possible that the precursors of cortisol do not accumulate in the zebrafish brain.
Nonetheless, after the observation that cortisol is present in the adult zebrafish brain, we next analyzed whether glucocorticoids can be *de novo* synthesized locally in the adult zebrafish brain. Thus, we treated zebrafish brains with [^{3}H]-pregnenolone for 1, 2, 3 or 4 h and measured the biosynthesis of [^{3}H]-cortisol and also the neurosteroid [^{3}H]-THDOC after treatment using reversed-phase high-performance liquid chromatography (RP-HPLC). Our results clearly demonstrated *de novo* synthesis of [^{3}H]-cortisol and [^{3}H]-THDOC in the adult zebrafish brain with a peak in biosynthesis after 2 h of [^{3}H]-pregnenolone treatment (Fig. 6 B, C). The synthesis of other precursors and neurosteroids including 17OH-pregnenolone, dehydroepiandrosterone, androstenedione, testosterone, dihydro-testosterone, estrone, estradiol, progesterone, and dihydro- and tetrahydro-progesterone has been described previously (DIOTEL REF). Taken together, the zebrafish brain has the capacity for *de novo* synthesis of glucocorticoids and neurosteroids relying on enzymes involved in this pathway. This suggests that the steroidogenic enzymes of glucocorticoid biosynthesis in the adult zebrafish brain are part of functional pathways of *de novo* steroid hormone and neurosteroid synthesis in the adult brain of zebrafish.

Discussion

Expression studies during zebrafish development
Here, we report a comprehensive study analyzing the expression profiles of the steroidogenic enzymes involved in glucocorticoid biosynthesis as well as the mitochondrial electron-

providing co-factors ferredoxin (*fdx1*, *fdx1b*) in zebrafish. Our results provide clear evidence that steroidogenic genes of glucocorticoid biosynthesis and the *fdx* co-factors are expressed during the first days of zebrafish development. In fact, transcript levels of all genes are already detected within the first 24 h of development, suggesting an early developmental function of these genes in the zebrafish embryo. Indeed, *cyp11a1*-mediated pregnenolone biosynthesis is required to enable the first embryonic cell movement in the embryo, namely epiboly, by stabilizing yolk microtubules (37, 41). The current literature suggests important functions of cortisol, including craniofacial development (42), mesoderm formation and muscle development (43). Furthermore, during later embryonic stages, cortisol biosynthesis has been suggested to play an important role in hatching and swimming activity (44), stress-response (45) and even in larval food consumption (46).

The zebrafish interrenal gland is the equivalent organ of the mammalian adrenal gland (23, 24). In zebrafish, it produces cortisol starting at 48 hpf (25, 47). However, endogenous release of cortisol in response to various stressors leading to downstream gene transcription occurs later during embryonic development, between 96 and 120 hpf (25, 26, 48). The *cyp11c1* transcript can be identified in all stages examined with a peak after the long-pec (48 hpf) stage and is also expressed in the interrenal gland. *cyp11c1* is known to catalyze the final step of glucocorticoid biosynthesis from 11-deoxycortisol to cortisol in zebrafish (44). Transcript levels of *cyp11a2*, *hsd3b1* and *fdx1b* compared to their respective paralogs is high at larval stages including 120 hpf, which is also the case for *cyp21a2*. Importantly, *cyp11a2*, *hsd3b1*, *cyp21a2* and *fdx1b* are expressed in the larval interrenal gland. Overall, our data suggest complementary temporal expression of the duplicated steroidogenic genes of glucocorticoid biosynthesis and the *fdx* co-factors. Thus, it is tempting to speculate that only the later highly expressed genes, which are also expressed in the larval interrenal gland, have similar function as in mammals in glucocorticoid biosynthesis during larval stages. Indeed, recent studies have demonstrated that *cyp11a2*, *fdx1b* and *hsd3b1*, but not their corresponding paralogs, are involved in cortisol synthesis in zebrafish (33-35). In addition, we have recently shown a key role for *cyp21a2* in glucocorticoid biosynthesis in zebrafish (49).

cyp17a1 and *cyp17a2* transcripts were found in all analyzed stages, but overall *cyp17a1* expression levels are higher compared to *cyp17a2* expression levels at the stages examined. *cyp17a2* transcript patterns, however, behave similar as the other genes, which are specifically expressed in the larval interrenal gland (with an increase in transcript levels again at larval stages including 120 hpf). Furthermore, *cyp17a2* is the only *cyp17* gene that can be detected in the interrenal gland at 120 hpf, whereas *cyp17a1* cannot be visualized in any specific larval organ, suggesting a less specific distribution of this transcript in larvae. Importantly, both zebrafish *cyp17* genes have 17α-hydroxylation reaction function, which is required for the biosynthesis of glucocorticoids (36). However, in fish *cyp17a2* promotes 17α-hydroxylation of progesterone and pregnenolone more efficiently, whereas *cyp17a1* in addition possesses 17,20-lyase activity, required for the biosynthesis of androgens (36). Therefore, it is highly likely that *cyp17a2* is involved in the biosynthesis of glucocorticoids during larval stages, whereas *cyp17a1* might be important for steroidogenesis later during development, when the gonads are developed (50-52) and sex steroid synthesis in fish is also required. Supportive for this assumption are the observations that androgen precursor androstenedione cannot be detected in

120 hpf zebrafish larvae (49) and both *cyp17* genes can be detected in the kidney head of adult zebrafish with much higher expression levels for *cyp17a1* (28 fpkm) than for *cyp17a2* (0.3 fpkm) (ArrayExpress database, http://www.ebi.ac.uk/arrayexpress, accession number E-MTAB-460).

Expression studies in the brain of adult zebrafish

Little is known about the expression of steroidogenic enzymes and the *fdx* co-factors in adult zebrafish brain. Previous studies have analyzed the spatial expression and activity of three steroidogenic enzymes (Cyp11a, Hsd3b and Cyp17) in the zebrafish adult brain (8, 32). However, a further depth study analyzing the expression of the whole set of the steroidogenic genes of glucocorticoid biosynthesis and the *fdx* co-factors in the different brain regions did not exist. Such data will allow to further analyze the potential role of steroidogenic enzymes involved in the biosynthesis of glucocorticoids and their derivates in respective brain regions. Moreover, in contrast to the previous studies our present work distinguishes between the zebrafish paralogs (*cyp11a1* vs. *cyp11a2*, *hsd3b1* vs. *hsd3b2*, *cyp17a1* vs. *cyp17a2*) and provides additional data by analyzing the expression of other important steroidogenic genes of glucocorticoid biosynthesis (*cyp11c1*, *cyp21a2*) and the ferredoxin (*fdx1*, *fdx1b*) co-factors.

Here, we show that the steroidogenic genes of glucocorticoid biosynthesis and the *fdx1* gene are widely expressed in the adult brain, namely in the telencephalon, diencephalon and rhombencephalon. Interestingly, the expression patterns of all studied enzymes clearly overlap, suggesting the existence of true steroidogenic regions in the adult brain. In humans, expression of the steroidogenic enzymes *CYP11A1, CYP17A1, HSD3B* and *CYP21A2* has been reported in several regions of the human brain including the amygdala, caudate nucleus, corpus callosum, hippocampus, cerebellum and thalamus (53). *CYP11B1* was detected in the thalamus (53) and *CYP11A1*, *HSD3B2* and *CYP21A2* in the hippocampus and cerebellum (54). Similarly, in rodents *Cyp17a1* and *Hsd3b* expression were described in various brain regions including the cortex, the hypothalamus, the thalamus and the cerebellum (55, 56). Thus, there seem to be some conserved distribution in regions such as the hypothalamus, the thalamus and the cerebellum of steroidogenic gene expression between fish and mammals. Previous semi-quantitative based PCR expression studies using whole adult zebrafish brain tissue have reported that only *cyp11a2*, and not *cyp11a1*, is expressed in the whole zebrafish brain (33). We detected expression of both *cyp11a1* and *cyp11a2* in the adult brain with two independent techniques (quantitative RT-PCR and whole-mount *in situ* hybridization). This discrepancy might be explained by the overall higher sensitivity of our used techniques compared to semi-quantitative PCR.

Neurosteroids are known to be important for brain development and homeostasis (6), neuroprotection (7) and neurogenesis (8, 9). They can also act as allosteric modulators of neurotransmitter receptors such as GABA-A (57-59). We showed that cortisol is present in the adult zebrafish brain and that cortisol and THDOC can be biosynthesized *de novo* from radiolabeled pregnenolone. The production of THDOC as the previous report of de novo synthesis of dihydrotestosterone (REF) points towards enzymatic 5 alpha reductase activity in the adult zebrafish brain. Interestingly, previous reports suggest a role of 11-hydroxysteroid dehydrogenase type 2 in the regulation of the response to stress in the brain, whereas the

expression of the cortisol inactivating 20-beta hydroxysteroid dehydrogenase has not been analysed yet. THDOC is a well-described potent positive allosteric modulator of GABA-A receptors (60-62). Furthermore, the distribution of GABAergic neurons in the brain overlaps with the expression of our examined steroidogenic genes and *fdx1* co-factors. For example, the GABA-synthesizing enzyme *gad67* is widely expressed in the zebrafish telencephalon (subpallium and pallium), the preoptic area, the thalamus, the hypothalamus and the optic tectum (63-65), while *gad65* is expressed in the cerebellum (66). In addition, *dlx5*, a gene that regulates the production of GABA-synthesizing enzymes (67) has been shown to be expressed in the subpallium of adult zebrafish (68). There is a clear overlap in expression of the steroidogenic enzymes of glucocorticoid biosynthesis and *fdx* co-factors with the distribution of GABA-synthesizing enzymes in the adult zebrafish brain. Thus, we hypothesize that neurosteroids in zebrafish might modulate the activity of neurotransmitters system as it is the case in mammals. Future functional studies will be required to elucidate the role of the steroidogenic genes and *fdx* co-factors in neurobiology of zebrafish.

The spatial distribution of the steroidogenic genes involved in glucocorticoid biosynthesis and the *fdx* co-factors suggest expression of these steroids in neurons. In particular their expression along the ventricular and periventricular layers, known to correspond to neurogenic regions, indicates a potential expression of these genes in in neural stem cells and/or neural progenitors, as it was previously described for the sex steroid synthesizing enzyme aromatase B (*cyp19a1b*) and for other steroidogenic enzymes (8, 69-72). This is in line with the idea that radial glia cells in fish have a wide steroidogenic capacity (72, 73). The distribution of steroidogenic genes of glucocorticoid biosynthesis and the *fdx1* gene along the ventricular/periventricular layer suggests a role for locally-synthesized steroids in the regulation of adult neurogenesis and the cerebral homeostasis. Indeed, locally synthesized steroids and/or peripheral steroids can modulate neural progenitor activity, as it was previously demonstrated for *cyp19a1b* and estrogens (74, 75). It will be interesting to determine if zebrafish neurosteroids can modulate synaptic plasticity and display neuroprotective effects, or they can play regenerative roles in stroke and neurodegenerative disease as it is the case in mammals (76-79). In zebrafish, it has previously been shown that the synthetic glucocorticoid dexamethasone decreases injury-induced proliferation of radial glial cells after stab wounding of the telencephalon, but it does not have any effect on constitutive neurogenesis at the proliferative step (80). However, to the best of our knowledge no data are available about a potential effect of dexamethasone on migration, differentiation and cell survival of newborn neurons. This observation, however, might also be explained by drug specific properties of dexamethasone or by the fact that other neurosteroids are involved in such processes.

Although the analyzed genes are essential for *de novo* glucocorticoid biosynthesis in other tissues, it has to be emphasized that their catalytic function might not necessarily be restricted to *de novo* glucocorticoid biosynthesis in the adult brain. Despite the 21-hydroxylase activity in human adult brain (81), human CYP21A2 is only expressed at low levels (82), thus leading to the idea that enzymes other than CYP21A2 might confer 21-hydroxylase activity in the adult brain. Indeed, a study in rat brain microsomes has suggested that the isoform CYP2D4 acts as a steroid 21-hydoxylase (83). Thus, it remains to be clarified if the expression of steroidogenic

enzymes in the brain are relevant to de novo centralnervous glucocorticoids synthesis or if they are involved in other pathways in the adult brain.

Taken together, our study lays a strong foundation for understanding the spatio-temporal distribution of the steroidogenic enzymes of glucocorticoid biosynthesis and the *fdx1* co-factors during development and in the adult zebrafish brain. Notably, it reports expression of the examined genes in areas known for adult neurogenesis and in GABAergic transmission, suggesting a potential role for those genes in neurogenesis and GABA signaling. Furthermore, it shows that the zebrafish brain is capable of *de novo* synthesis of glucocorticoids or other neurosteroids from pregnenolone. Future studies will help to increase the understanding of the role of the examined genes in those brain regions, especially for adult neurogenesis and brain homeostasis.

Materials and Methods

Zebrafish husbandry

Adult zebrafish (AB wild-type strain) were raised and bred according to standard methods with a 14-hour light/10-hour dark photoperiod and a temperature of 28.5° C in recirculation systems. Embryos were obtained by natural spawning and incubated at 28.5 °C in 1x E3 medium (5 mmol/L NaCl, 0.17 mmol/L KCl, 0.33 mmol/L CaCl2, 0.33 mmol/L MgSO4, 0.1% methylene blue). The developmental stages were determined according to hours post-fertilisation (hpf) as previously described (84). All procedures were approved by the Home Office, United Kingdom and carried out line with the Animals (Scientific Procedures) Act 1986 and were conducted in accordance with the regulations of the Nestlé ethical committee and the veterinary office of the Canton of Vaud (VD 3177).

Phylogenetic analysis

The protein sequences of the examined genes (Table S1) were identified from a literature search and through ENSEMBL v84 (GRCz10). The phylogenetic analysis was carried out with the corresponding protein sequences of the examined genes in the human (*Homo sapiens*, hs), mouse (*Mus musculus*, mm) and zebrafish (*Danio rerio*, dr) on the Phylogeny.fr platform (http://www.phylogeny.fr/) as previously reported (85), using the standard settings of the "advanced" phylogeny analysis option.

Transcriptome studies

Raw count data from the RNA-Seq/DeTCT based study to assess gene expression during zebrafish development (39) was retrieved from expression atlas (ebi.ac.uk/gxa/; accession number: E-ERAD-475). Using DESeq2 (86) raw count data was normalized by size factor and fpkm (fragments per kilobase per million) was calculated for each gene. The mean of each developmental stage represents 20 biological replicates of 8-12 embryos/larvae.

Quantitative Real-time PCR

Brain tissue (one brain per sample, n= 6) were sampled in liquid nitrogen and then homogenized with micropestles (Eppendorf, #Z317314-1PAK) in 1 ml Trizol reagent (LifeTechnologies, #15596-026) before being stored overnight at -80°C. Samples were passed several times

through a syringe (BD Microlance, 25 G 1 0.5 x 25 mm, #3086982) and RNA extraction was carried out according to the manufacturer's protocol. cDNA synthesis was carried out with 1 µg RNA using the SuperScript VILO cDNA Synthesis Kit (LifeTechnologies, #11754-050). Analysis of the genes of interest was carried out with SYBRGreen PCR Master Mix (Applied Biosystems, #4309155) in a total volume of 10 µl, with 150 nM primer (Table S2) and 1 µL of cDNA (1:5 dilution), using an ABI 7500 sequence detection system (Applied Biosystems). *β-actin* was used as a normalization control (Table S2).

Fixation of embryos and adult brain organs
Larvae (120 hpf) were fixed overnight at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4). The next day, they were stepwise dehydrated in a methanol/PBS concentration series before being stored at -20°C in 100% methanol until use.
Male fish (6 to 12 months of age) were first anesthetized in 0.02% tricaine methanesulfonate (MS-222, Sigma, pH7) before being sacrificed. Heads were cut, washed in 1x PBS and then fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) overnight at 4°C. The following day, heads were transferred in petri dishes with 1x PBS and brains were dissected. The dissected brains were then stepwise dehydrated in a methanol/PBS concentration series and stored at -20°C in 100% methanol until use (87).

Generation of probes and whole-mount in situ hybridization
The templates for DIG-labeled RNA probe synthesis were linearized plasmids. The whole-mount *in situ* templates were cloned by performing a standard PCR using a cDNA mixture of embryonic (24 hpf) and larval cDNA (120 hpf) and gene-specific primers (listed in Table S3). The resulting PCR products were subcloned into the pGEMT-Easy vector (Promega) as described by the manufacturer. For DIG-labeled RNA synthesis, the templates were linearized with the respective digestion enzymes to obtain sense and anti-sense direction. Digoxigenin (Roche, #11277073910) labelled probe synthesis and *in situ* hybridization as a spatial analysis on 120 hpf larvae (n≥ 10) and whole adult brain vibratome slices (n=3 brains; 50 µm thickness) were performed as previously described (87). While staining time of the respective sense and anti-sense probes for a gene of interest was identical, staining time between the analysed genes varied to allow the best possible staining of each gene. Sense probes are not considered to be a proper control for all genes as they can result in staining due to antisense transcription at the targeted genomic locus (88, 89). In the case of that the corresponding sense probe was leading to a staining, an alternative non-overlapping anti-sense probe was used for the same gene to verify the specificity of the staining.
For bright field microscopy, pictures were acquired using a Leica compound microscope (DFC29D). Pictures were adjusted for brightness and contrast in Adobe Photoshop CS5.

Steroid measurements
Steroid measurements in adult zebrafish were carried out as follows: Zebrafish brains (≥9 adult brains per sample) were freshly dissected washed with PBS, lyophilized and extracted as follows: Approximately 15-20 mg of brain tissue was transferred into a 2 ml microcentrifuge tube containing a single stainless steel metal bead (5 mm). 950 µl LC-MS grade water and 50

µl of a solution containing a mix of deuterated steroids in methanol were added to each tube prior to homogenized using a Bead Ruptor (Omni International, GA, USA) for 90 s. Samples were subsequently centrifuged (8000xg) at 4 °C for 5 min. The supernatant was transferred to a clean glass tube and the steroids extracted with 3 ml Methyl tert-butyl ether (MTBE). The upper organic phase containing the extracted steroids was then transferred into clean glass tube. The extraction step was repeated using an additional 3 ml MTBE and the organic phase transferred to the corresponding glass tube. An additional 1 ml of water was also added to the original sample and the homogenization, centrifugation and extraction steps were repeated. The pooled solvent from the three extractions were subsequently evaporated under a stream of nitrogen and the dried steroids were then resuspended in 1 ml methanol. The suspension was subsequently eluted through a solid phase extraction phospholipid removal column (Phree, Phenomenex, CA, USA). An additional 1 ml of methanol was used to rinse the glass tube and was then eluted through the corresponding solid phase extraction phospholipid removal column. The methanol was subsequently evaporated under a stream of nitrogen and the dried steroids were then resuspended in 150 µl 50% methanol prior to analysis. Steroids were separated and quantified using an ACQUITY UPLC system (Waters, Milford, USA) coupled to Xevo TQ-S tandem mass spectrometer (Waters, Milford, USA). Chromatographic separation was achieved using a UPLC high strength silica (HSS) T3 column (2.1 mm x 50 mm, 1.8 µm) (Waters, Milford, USA) as previously described (90).
The analysis of *de novo* synthesis of cortisol and THDOC in zebrafish brain was carried out as previously described (8). Briefly, brains of 12 months old zebrafish (14 brains/sample) were freshly dissected and incubated with [^{3}H]-pregnenolone for up to 4 h and newly synthesized tritiated metabolites were analysed by RP-HPLC.

Conflict of interest

BDW and PG are employees of Nestlé Institute of Health Sciences SA.

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Figure legends

Figure 1. Glucocorticoid biosynthesis in human and zebrafish
(A) Schematic illustrates the different steps of glucocorticoid biosynthesis and its catalyzing cytochrome P450 (CYP) and hydroxysteroid dehydrogenase (HSD) enzymes (dark grey) in the human. Asterisks indicate the steps dependent on Ferredoxin (FDX1) mediated electron transfer. (B-D) Phylogenetic analysis of protein sequences of the enzymes involved in glucocorticoid biosynthesis in the human (*Homo sapiens*, hs), mouse (*Mus musculus*, mm) and zebrafish (*Danio rerio*, dr). (B) Phylogenetic tree of *cyp* genes. (C) Phylogenetic tree of *hsd* genes. (D) Phylogenetic tree of *fdx1* genes. (E-N) Transcript levels of *cyp11a1* (E), *cyp11a2* (F), *cyp17a1* (G), *cyp17a2* (H), *hsd3b1* (I), *hsd3b2* (J), *cyp21a2* (K), *cyp11c1* (L), and the ferredoxin genes *fdx1* (M) and *fdx1b* (N) in whole embryos/larvae during zebrafish development between zygote stage (1-cell) to 120 hours post fertilization (hpf).

Figure 2. Spatial expression of the main glucocorticoid biosynthesis genes and the *fdx* co-factors in zebrafish larvae
(A1-J2) Spatial expression of *cyp* (*cyp11a1*, *cyp11a2, cyp11c1, cyp17a1, cyp17a2, cyp21a2), hsd3 (hsd3b1*, *hsd3b2)* and ferredoxin genes (*fdx1* and *fdx1b)* in 120 hpf larvae from lateral view. Arrowhead indicates the interrenal gland. Specific staining of the interrenal gland is detected for *cyp11a2*, *cyp11c1*, *cyp17a2*, *cyp21a2*, *hsd3b1*and *fdx1b*. Expression of *fdx1* is detected in the gut and the liver. No specific staining is observed for the *cyp11a1*, *cyp17a1* and *hsd3b2* probes. Corresponding sense probes were used to verify the specificity of the staining. Scale-Bar: 0.5 mm.

Figure 3. Gene expression analysis of glucocorticoid biosynthesis and *fdx* genes in the adult zebrafish brain
(A1-A5) Schematic indication of anatomical subdomains at the levels of the sections in (B-K) [modified from (91); a higher magnification of the different brain regions and an overview about the nomenclature can be found in Fig. S3]. Indicated in red are zones of proliferation revealed by PCNA (proliferative cell nuclear antigen) and/or BrdU (24 h exposure followed by sacrifice) labelling (70, 92). (B1-K5) *In situ* hybridizations with anti-sense probes for the glucocorticoid biosynthesis genes (*cyp11a1*, *cyp11a2, cyp11c1, cyp17a1, cyp17a2, cyp21a2, hsd3b1*, *hsd3b2*) and for the *fdx* co-factors (*fdx1, fdx1b*) in representative transverse sections through the olfactory bulb/telencephalon junction (first column), the telencephalon (second column), the posterior part of the preoptic area (third column), the mediobasal (fourth column) and the caudal (last column) hypothalamus. Transcripts of the examined steroidogenic enzymes and the *fdx1* co-factors are widely expressed in the brain, notably in the telencephalon, the hypothalamus, and the tectum, and display overlapping distribution. Scale bar: A1-K1 (350 µm), A2-K2 (300 µm), A3-K3 (260 µm), A4-K4 (360 µm), A5-K5 (380 µm).

Figure 4. Higher magnification images of glucocorticoid biosynthesis and *fdx1* genes in the adult zebrafish brain

(A, B) Schematic indication of anatomical subdomains at the levels of the sections in (C-L) [modified from (91)]. (C-L) High magnification views of the respective numerated boxes in A and B showing the dorsomedian telencephalon (Dm in 1), the ventral nucleus of the ventral telencephalic area (Vv in 2), the periglomerular grey zone of the optic tectum (TeO in 3), and the mediobasal hypothalamus, where the lateral recess opens (Hv LR in 4). A higher magnification of the different brain regions and an overview about the nomenclature can be found in Fig. S3. Scale Bar: A (180 µm), B (320 µm), C - L (150 µm).

Figure 5. Schematic distribution of glucocorticoid biosynthesis and *fdx* genes in the adult zebrafish brain

Mapping of steroidogenic enzymes and *fdx* mRNA-expressing cells in representative transversal brain sections taken from the zebrafish brain atlas [adapted from (91)] through the junction between the olfactory bulbs and the dorsal telencephalon (A), the telencephalon (B), the anterior (C) and the posterior (D) parts of the preoptic area, the anterior (E) and mediobasal (F) hypothalamus, the posterior and lateral recess of the hypothalamus (G and H), and the cerebellum (I). A higher magnification of the different brain regions and an overview about the nomenclature can be found in Fig. S3. The steroidogenic enzymes as well as *fdx* co-factors are widely expressed in the brain, notably in the telencephalon, diencephalon and rhombencephalon, and they exhibit overlapping distributions as clearly evidenced by the schematic distribution. The first column shows the distribution of aromatase B-positive radial glial cells [adapted from Menuet et al. (69)], acting as neural stem cells, and highlights the overlap with other steroidogenic enzymes and *fdx1* along the ventricular layer (69-71). *Cyp19a1b* gene expression has been reported by (69). Note that the decreased size of the red dots around the LR and PR is only for readability and does not reflect a lower expression of the corresponding transcripts. Scale Bar: A (350 µm), B (420 µm), C (460 µm), D-H (600 µm).

Figure 6. *De novo* synthesis of cortisol and THDOC in the adult zebrafish brain

(A) Steroid measurements in adult zebrafish brain tissue with UHPLC-MS/MS. Chromatogram of cortisol. Cortisol was detected in the adult zebrafish brain at a concentration of 0.09 ± 0.02 ng/mg dry mass. The other examined steroids [i.e., pregnenolone, progesterone, 17-hydroxypregnenolone, 17-hydroxyprogesterone, 11-deoxycortisol, 11-deoxycorticosterone, corticosterone, cortisone, tetrahydrodeoxycorticosterone (THDOC)] were below the detection limit (n.d., not detected). (B, C) Kinetics of conversion of [^{3}H]-pregnenolone into tritiated cortisol (B) and THDOC (C) by zebrafish brains using RP-HPLC. Incubation of brains with [^{3}H]-pregnenolone were carried out for 1 h, 2 h, 3 h and 4 h.

Figure S1. Whole-mount *in* situ hybridization staining in zebrafish larvae

Anti-sense and sense probes for *cyp* (*cyp11a1, cyp11a2, cyp11c1, cyp17a1, cyp17a2, cyp21a2*), *hsd3b* (*hsd3b1, hsd3b2*) and *ferredoxin* genes (*fdx1 and fdx1b*) at 120 hpf larvae from dorsal view.

Figure S2: Expression of glucocorticoid biosynthesis and *fdx* genes in whole adult brain tissue

Determination of transcript levels of *cyp11a1, cyp11a2, cyp11c1, cyp17a1, cyp17a2, cyp21a2*), *hsd3b* (*hsd3b1, hsd3b2*) and *ferredoxin* genes (*fdx1 and fdx1b*) by quantitative RT-PCR in whole adult zebrafish brain tissue (n=6).

Figure S3. High resolution schematics of zebrafish sections with nomenclature
(A-I) Schematic indication of anatomical subdomains at the levels of the sections [modified from (91)]. Indicated in red are zones of proliferation revealed by PCNA (proliferative cell nuclear antigen) and/or BrdU (24 h exposure followed by sacrifice) labeling (70, 92). Transversal brains section through the junction between the telencephalon and the olfactory bulbs (A), the telencephalon (B), the anterior part of the preoptic area (C), the posterior part of the pre-optic area (D), the anterior part of the hypothalamus (E), the mediobasal hypothalamus (F), the caudal hypothalamus (G and H) and through medulla oblongata (I). Abbreviations: A, anterior thalamic nucleus; APN, accessory pretectal nucleus; ATN, anterior tuberal nucleus; CCe, corpus cerebelli; Chab, habenular commissure; Chor, horizontal commissure; CM, corpus mamillare; CP, central posterior thalamic nucleus; CPN, central pretectal nucleus; Cpop, postoptic commissure; Cpost, posterior commissure; D, dorsal telencephalic area; Dc, central zone of dorsal telencephalic area; Dl, lateral zone of dorsal telencephalic area; Dm, medial zone of dorsal telencephalic area; DOT, dorsomedial optic tract; Dp, posterior zone of dorsal telencephalic area; DP, dorsal posterior thalamic nucleus; DTN: dorsal tegmental nucleus; ECL, external cellular layer of olfactory bulb; EG, eminentia granularis; ENv, entopendoncular nucleus, ventral part; FR, fasciculus retroflexus; GL, glomerular layer of olfactory bulb; Had, dorsal habenular nucleus; Hav, ventral habenular nucleus; Hc, caudal zone of periventricular hypothalamus; Hd, dorsal zone of periventricular hypothalamus; Hv, ventral zone of periventricular hypothalamus; ICL, internal cellular layer of olfactory bulb; IL, inferior lobe; LH, lateral hypothalamic nucleus; LLF: lateral longitudinal fascicle; LR, lateral recess of diencephalic nucleus; MLF, medial longitudinal fascicle; NIII, oculomotor nucleus; NLV, nucleus lateralis valvulae; NMLF, nucleus of medial longitudinal fascicle; PG, preglomerular nucleus; PGa, anterior preglomerular nucleus; PGl, lateral preglomerular nucleus; Pit, pituitary; PO, posterior pretectal nucleus; PP, periventricular pretectal nucleus; PPa, parvocellular preoptic nucleus, anterior part; PPp, parvocellular preoptic nucleus, posterior part; PR, posterior recess of diencephalic ventricle; PSp, parvocellular superficial pretectal nucleus; PTN, posterior tuberal nucleus; R, rostrolateral nucleus; RF, reticular formation; SC, suprachiasmatic nucleus; SD, saccus dorsalis; SO, secondary octaval population; TeO, tectum opticum; TL, torus longitudinalis; TLa, torus lateralis; TPp, periventricular nucleus of posterior tuberculum; TS, torus semicircularis; V3, third ventricle; VII, sensory root of the facial nerve; VIII, octaval nerve; VCe, valvula cerebelli; Vd, dorsal nucleus of ventral telencephalic area; VL, ventrolateral thalamic nucleus; VM, ventromedial thalamic nucleus; VOT, ventrolateral optic tract; Vp, postcommissural nucleus of ventral telencephalic area; Vv, ventral nucleus of dorsal telencephalic area; ZL, zona limitans. Scale Bar: A (350 µm), B (150 µm), C-D (300 µm), E-H (350 µm), I (600 µm).

Figure S4. Specificity of whole-mount *in situ* probes in adult zebrafish brain

(A, B) Whole-mount *in situ* hybridization of adult zebrafish brain demonstrate the specificity of the used probes. (A) Anti-sense probes for *cyp11a1*, *cyp11c1*, *cyp17a2*, *cyp21a2*, *hsd3b1*, *hsd3b2*, *fdx1* and *fdx1b* show a clear staining of different regions and cerebral nuclei of the adult zebrafish brain, as shown here as an example for the dorsomedial telencephalon (Dm) and the dorsal nuclei of the ventral telencephalon (Vd). In contrast, their corresponding sense probes do not lead to any staining. (B) In case of *cyp11a2* and *cyp17a1* specificity was verified with a second non-overlapping anti-sense probes. Both probes show similar expression patterns. Abbreviations: Vv, ventral nucleus of the ventral telencephalic area; Vc, central nucleus of the ventral telencephalic area. Scale bar: A (85 µm), B (200 µm).

Table S1. Protein sequences

Table S2. Quantitative RT-PCR primers

Table S3. Whole-mount *in situ* probe primers

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