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The P450 side-chain cleavage enzyme Cyp11a2 facilitates steroidogenesis in zebrafish

Nan Li^{1,2}, James A Oakes^{1,2}, Karl-Heinz Storbeck³, Vincent T Cunliffe^{2,4,*}, and Nils P Krone^{1,2,5,*}

¹Department of Oncology & Metabolism, School of Medicine, University of Sheffield, Sheffield, UK

²The Bateson Centre, Firth Court, Western Bank, Sheffield, UK

³Department of Biochemistry, Stellenbosch University, Stellenbosch, Matieland, South Africa

⁴Department of Biomedical Science, Firth Court, Western Bank, Sheffield, UK

⁵Department of Medicine III, University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany

Correspondence should be addressed to N P Krone: n.krone@sheffield.ac.uk

*(V T Cunliffe and N P Krone contributed equally to this work)

Abstract

Cytochrome P450 side-chain cleavage enzyme, encoded by the *CYP11A1* gene, catalyzes the first and rate-limiting step of steroid hormone biosynthesis. Previous morpholino-knockdown studies in zebrafish suggested *cyp11a2* is a functional equivalent of human *CYP11A1* and is essential for interrenal steroidogenesis in zebrafish larvae. The role of Cyp11a2 in adult zebrafish, particularly in gonadal steroidogenesis, remains elusive. To explore the role of Cyp11a2 in adults, we developed zebrafish mutant lines by creating deletions in *cyp11a2* using the CRISPR/Cas9 genomic engineering approach. Homozygous *cyp11a2* mutant zebrafish larvae showed an upregulation of the hypothalamic–pituitary–interrenal axis. Furthermore, these Cyp11a2-deficient zebrafish demonstrated profound glucocorticoid and androgen deficiencies. *Cyp11a2* homozygotes only developed into males with feminized secondary sex characteristics. Adult *cyp11a2*^{−/−} mutant fish showed a lack of natural breeding behaviors. Histological characterization revealed disorganized testicular structure and significantly decreased numbers of mature spermatozoa. These findings are further supported by the downregulation of the expression of several pro-male genes in the testes of *cyp11a2* homozygous zebrafish, including *sox9a*, *dmrt1* and *amh*. Moreover, the spermatogonia markers *nanos2* and *piwil1* were upregulated, while the spermatocytes marker *sycp3* and spermatids marker *odf3b* were downregulated in the testes of *cyp11a2* homozygous mutants. Our expression analysis is consistent with our histological studies, suggesting that spermatogonia are the predominant cell types in the testes of *cyp11a2* homozygous mutants. Our work thus demonstrates the crucial role of Cyp11a2 in interrenal and gonadal steroidogenesis in zebrafish larvae and adults.

Key Words

- Cyp11a2
- glucocorticoid
- androgen
- zebrafish
- CRISPR/Cas9

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Introduction

Zebrafish has been established as vertebrate model to elucidate gene function in development and disease. Despite their evolutionary distances, zebrafish share extensive conservation with humans at both genomic

and functional levels. In particular, molecular pathways controlling steroidogenesis and steroid signaling are highly conserved (Lohr & Hammerschmidt 2011, Tokarz *et al.* 2013, 2015). Similar to humans, cortisol is

the main glucocorticoid in zebrafish and is produced in the interrenal (equivalent of the mammalian adrenal gland) (Dickmeis 2009). The production of cortisol is under the control of hypothalamus and pituitary, forming the hypothalamus-pituitary-interrenal (HPI) axis (Liu 2007, To *et al.* 2007). In addition to the interrenal, steroidogenesis in zebrafish also occurs in other tissues such as gonads and brain (Tokarz *et al.* 2013).

The cytochrome P450 side-chain cleavage enzyme, encoded by the *CYP11A1* gene, catalyzes the first and rate-limiting step of steroidogenesis and is a prerequisite for all steroid hormone biosynthesis (Miller & Auchus 2011). Previous studies identified two *cyp11a* genes in zebrafish, *cyp11a1* and *cyp11a2* (Goldstone *et al.* 2010, Parajes *et al.* 2013). Our previous work assumed a paralog-specific role for each *cyp11a* gene in zebrafish larvae (Parajes *et al.* 2013). During early development, *cyp11a1* is expressed from zygote to segmentation periods (22–24 hpf, hours post fertilization), while the expression of *cyp11a2* becomes prominent just before the onset of *de novo* (48 hpf) and HPI-mediated (96 hpf) cortisol biosynthesis. Morpholino-mediated mRNA knockdown revealed that *cyp11a1* is required for early embryonic development, whereas its paralog *cyp11a2* represents the functional equivalent of human *CYP11A1* and is essential for interrenal steroidogenesis in larvae (Parajes *et al.* 2013).

However, loss-of-function studies using morpholinos only transiently reduce expression levels of target genes, and the physiological role of Cyp11a2 in adult zebrafish remains unclear. To define the function of Cyp11a2 in adult zebrafish, we developed zebrafish *cyp11a2*-null alleles using a CRISPR/Cas9 approach. Cyp11a2-deficient zebrafish had a phenotype resembling severe combined interrenal and gonadal insufficiency, including decreased expression of glucocorticoid target genes, altered steroid hormone profiles, reduced fertility, disorganized gonadal tissues and all-male fish with feminized secondary sex characteristics. Thus, we demonstrate that *cyp11a2* is the functional P450 side-chain cleavage enzyme paralog in zebrafish, facilitating the primary step of interrenal and gonadal steroidogenesis.

Materials and methods

Zebrafish maintenance

The zebrafish strains were maintained in a recirculating system (ZebTECTM, Techniplast®, Kettering, UK and Sheffield, UK) at 28.5°C in a 10-h dark, 14-h light

photoperiod. Embryos were obtained by natural spawning and incubated at 28.5°C in E3 medium (5 mmol/L NaCl, 0.17 mmol/L KCl, 0.33 mmol/L CaCl₂, 0.33 mmol/L MgSO₄) containing 0.1% (v/v) methylene blue. The developmental stages were determined according to hours post fertilization (hpf), and days post fertilization (dpf). All procedures were approved by the United Kingdom Home Office and carried out in line with the Animals (Scientific Procedures) Act 1986.

Generation of *cyp11a2* mutants by CRISPR/Cas9

Gene-specific guide RNA (gRNA) was designed against an optimal CRISPR site targeting exon 5 of *cyp11a2* (ENSDARG00000092696) following an adapted strategy described previously (Hruscha *et al.* 2013, Talbot & Amacher 2014). gRNA antisense DNA sequence was first amplified using 2.5 µL gRNA primer 1 (10 µM, 5'-GCGTAATACGACTCACTATAG-3'), 2.5 µL gRNA primer 2 (10 µM, 5'-AAAAAAGCACCGACTCGGTGCCAC-3'), 2 µL gRNA guide oligo (1 µM, oligonucleotide sequence is listed in Supplementary Table 1, see section on [supplementary materials](#) given at the end of this article), 20 µL 5× FIREPol® Master Mix (Solis Biodyne, Tartu, Estonia), and 73 µL MilliQ H₂O. The whole PCR product was loaded on a 2.5% (w/v) agarose gel and extracted using MinElute Gel Extraction Kit (Qiagen) following the manufacturer's protocol. The subsequent *in vitro* transcription was performed using MEGashortscript T7 kit (Life Technologies), using 1 µL as a template. Fertilized eggs were injected at one-cell stage with 1 nL of a solution containing 2 µM Cas9 protein (NEB, Hitchin, UK), 2.4 ng/nL gRNA and 0.1% (v/v) phenol red. Genomic DNA was extracted from injected individuals at 5 dpf to verify the presence of mutations and confirm the activity of the gRNA. Injected embryos were raised to adulthood (F0 generation). To confirm germline transmission of the mutation, a pool of 20 embryos collected from out-crosses between the F0 and wild-type were genotyped. F0 fish with successful germline transmission were kept as F0 founders. Embryos from out-crosses were raised to generate the F1 generation and genotyped for heterozygous mutations using fin clips. F1 heterozygotes were then out-crossed to generate the F2 heterozygotes which were in-crossed to characterize the *cyp11a2* mutations.

Genotyping *cyp11a2* mutants

Adult fish were anesthetized in tricaine methanesulfonate (MS222, Sigma-Aldrich) and one-third of caudal fin was

cut with a sharp blade. Genomic DNA was extracted from clipped caudal fin tissue or from whole larvae. Samples were lysed in 20 μ L of 50 mM NaOH and boiled for 10 min at 98°C and 1/10 volume of 100 mM Tris-HCl pH8.0 was added to buffer the reaction. PCR amplification of *cyp11a2* exon 5 was carried out in 20 μ L reaction with 1 μ L genomic DNA template, 1 μ L forward primer (10 μ M, 5'-TTTAAGACCACCTCGCCCAT-3') and reverse primer (10 μ M, 5'-GAGCCAGATTCAAACCAGCA-3') using FIREPol® Master Mix system (Solis Biodyne). The PCR product was analyzed on a 2.5% (w/v) agarose gel for any indel mutations.

Analysis of visual background adaptation (VBA)

To identify *cyp11a2*^{-/-} mutants for defective VBA behavior, larvae were analyzed at 96 hpf as described previously (Griffin *et al.* 2016). Briefly, larvae were maintained in a dark environment for at least 60 min, followed by exposure to bright illumination for 30 min. Larvae were then scored using a stringent criterion for lack of melanophore contraction, as an indicator of defective VBA. Only larvae with strongly dark pigmentation were designated as VBA-negative (VBA-) while only the lightest ones were designated as VBA-positive (VBA+) (Fig. 1C). Larvae with

ambiguous pigmentation level were discarded. The groups of larvae with either dark or light pigmentation were then genotyped to confirm the accuracy of VBA analysis.

Steroid hormones measurements by LC-MS/MS

Since over 90% VBA- larvae were confirmed to be *cyp11a2*^{-/-} mutants (Supplementary Table 2), we used this method to identify homozygous mutants and wild-type siblings for steroid hormone measurements in larvae. Three clutches of 150 VBA+ larvae and 150 VBA- larvae were collected at 120 hpf into a silanized tube and snap frozen on dry ice. 1 mL of 1× PBS was added to the samples, and the samples were lysed through four rounds of freeze-thawing. After lysis the samples were homogenized with a pestle homogenizer and freeze-dried. The whole bodies of adult wild-type siblings and *cyp11a2*^{-/-} mutants at 180 dpf (eight biological replicates each) were snap frozen on dry ice. The adult samples were homogenized using a Mikro-Dismembrator S (Sartorius, Gottingen, Germany) and then freeze-dried. Approximately 100 mg of the dried samples were transferred to a glass test tube and resuspended in 900 μ L MilliQ water and 100 μ L MilliQ water containing internal standard (15 ng D4-cortisol, D7-11 β -hydroxyandrostenedione, D7-androstenedione and 1.5 ng D2-testosterone). The steroids were extracted twice using 3 mL methyl tert-butyl ether (MTBE). Following centrifugation for 5 min at low speed the MTBE fractions for each sample were pooled and dried under a stream of nitrogen at 45°C. The dried residue was resuspended in 150 μ L 50% (v/v) methanol prior to analysis. Steroids were separated and quantified using an Acquity UPLC System (Waters, Milford, CT, USA) coupled to a Xevo TQ-S tandem mass spectrometer (Waters) as previously described (O'Reilly *et al.* 2017).

Figure 1

Generation of *cyp11a2* null-allele using CRISPR/Cas9 strategy. (A) Exon 5 of *cyp11a2* was targeted for genetic disruption by CRISPR/Cas9. *Cyp11a2*^{SH565} has a 161 bp deletion with the whole exon 5 deleted, resulting in a truncated protein of 282aa. *Cyp11a2*^{SH567} has a deletion of 144 bp leading to a truncated protein of 464aa. (B) Gel electrophoresis shows genotyping of wild-type, heterozygotes and homozygotes by PCR. (C) *cyp11a2*^{-/-} mutant larvae (120 dpf) had impaired visual-mediated background adaptation. Scale bar: 300 μ m.

Induction of osmotic stress

Three clutches of 25 VBA+ and VBA- larvae were collected at 120 hpf. Larvae were treated in 250 mM sodium chloride (in E3 medium) for 20 min. Subsequently, larvae were washed and incubated in E3 medium for 4 h before sampling for RNA extraction (Griffin *et al.* 2016).

Comparative gene expression analysis by quantitative real-time PCR (qRT-PCR)

Three clutches of 25 VBA+ and VBA- larvae were collected at 120 hpf. Gonad, pituitary and liver were collected from 3 to 5 male adult individuals. All the samples were

snap frozen on dry ice before processing. Total RNA from whole larvae or adult tissues and RT were performed as previously described (Griffin *et al.* 2016). Oligonucleotide sequences are listed in Supplementary Table 3. The qRT-PCR reaction was performed in 10 μ L volumes on 384-well plates using PowerUP SYBR Green Master Mix (Applied Biosystems) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Expression levels for each gene were normalized to reference gene β -actin, and fold changes were calculated relative to wild-type siblings. Expression analysis of *pomca*, *fshb*, *lhb*, *fshr*, *lhcg*, *gh1* was performed using TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays (Applied Biosystems). Expression level was then normalized to reference gene *ef1a*.

Whole-mount RNA *in situ* hybridization (WISH)

Whole-mount RNA *in situ* hybridization (WISH) was carried out following a standard protocol (Thisse & Thisse 2008). Briefly, 40 wild-type siblings and 40 *cyp11a2*^{-/-} mutant larvae at 5 dpf were fixed in 4% (w/v) paraformaldehyde (PFA) in 1 $\times$ PBS overnight and the next day were hybridized with *pomca* probe at 70°C overnight. Larvae were then incubated with anti-digoxigenin-AP antibody solution (1:4000) overnight at 4°C and stained with NBT/BCIP until it reached the desired intensity. The stained larvae were imaged using Leica DFC420 camera (Leica Camera AG). The *pomca* probe was generated as previously described (Muthu *et al.* 2016).

Morphological analysis of adults

The gross morphology of wild-type siblings and *cyp11a2*^{-/-} mutants were compared at 180 dpf ($n=10$ for each genotype). The secondary sex characteristics were examined based on a well-established method involving body shape and coloration (Schilling 2002, Dranow *et al.* 2013). The characterization was further confirmed by other two independent researchers blinded to the genotype. The weight and length of adult zebrafish were measured after being killed. Subsequently, zebrafish were dissected, gonads were examined and photographed.

Breeding ability assessment and *in vitro* fertilization (IVF)

A *cyp11a2*^{-/-} mutant male or a wild-type sibling male was out-crossed to an unrelated wild-type female in a breeding

tank with a divider in the middle on the afternoon prior to a breeding trial. The following morning, the divider separating male and female fish was removed and they were allowed to mate. The number of fertilized eggs was counted after 3 h.

For IVF experiments, sperm were collected by dissecting the testes of killed males, which were then homogenized in 50 μ L Hank's balanced salt solution prepared as previously described (Kroeger *et al.* 2014). Eggs were collected from anesthetized wild-type females by gentle palpation of the abdomen. 20 μ L of sperm solution was mixed with the clutch of collected eggs, followed by 1 mL of E3 medium to activate the sperm. After 2 min, further E3 medium was added and eggs were incubated at 28.5°C for development (Westerfield 2000). Fertilization of eggs was confirmed by visualization under a dissecting microscope.

Mating behavior analysis

A *cyp11a2*^{-/-} mutant male or wild-type male sibling was paired with a wild-type female in the late afternoon with a divider in the middle of the breeding tank. The next morning, the divider was removed and breeding behaviors was recorded from above the tank using the Zebrelab software (Viewpoint Life Sciences, Montreal, Canada). Videos of the breeding behaviors were then analyzed. The frequency and duration of intimate contacts, including parallel swimming, chasing and spawning, were recorded.

Sperm counting

Sperm were collected by dissecting the testes of killed males, followed by homogenization in a 100 $\times$ mass:volume dilution with 600 mosmol/kg Hank's balanced salt solution prepared as described (Jing *et al.* 2009). 10 μ L of sperm solution was transferred to each counting chamber of a dual-chamber Improved Neubauer hemocytometer (Hawksley, Lancing, UK) and sperm from each sample were counted in duplicate according to the protocol specified in WHO Laboratory manual for the examination and processing of human semen (World Health Organization 2010). A minimum of 200 sperms were counted for each replicate. Sperm concentration was determined by calculating the concentration per nanolitre and multiplying this by the weight to volume dilution factor. The gonadosomatic index (GSI) was calculated using the formula (gonad weight/total tissue weight) $\times$ 100.

Hematoxylin and eosin (H&E) staining

Adult fish were fixed in 4% (w/v) paraformaldehyde (PFA) in 1× PBS for 4 days at 4°C followed by brief washing in 1× PBS. Fish were decalcified in 0.25 M EDTA pH 8.0 for 4 days at room temperature and then transferred to 70% (v/v) ethanol for storage at 4°C. The head, caudal fins and anal fins of the zebrafish were removed and the samples were transferred to a tissue processor (Leica TP2010) for dehydration and paraffin infiltration. After the pre-processing, the samples were embedded in paraffin wax and 5 µm sections were cut through the entire gonad. For H&E staining, samples were dewaxed using xylene and rehydrated by transferring through a series of ethanol baths. Samples were then stained with Gill's hematoxylin for 1 min before dehydration in ethanol. Samples were stained with 1% eosin in 95% (v/v) ethanol for 30 s and subsequently washed in absolute ethanol for three times. Samples were transferred to xylene and mounted using DPX mountant. The mounted samples were left dry for overnight and imaged using Leica DFC420 camera (Leica Camera AG).

Statistical analysis

Statistical analysis and graphics were performed using Graphpad Prism 7.0c (GraphPad Software). For the comparison of means between two samples, unpaired *t*-tests were used. For the comparison of means of more than two samples, one- or two-way ANOVA (ANOVA) and Sidak's multiple comparisons test was used.

Results

Generation of *cyp11a2*-null alleles in zebrafish with CRISPR/Cas9

To fully understand the function of Cyp11a2 in zebrafish larvae and adults, we disrupted the *cyp11a2* gene employing a CRISPR/Cas9 strategy. gRNA was designed to target a 18 bp site (AATATCTACAGACAATTG) in exon 5 for generation of a 5-prime disruption within the *cyp11a2* gene. Genomic disruption in the germline of injected embryos was detected by conventional PCR. Sanger sequencing of the F1 generation identified three different heritable alleles within the targeted region. Two lines were subsequently maintained (*cyp11a2*^{SH565}, *cyp11a2*^{SH567}). The *cyp11a2*^{SH565} line has a 161 bp deletion

(c.818_978del) spanning the entire exon 5, introducing a frameshift with a premature stop at amino-acid position 282 (p.A273DfsX10) (Fig. 1A and B). The second-line *cyp11a2*^{SH567} carried a 144 bp deletion (c.821_964del), subsequently causing a 48 amino-acid in-frame deletion (p.274_321del) (Fig. 1A and B). Thus, both mutations are predicted to abolish Cyp11a2 activity.

The phenotypes of *cyp11a2* homozygous mutant larvae were characterized before 5 dpf. *cyp11a2*^{SH565} and *cyp11a2*^{SH567} homozygous mutants were morphologically similar to control siblings during early development. The *cyp11a2*^{SH565} line was used for subsequent analysis and will be named *cyp11a2*^{-/-} hereafter.

Background adaptation in zebrafish has been associated with impaired glucocorticoid biosynthesis and signaling (Griffin *et al.* 2016, Eachus *et al.* 2017). Larvae from a heterozygous in-cross were analyzed by VBA assessment at 96 hpf and sorted into groups with either dark or light pigmentation (Fig. 1C). Subsequent genotyping revealed over 90% of VBA-negative larvae were *cyp11a2*^{-/-} mutants (Supplementary Table 2). VBA-positive larvae were either wild-type or *cyp11a2*^{+/-}, confirming that *cyp11a2*^{-/-} mutants are reliably identified by VBA assessment.

Impaired steroid biosynthesis and systemic changes in *cyp11a2*^{-/-} mutant larvae

To assess the importance of Cyp11a2 in steroidogenesis, we measured cortisol concentrations by LC-MS/MS in *cyp11a2*^{-/-} larvae. At 96 hpf, larvae from a heterozygous in-cross were sorted by VBA assessment into VBA+ and VBA- groups (150 larvae in each group, 3 replicates). Steroid hormone profiles were analyzed at 120 hpf. The cortisol concentration was significantly reduced in *cyp11a2*^{-/-} mutants compared to their siblings (*P*=0.0143) (Fig. 2A).

To further analyze the loss of Cyp11a2 on glucocorticoid action, qRT-PCR was used to assay the transcriptional responses of glucocorticoid-inducible genes after exposure to osmotic stress. Transcription of the glucocorticoid-responsive genes *fkbp5* (*P*<0.0001) and *pck1* (*P*=0.0135) was significantly decreased in *cyp11a2*^{-/-} mutant larvae compared to siblings, when maintained under basal conditions (Fig. 2B and C). Exposure to osmotic stress resulted in a significant increase in the expression of both *fkbp5* (*P*<0.0001) and *pck1* (*P*=0.0088) in wild-types, but this treatment had no significant effect

Figure 2

Zebrafish *cyp11a2*^{-/-} mutant larvae are glucocorticoid deficient. (A) LC-MS/MS showed significantly reduced level of cortisol in *cyp11a2* homozygous mutant larvae at 120 hpf (unpaired *t*-test). Analysis of baseline and stress-induced transcript levels of (B) *fkbp5* and (C) *pck1* using qRT-PCR in 120 hpf siblings and homozygous larvae (two-way ANOVA). **P* < 0.05; ***P* < 0.005; *****P* < 0.0001.

on expression levels of these genes in *cyp11a2*^{-/-} larvae (Fig. 2B and C).

Moreover, impaired cortisol biosynthesis led to significantly higher levels of *pomca* expression measured by qRT-PCR in *cyp11a2*^{-/-} larvae (*P* = 0.001) (Fig. 3A), suggesting activation of the HPI axis due to lack of negative feedback. This finding was confirmed by WISH showing increased staining of a *pomca* probe in pituitary tissue in *cyp11a2*^{-/-} larvae compared with wild-type siblings (Fig. 3B).

Figure 3

Zebrafish *cyp11a2*^{-/-} mutants have a dysregulated HPI axis at 120 hpf. (A) qRT-PCR analysis of the expression of *pomca* was significantly upregulated in *cyp11a2*^{-/-} mutant larvae (unpaired *t*-test, ****P* = 0.001). (B) WISH analysis showed the expression of *pomca* was increased in *cyp11a2*^{-/-} mutant larvae compared to wild-type. Scale bar: 0.3 mm.

Cyp11a2 deficiency causes the development of only male fish with partly feminized secondary sex characteristics

Despite their profound glucocorticoid deficiency, *cyp11a2*^{-/-} zebrafish develop into viable adults. Typically, wild-type adult male zebrafish have a slender body shape with pinkish-yellow coloration, whereas wild-type females have an egg-filled abdomen of bluish-silver color (Schilling 2002, Dranow *et al.* 2013). The gross morphology of wild-type siblings and *cyp11a2*^{-/-} mutants was compared at 180 dpf (*n* = 10 for each genotype). All *cyp11a2*^{-/-} animals had female-like bluish-silver body pigmentation and an abdomen rounder than that of wild-type males. Wild-type males had a dark yellow anal fin, compared to a light-yellow color of this structure in wild-type females. All *cyp11a2*^{-/-} mutant animals had a dark yellow-pigmented anal fin. However, similar to wild-type females, *cyp11a2*^{-/-} mutants displayed wider blue and narrower yellow stripes than wild-type males (Fig. 4A). Taken together, *cyp11a2*^{-/-} mutants showed mixed secondary sex characteristics with more pronounced female secondary sex characteristics. Interestingly, the genital papilla was not apparent in all *cyp11a2*^{-/-} fish, suggesting that their gonadal sex is male (Yossa *et al.* 2013).

Figure 4

Depletion of Cyp11a2 leads to all-male homozygous fish. (A) Cyp11a2 deficiency caused fish to develop only into males as seen with the presence of testes and an apparent absence of the genital papilla. The *cyp11a2*^{-/-} fish mainly exhibited feminized secondary sex characteristics. Scale bar: 1 mm. Adult *cyp11a2*^{-/-} zebrafish exhibited increased (B) weight and (C) length compared to wild-type siblings. (D) Expression of *gh1* was significantly increased in the testes of *cyp11a2*^{-/-} mutant adults compared to that of wild-type adult males ($n = 5$). (E) Expression of *gh1* was unchanged in the pituitary of *cyp11a2*^{-/-} mutant adults compared to wild-type adult males ($n = 5$). An unpaired *t* test was used for the analysis. * $P < 0.05$; ** $P < 0.005$.

Further anatomical examination demonstrated that wild-type animals with male or female secondary sex characteristics possessed testes or ovaries at similar proportions ($n = 20$). By contrast, all *cyp11a2*^{-/-} mutants had testes and none had ovaries ($n = 20$) (Fig. 4A). Interestingly, *cyp11a2*^{-/-} mutant fish were found to be significantly heavier ($P = 0.0132$) and longer ($P = 0.001$) than their wild-type male and female siblings

Figure 5

Zebrafish *cyp11a2*^{-/-} mutant adults have impaired interrenal and gonadal steroid hormone biosynthesis. The concentration of (A) cortisol, sex steroids and their precursors (B) androstenedione, (C) 11-ketoandrostenedione, (D) testosterone, and (E) 11-ketotestosterone were significantly decreased in *cyp11a2*^{-/-} adult males compared to wild-type siblings ($n = 8$). Transcript levels of (F) *fkbp5* and (G) *pck1* were also significantly decreased in *cyp11a2*^{-/-} adult liver compared to the livers of wild-type siblings ($n = 5$). (H) Expression of the androgen-responsive gene *cyp2k22* was almost completely extinguished in the liver of *cyp11a2*^{-/-} adult males ($n = 5$). An unpaired *t*-test was used for all analysis. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$; **** $P < 0.0001$.

(Fig. 4B and C). Further analysis on *gh1* (growth hormone 1) showed significantly increased expression ($P = 0.001$) in the testis of *cyp11a2*^{-/-} mutant adults ($n = 5$) but no significant change in *gh1* expression in the pituitary ($P = 0.1236$) compared to wild-type siblings ($n = 5$) (Fig. 4D and E).

cyp11a2^{-/-} mutant adults are both glucocorticoid and androgen deficient

To determine steroid hormone concentrations in adults, the whole bodies of wild-type siblings and *cyp11a2*^{-/-} mutants at 180 dpf (eight biological replicates) were used for LC/MS-MS analysis. Mutation of *cyp11a2* resulted in reduced cortisol concentrations ($P < 0.0001$) (Fig. 5A). Systemic glucocorticoid deficiency was confirmed by significantly decreased expression of *fkbp5* ($P = 0.0006$) and *pck1* ($P = 0.0005$) in the livers of *cyp11a2*^{-/-} adult animals, compared to their siblings (Fig. 5F and G). Furthermore, loss of *cyp11a2* function caused significant reductions in the concentration of androstenedione ($P < 0.0001$), 11-ketoandrostenedione ($P = 0.0009$), testosterone ($P = 0.0128$) and the active zebrafish androgen, 11-ketotestosterone ($P = 0.0038$) (Fig. 5B, C, D and E). Transcription of the known

androgen-responsive gene *cyp2k22* was also markedly decreased in the livers of *cyp11a2*^{-/-} males compared to their wild-type siblings ($P=0.0003$) (Fig. 5H).

Cyp11a2 deficiency causes activation of the hypothalamic-pituitary-gonadal axis (HPG axis)

In response to the impaired sex steroid biosynthesis, significantly higher levels of *fshb* ($P=0.0053$) and *lhb* ($P=0.0404$) transcripts were detected in the pituitary of *cyp11a2*^{-/-} mutant adults ($n=5$) compared to wild-type siblings ($n=5$) (Supplementary Fig. 1A and B), which may be caused by lack of negative feedback due to androgen deficiency.

Cyp11a2^{-/-} homozygous adults are infertile

Fertility of *cyp11a2*^{-/-} fish was first assessed by out-crossing *cyp11a2*^{-/-} mutants or their wild-type male siblings with an unrelated wild-type female. In four independent experiments, we found that wild-type males ($n=10$) could successfully fertilize eggs produced by the females at a frequency of between 70–90%, whereas none of the pairs that included a *cyp11a2*^{-/-} mutant ($n=10$) could produce fertilized eggs (Supplementary Fig. 2A). We also examined the breeding behavior of wild-type male siblings ($n=6$) and *cyp11a2*^{-/-} mutants ($n=6$) out-crossed with unrelated wild-type females. Wild-type males exhibited typical breeding behaviors, that is chasing wild-type females, making intimate contacts and inducing spawning (Supplementary Fig. 2B and C). However, *cyp11a2*^{-/-} mutants exhibited greatly reduced levels of chasing and intimate contact with wild-type females (Supplementary Fig. 2B and C). Consequently, this resulted in failure of spawning induction in females.

Sperm concentration was then examined and found significantly decreased in *cyp11a2*^{-/-} mutants compared to wild-type siblings ($P=0.0071$) (Fig. 6A). Fertility of *cyp11a2*^{-/-} mutants was further assessed by IVF. Interestingly, sperm from the dissected testis ($n=5$) could not fertilize any wild-type eggs ($P<0.0001$), compared to 50–90% fertilization rate by wild-type sperm (Fig. 6B).

Taken together, our results suggested that loss of *cyp11a2* function considerably impairs the fertility of adult males.

Homozygous *cyp11a2*^{-/-} mutant fish exhibit defective spermatogenesis and disorganized testes

The finding of a low sperm count and the absence of breeding behaviors in *cyp11a2*^{-/-} mutants led us to examine

testicular structures and functions. Histological analysis using H&E staining showed well-organized testes in wild-type siblings. These testes had well-defined seminiferous tubules separated by thin strands of interstitial connective tissue. Spermatozoa were located in the center of each tubular lumen, with spermatids, spermatocytes and

Figure 6

Cyp11a2^{-/-} homozygous male adults are infertile. (A) Sperm concentration was markedly reduced in *cyp11a2*^{-/-} mutant males ($n=4$). (B) Sperm from the dissected testis of the *cyp11a2*^{-/-} mutants ($n=5$) could not fertilize any wild-type eggs, compared to 50–90% fertilization rate by wild-type sperm. (C, D, E and F) Histological analysis of testes at 180 dpf showed that *cyp11a2*^{-/-} mutants (E and F) had disorganized seminiferous tubules with a barely distinguishable central lumen compared to the testes of wild-type males (C and D). Spermatogonia were the main cell type found in the mutant testes, with some spermatocytes and a very few spermatozoa also present. SG, spermatogonia; SC, spermatocyte; SZ, spermatozoa. Scale bar: 10 μ m. An unpaired *t*-test was used for the analysis. ** $P<0.005$, **** $P<0.0001$.

spermatogonia located more peripherally, toward the basement membrane (Fig. 6C and D).

By contrast, the testes of *cyp11a2*^{-/-} mutants were markedly disorganized and adjacent seminiferous tubule cross-sections were difficult to distinguish from one another. For each tubule cross-section, the central lumen was difficult to identify, and mature spermatozoa were very rarely found within the testis. Spermatogonia were the main cell type found in the testes of *cyp11a2*^{-/-} mutants, with spermatocytes being the second dominant cell type (Fig. 6E and F). Interestingly, there was no difference in gonadosomatic index between *cyp11a2*^{-/-} mutant and wild-type siblings at 180 dpf (Supplementary Fig. 2D).

Cyp11a2 deficiency causes downregulation of key genes involved in testis development and spermatogenesis

To investigate the molecular mechanisms underlying the phenotypic defects observed, we used qRT-PCR to examine the transcription of genes involved in testis development and spermatogenesis.

Doublesex and mab-3-related transcription factor 1 (*dmrt1*) is a key regulator of testis development, and anti-Müllerian hormone (*amh*) is a pro-testis hormone during zebrafish development. Interestingly, the expression of both genes was decreased in the testes of *cyp11a2*^{-/-} mutants (*dmrt1*, $P=0.0151$; *amh*, $P=0.0002$) (Fig. 7A and B). Furthermore, SRY-box 9a (*sox9a*), a pro-male transcription factor involved in ovary-testis transition in zebrafish, is also significantly downregulated ($P=0.0249$) (Fig. 7C).

Figure 7

Expression of pro-testis and spermatogenic genes was downregulated in the testes of *cyp11a2*^{-/-} zebrafish. (A, B, C, D, E and F) Relative expression of *dmrt1*, *amh*, *sox9a*, *igf3*, *insl3* and *inha* were significantly decreased in the testes of homozygous mutants compared to wild-type siblings ($n=5$). An unpaired *t*-test was used for all analysis. * $P<0.05$; ** $P<0.005$; *** $P<0.0005$, **** $P<0.0001$.

Insulin-like growth factor 3 (*igf3*) and insulin-like peptide 3 (*insl3*) are both involved in the proliferation and differentiation of type A spermatogonia in the early stages of spermatogenesis (Crespo *et al.* 2016, Safian *et al.* 2016). Significant downregulation of *igf3* ($P=0.001$) (Fig. 7D) and *insl3* ($P=0.0006$) were found in *cyp11a2*^{-/-} testes (Fig. 7E), consistent with the findings of decreased sperm quantity. The transcript level of inhibin alpha (*inha*) was almost completely extinguished in testes from *cyp11a2*^{-/-} mutants ($P<0.0001$) (Fig. 7F).

To further investigate molecular mechanisms of the decreased expression of *igf3* and *insl3*, we examined the expression of *fshr* and *lhcr* in the testes of both mutant and wild-type sibling adults. However, no significant difference was detected for either *fshr* ($P=0.2481$) or *lhcr* ($P=0.1372$) (Supplementary Fig. 1C and D).

Differential expression of spermatogenesis markers in *cyp11a2* homozygous mutants

To investigate how loss of Cyp11a2 function impacts spermatogenesis, we measured the expression of germ cell markers at several stages of spermatogenesis. *nanos2* is expressed in type A undifferentiated spermatogonia, while *piwil1* is expressed in all type A spermatogonia (Beer & Draper 2013, Safian *et al.* 2016). We found a significant upregulation of *nanos2* ($P=0.0004$) and *piwil1* ($P=0.022$) expression in the testes of *cyp11a2*^{-/-} fish. No change was found in the expression of *dazl*, a marker of type B spermatogonia ($P=0.8219$) (Safian *et al.* 2016). Interestingly, expression of spermatocyte marker *sycp3* ($P=0.0012$) and spermatid marker *odf3b* ($P=0.0003$) was significantly downregulated (Fig. 8A, B, C, D and E) (Ozaki *et al.* 2011, Tang *et al.* 2018). The expression profile is consistent with the assessment of sperm quality and quantity, suggesting decreased numbers of late stage germ cells and accumulation of germ cell precursors.

Discussion

Previous data by our group and others (Hsu *et al.* 2009, Parajes *et al.* 2013) suggested that *cyp11a1* is expressed during early development stages from zygote to 24 hpf, while the expression of *cyp11a2* becomes continuously prominent just before *de novo* (48 hpf) and HPI-mediated (96 hpf) cortisol synthesis commences (Parajes *et al.* 2013, Weger *et al.* 2018). Further *in situ hybridization* analysis showed the expression of *cyp11a2* is restricted to the interrenal at 120 hpf, whereas no expression of *cyp11a1*

Figure 8

Spermatogenesis markers were differentially expressed in the testes of *cyp11a2*^{-/-} mutant and wild-type adults. Expression of (A) *nanos2* and (B) *piwil1*, which are markers for type A_{und} spermatogonia and all type A spermatogonia, respectively, was significantly increased in the testes of homozygous mutants (*n* = 5). (C) Expression of *dazl*, a marker of type B spermatogonia, was not changed (*n* = 5). Expression of the spermatocyte marker (D) *sycp3*, and the spermatid marker, (E) *odf3b* was significantly downregulated (*n* = 5). An unpaired *t*-test was used for all analysis.

P* < 0.05; *P* < 0.005; ****P* < 0.0005.

was found (Weger *et al.* 2018). Functional analysis using transient morpholino knockdown in larvae revealed distinct roles between the two genes. *cyp11a1* is required for early embryonic development, which is consistent with observations in other reports (Goldstone *et al.* 2010). *cyp11a2* is essential for *de novo* steroidogenesis in zebrafish larvae and appeared to be the functional equivalent of human CYP11A1 (Parajes *et al.* 2013). In this study, we confirmed previously reported results and clarified the role of Cyp11a2 in adult zebrafish using stable null-allele lines.

Disruption of *cyp11a2* leads to glucocorticoid deficiency

Previous morpholino knockdown of *cyp11a2* showed various levels of phenotypic abnormalities including craniofacial defects, pericardiac oedema and absence of swim bladder (Parajes *et al.* 2013). Similar phenotypes were found in loss of *ff1b* (ortholog of mammalian steroidogenic factor 1) or *dax1*, but all the studies were based on morpholinos (Chai *et al.* 2003, Zhao *et al.* 2006). In contrast, these phenotypes were absent in our newly generated *cyp11a2*^{-/-} knockout lines and such phenotypes have also not been observed in our published glucocorticoid-deficient lines due to 21-hydroxylase (Eachus *et al.* 2017) or ferredoxin deficiency (Griffin *et al.* 2016). Morpholinos are well known for their off-target effects (Eisen & Smith 2008) and thus the previously described changes attributed to glucocorticoid deficiency

(Parajes *et al.* 2013) are more likely caused by morpholino toxicity (Stainier *et al.* 2017).

Our *cyp11a2*^{-/-} mutants displayed typical characteristics of glucocorticoid deficiency. VBA is a glucocorticoid-dependent neuroendocrine response mediated via the glucocorticoid receptor (Muto *et al.* 2005, 2013). Impaired VBA has been previously described in several zebrafish lines with defective glucocorticoid signaling (Griffin *et al.* 2016, Eachus *et al.* 2017, Facchinello *et al.* 2017). The VBA analysis of larvae at 120 hpf revealed that about 93% of the strongly dark pigmented larvae were *cyp11a2*^{-/-} mutants, suggesting impaired glucocorticoid biosynthesis.

Adrenal insufficiency causes an upregulation of the hypothalamus-pituitary-adrenal axis in humans due to a disruption of the negative HPA feedback (Liyanarachchi *et al.* 2017). Our *cyp11a2*^{-/-} mutants exhibited an upregulation of the HPI axis as indicated by increased *pomca* expression (Fig. 3). This is consistent with other zebrafish models of glucocorticoid resistance (Ziv *et al.* 2013, Lin *et al.* 2015) and glucocorticoid deficiency (Griffin *et al.* 2016, Eachus *et al.* 2017) due to loss of negative feedback to the pituitary and hypothalamus.

The significantly decreased concentration of cortisol in the VBA- larvae clearly demonstrates the requirement for Cyp11a2 to enable glucocorticoid biosynthesis, which is consistent with our previous morpholino knockdown study (Parajes *et al.* 2013). However, some baseline cortisol production was detected in VBA- larvae. The most plausible explanation is that a small proportion of wild-type or heterozygous siblings are also VBA- but designated as *cyp11a2*^{-/-} mutant larvae. This limitation to the use of VBA as a genotyping proxy has been highlighted previously (Eachus *et al.* 2017). However, the lack of detectable concentrations of cortisol in *cyp11a2*^{-/-} adults confirmed that they are glucocorticoid deficient. To further validate this model of glucocorticoid deficiency, we subjected the larvae to osmotic stress, which has previously been used to induce cortisol production (Weger *et al.* 2012). However, there was no transcriptional increase of either *fkbp5* or *pck1* in the *cyp11a2*^{-/-} mutant larvae under osmotic stress, further demonstrating a block in glucocorticoid biosynthesis.

Disruption of *cyp11a2* leads to sex steroid deficiency and all-male fish with feminized secondary sex characteristics (SSCs)

Sex steroids are required for sexual development and are also responsible for the maintenance of established sex phenotypes (Goppert *et al.* 2016). We found

significantly reduced concentrations of the sex steroid precursor androstenedione, the active zebrafish androgen 11-ketotestosterone, and testosterone in *cyp11a2*^{-/-} adult mutants. All *cyp11a2*^{-/-} mutant zebrafish developed testes. Previous studies on *cyp19a1a* and *cyp17a1* in zebrafish have shown all-male phenotypes (Lau *et al.* 2016, Zhai *et al.* 2018). Therefore, our results suggest that the impaired biosynthesis of sex steroids in *cyp11a2*^{-/-} mutant zebrafish is responsible for their all-male phenotype.

Cyp11a2-deficient male zebrafish mainly exhibited feminized SSCs, including female body pigmentation and a rounded body shape. In teleost fish, androgens, especially 11-ketotestosterone, are important for testicular development and for the development of SSCs (Borg 1994). Both testosterone and 11-ketotestosterone have high affinity and transactivation potency for the androgen receptor (Ar) in zebrafish (de Waal *et al.* 2008, Hossain *et al.* 2008). Similarly, Ar-deficient zebrafish males also exhibited female-type coloration and body shape (Crowder *et al.* 2018, Yu *et al.* 2018). Moreover, feminized SSCs were also demonstrated in the Cyp17a1-deficient zebrafish males; however, this androgen-deficient line exhibited a slender body shape as found in wild-type males (Zhai *et al.* 2018). Taken together, these results suggest that the development of male SSCs requires appropriate androgen exposure. However, the cause of observed phenotypic variability in different androgen-deficient lines remains to be elucidated.

cyp11a2^{-/-} mutants exhibit male infertility

Our *cyp11a2*^{-/-} mutants failed to exhibit male-typical chasing activity and the ability to induce female spawning during natural breeding. Similarly, Ar-deficient zebrafish and androgen-deficient *cyp17a1*-knockout zebrafish exhibit defective breeding behaviors (Crowder *et al.* 2018, Tang *et al.* 2018, Yu *et al.* 2018, Zhai *et al.* 2018). Ar is necessary for zebrafish fertility, and male mating behaviors are likely to be regulated by androgen signaling. Further fertility assessment by sperm counting revealed significantly reduced sperm concentrations in *cyp11a2*^{-/-} mutants, indicating impaired spermatogenesis. A lower amount of mature spermatozoa was also observed in the testes of zebrafish *ar*^{-/-} mutants (Crowder *et al.* 2018, Tang *et al.* 2018, Yu *et al.* 2018). Interestingly, normal spermatogenesis was found in Cyp17a1-deficient zebrafish mutants, possibly due to compensation by Cyp17a2 (Zhai *et al.* 2018). However, biochemical analysis

suggested zebrafish Cyp17a2 cannot catalyze the 17,20-lyase reactions that yield to DHEA and androstenedione (Pallan *et al.* 2015). Progesterone has been suggested to function in spermatogonial cell proliferation and spermatogenesis in teleosts (Liu *et al.* 2014, Wang *et al.* 2016). Moreover, the specificity of the interactions between Ar and its ligands are not fully characterized in teleosts. The development of relatively normal testes with mature spermatozoa in *cyp17a1* mutant animals might be explained by some affinity of Ar for progesterone (de Waal *et al.* 2008).

In mammals, androgen signaling is believed to be required for testis development and spermatogenesis. A murine *Cyp11a1* deletion model had testes with disorganized seminiferous tubules containing only spermatocytes arrested at the meiotic stage (Hu *et al.* 2002). Our histological analysis of the testes from *cyp11a2*^{-/-} mutants showed poorly defined seminiferous tubules without a distinguishable central lumen. Moreover, the seminiferous tubules mainly contained spermatogonia and very few spermatocytes or mature spermatozoa. Similarly, zebrafish *ar*^{-/-} mutants also exhibit disorganized seminiferous tubules replete with spermatogonia and spermatocytes but deficient in mature spermatozoa (Crowder *et al.* 2018, Yu *et al.* 2018).

Whilst the molecular mechanism underlying the severe disruption of spermatogenesis in Cyp11a2-deficient zebrafish remains elusive, our observations of markedly reduced *igf3* and *insl3* expression may contribute to the accumulation of early spermatogonial cells as Igf3 and Insl3 are both involved in the proliferation and differentiation of type A spermatogonia (Assis *et al.* 2016, Crespo *et al.* 2016, Safian *et al.* 2016). Downregulation of *igf3* and *insl3* was previously reported in *ar*-null mutants (Tang *et al.* 2018). Moreover, unchanged level of *dazl* expression, a marker of type B spermatogonia, suggests normal differentiation from type A to type B spermatogonia. On the other hand, the reduced expression of the spermatocyte marker *sycp3* and spermatid marker *odf3b* indicate that the transition into and/or completion of meiosis are compromised.

In this study, we generated *cyp11a2*-knockout animals by employing CRISPR/Cas9 gene editing technology. These *cyp11a2*-null mutant zebrafish are deficient in both glucocorticoid and sex steroids. We demonstrated the phenotypic consequences of the combined steroid hormone deficiencies. This work thus establishes a requirement for Cyp11a2 in adrenal and gonadal steroidogenesis in zebrafish.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-19-0384>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

V T Cunliffe and N P Krone contributed equally to this work.

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