Transgenerational hypocortisolism and behavioral disruption are induced by the antidepressant fluoxetine in male zebrafish *Danio rerio*

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The global prevalence of depression is high during childbearing. Due to the associated risks to the mother and baby, the selective serotonin reuptake inhibitor fluoxetine (FLX) is often the first line of treatment. Given that FLX readily crosses the placenta, a fetus may be susceptible to the disruptive effects of FLX during this highly plastic stage of development. Here, we demonstrate that a 6-day FLX exposure to a fetus-relevant concentration at a critical developmental stage suppresses cortisol levels in the adult zebrafish (F_0). This effect persists for three consecutive generations in the unexposed descendants (F_1 to F_3) without diminution and is more pronounced in males. We also show that the in vivo cortisol response of the interrenal (fish "adrenal") to an i.p. injection of adrenocorticotropic hormone was also reduced in the males from the F_0 and F_3 FLX lineages. Transcriptomic profiling of the whole kidney containing the interrenal cells revealed that early FLX exposure significantly modified numerous pathways closely associated with cortisol synthesis in the male adults from the F_0 and F_3 generations. We also show that the low cortisol levels are linked to significantly reduced exploratory behaviors in adult males from the F_0 to F_2 FLX lineages. This may be a cause for concern given the high prescription rates of FLX to pregnant women and the potential long-term negative impacts on humans exposed to these therapeutic drugs.

transgenerational | fluoxetine | stress | epigenetic | zebrafish

Pregnancy and the postpartum period are accompanied by an increase in vulnerability to depression and anxiety (1). Psychiatric disorders such as these during pregnancy are associated with preterm delivery and numerous adverse neonatal outcomes, including impairments in cognitive and physical abilities and increased risk to develop neuropsychiatric disorders (2–5). A variety of pharmacological agents are used to treat these affective disorders. The selective serotonin [5-hydroxytryptamine (5-HT)] reuptake inhibitor (SSRI) family of antidepressants, especially fluoxetine (FLX), the active ingredient in well-known drugs such as Prozac, is generally the first line of pharmacological treatment for pregnant women (6). The SSRIs exert their therapeutic actions by enhancing serotonergic neurotransmission through inhibition of 5-HT reuptake transporters on presynaptic neurons (7).

Given that SSRIs are transferred from the treated mother to the fetus across the placenta (8–10), concerns have been raised regarding the neurobehavioral outcomes in children following prenatal SSRI exposure during developmentally sensitive periods. Critically, during brain development, 5-HT acts as a neurotrophic factor regulating neuronal proliferation, differentiation, migration, and synaptogenesis (11, 12) in addition to its prominent role in the programing of the stress axis (13, 14), also known as the hypothalamic–pituitary–adrenal (HPA) axis in mammals, which is highly plastic during development (15, 16). Furthermore, dysregulation in the physiological response of the HPA axis (changes in cortisol levels) in children and in adolescent rats follows prenatal exposure to SSRI medications via maternal treatment (17–20). Even though evidence exists for SSRI-induced disruption of the HPA axis following prenatal exposure, critically missing is any knowledge about the long-term consequences manifested in adulthood and in future generations.

We report here on the transgenerational disruption of the stress response and of behaviors following early developmental FLX exposure to concentrations within the lower range detected in the cord blood of FLX-treated pregnant women (54 $\mu g \cdot L^{-1}$) (8–10) and to an environmentally relevant concentration (0.54 $\mu g \cdot L^{-1}$) (21). We also provide insights on the potential mechanisms underlying the observed effects by means of global transcriptional analysis. We used zebrafish (ZF) *Danio rerio* as an amenable model system to determine the effects of FLX, since embryos develop external to the mother and can be directly exposed to specific concentrations of the studied chemical. Additionally, ZF have high physiological and genetic homologies to

Significance

Due to the high incidence of depression during childbearing, antidepressants such as fluoxetine (FLX) are highly prescribed during pregnancy, yet the risks to offspring are unknown. We report that a 6-day FLX exposure during early zebrafish development induces hypocortisolism for at least three generations. Gene expression analysis indicates that pathways controlling cortisol synthesis are altered in the descendants in the third generation. This FLX-induced low-cortisol phenotype is more prominent in males and is associated with significantly reduced exploratory behaviors for two generations. This is an important demonstration that, in an animal model, even a brief ancestral exposure to a common antidepressant modifies the stress response and critical coping behaviors for several generations.

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humans and the ease of genetic manipulations have rendered ZF a suitable model in stress research (22) and for other human-related brain disorders (23, 24).

Results

Cortisol Response to an Acute Stressor Is Reduced by FLX. Our first objective was to determine whether early developmental exposure to FLX during the first 6 d of life induces disruptive transgenerational effects on the stress axis, also known as the hypothalamic–pituitary–interrenal axis in teleosts. We examined whole-body cortisol levels as a stress response indicator in adult female and male ZF in the exposed F_0 generation and the unexposed F_1 to F_3 following a brief, standardized net handling stressor. A two-way ANOVA was conducted to examine the effects of the stressor and FLX treatments on cortisol levels. In the control (CTR) and in each of the treatment groups [low-FLX lineage (LFL), 0.54 $\mu g \cdot L^{-1}$, and high-FLX lineage (HFL), 54 $\mu g \cdot L^{-1}$], the cortisol response to the acute stressor was significantly elevated relative to basal (nonstressed) levels in males from each generation: F_0 [$F_{(1,42)} = 286.537$, $P < 0.001$], F_1 [$F_{(1,48)} = 965.475$, $P < 0.001$], F_2 [$F_{(1,48)} = 95.288$, $P < 0.001$], and F_3 [$F_{(1,46)} = 358.778$, $P < 0.001$]. However, total cortisol levels were significantly reduced in the FLX lineages from F_0 [38% and 57% reduction for LFL and HFL, respectively; $F_{(2,42)} = 51.618$, $P < 0.001$], F_1 [34% and 37%; $F_{(2,48)} = 11.392$, $P < 0.001$], F_2 [42% and 56%; $F_{(2,48)} = 12.596$, $P < 0.001$], and F_3 [35% and 52%; $F_{(2,46)} = 47.778$, $P < 0.001$] compared with their matched CTR group (Fig. 1*A*). The total cortisol content at both basal levels and following stress significantly varied across the FLX treatments (interactions) within generations F_0 [$F_{(2,42)} = 5.917$, $P = 0.005$], F_1 [$F_{(2,48)} = 7.144$, $P = 0.002$], and F_3 [$F_{(2,46)} = 16.162$, $P < 0.001$]. However, these interactions were not significant in the F_2 [$F_{(2,48)} = 0.003$, $P = 0.997$].

A two-way ANOVA also revealed that the females (*SI Appendix*, Fig. S1*A*) in all treatments from generations F_0 [$F_{(1,41)} = 64.037$, $P < 0.001$], F_1 [$F_{(1,46)} = 149.006$, $P < 0.001$], F_2 [$F_{(1,45)} = 168.748$, $P < 0.001$], and F_3 [$F_{(1,48)} = 170.433$, $P < 0.001$] also exhibited a cortisol response to the acute stressor. Their total cortisol levels were also significantly decreased in the FLX lineages of generations F_0 [13–30% reduction for LFL and HFL, respectively; $F_{(2,41)} = 4.008$, $P = 0.026$], F_2 [26% and 30%; $F_{(2,45)} = 10.798$, $P < 0.001$], and F_3 [31% and 37%; $F_{(2,48)} = 8.755$, $P < 0.001$] relative to their matched CTR groups. Generation F_1 was not affected by FLX [$F_{(2,46)} = 0.118$, $P = 0.889$]. The effects observed on the FLX lineages did not vary with the stressor [no interactions, F_0 ($F_{(2,41)} = 0.933$, $P = 0.402$); F_1 ($F_{(2,46)} = 2.968$, $P = 0.061$); F_2 ($F_{(2,45)} = 0.408$, $P = 0.667$); F_3 ($F_{(2,48)} = 1.974$, $P = 0.150$)].

Cortisol Response to an Intraperitoneal Injection of Adrenocorticotropic Hormone Is Reduced by FLX. The decreased basal and stress-induced cortisol production in FLX-exposed male ZF led us to predict a reduced responsiveness of the steroidogenic interrenal cells (equivalent to the cortical cells in the mammalian adrenal). We therefore administered adrenocorticotropic hormone (ACTH) to determine its impact on basal and stimulated cortisol production in the F_0 and F_3 generations. To control for handling/injection stress, we included both noninjected and saline-injected control groups. A two-way ANOVA was performed to assess the effects of the injection and the FLX concentrations on cortisol levels. The expected increase in total cortisol levels across the injected groups (noninjected, saline and ACTH groups) was observed in both the F_0 [$F_{(2,96)} = 157.436$, $P < 0.001$] and F_3 [$F_{(2,91)} = 154.809$, $P < 0.001$]. However, the interrenal cells of the males from the FLX lineages had a significantly attenuated response in each injection group compared with the CTR lineage in the F_0 [$F_{(2,96)} = 28.441$, $P < 0.001$] and F_3 [$F_{(2,91)} = 22.345$, $P < 0.001$] generations (Fig. 1*B*). In the F_0, the response of the interrenal cells to the treatments on the synthesis of cortisol significantly varied across the FLX lineages [interaction, $F_{(4,96)} = 3.121$, $P = 0.018$]. However, these interactions were not observed in the F_3 [$F_{(4,91)} = 1.493$, $P = 0.211$].

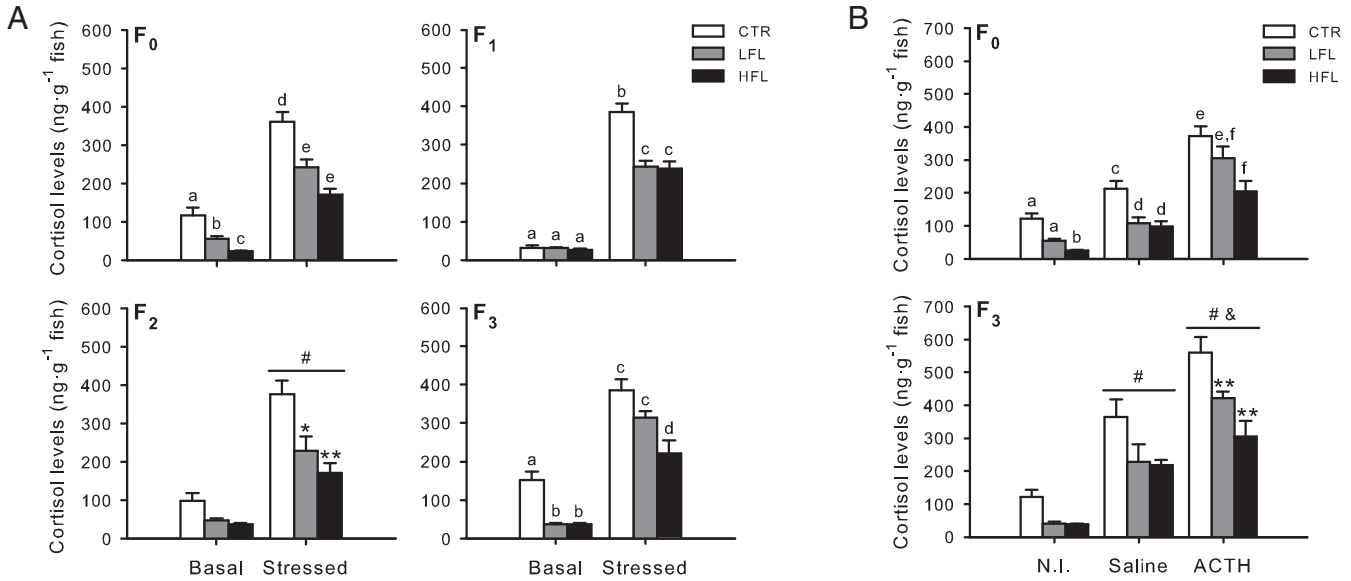

Fig. 1. Whole-body cortisol levels (nanograms per gram fish) in male adult ZF from the CTR and FLX lineages across generations. (*A*) Early developmental FLX exposure to the F_0 induced a transgenerational disruption of the stress axis manifested by a reduction of the basal and stress-induced cortisol levels in adult males. The stress response was instigated following a net handling stressor. The F_0 to F_3 denotes the different filial generations. $n = 7$–10 per group. (*B*) Male fish from the FLX lineages in the F_0 and F_3 generations exhibited a blunted cortisol production response following i.p. ACTH injection (0.0625 $IU \cdot g^{-1}$ fish). $n = 8$–17 per group. N.I., noninjected group. For *A* and *B*, the data are presented as mean ± SEM and analyzed by two-way ANOVA (on ranks for *A*, F_2; *B*, F_3); $P < 0.05$. The letters represent statistical difference when interactions are present. $^{\#}P < 0.001$ compared with the basal group for *A*, and compared with the N.I. group for *B*; $^{\&}P < 0.001$ compared with the saline group. The asterisks (*) represent significant difference in the FLX group compared with the CTR: $^{*}P = 0.004$ and $^{**}P < 0.001$.

Behavioral Responses to Novelty Are Reduced by FLX. Since disruption of the stress axis can elicit many behavioral alterations (25, 26), we investigated whether the blunted total cortisol levels produced by the females and males from the FLX lineages are linked to an altered behavioral response to novel environments. We conducted the novel-tank diving test (27) and tracked the locomotor and exploratory activities of each individual fish by video recordings analyzed using a validated in-house automated tracking (AT) Python script. We performed principal-component analysis (PCA) on 10 different behavioral metrics (*SI Appendix*, Table S1) obtained from the AT. Separate PCAs were performed on the female and male datasets. PCA yielded a single component (PC1) that strongly loaded most of the behavioral metrics (*SI Appendix*, Table S2) and explained 51% and 59% of the behavioral variance for males and females, respectively. The two variables that did not robustly contribute to PC1 were maximum speed and total distance traveled. Positive scores were associated with high exploratory and locomotor activities.

The locomotor and exploratory behaviors in males from the LFL group in F_0 [$t_{(26)} = 1.955$, $P = 0.061$], F_1 [$t_{(28)} = -0.407$, $P = 0.687$], F_2 [$t_{(25)} = 1.746$, $P = 0.093$], and F_3 ($U = 105$, $P = 0.983$) were not altered. This is in marked contrast to males in the HFL, where PC1 scores revealed that their exploratory and locomotor activities upon the novel-tank diving paradigm were significantly reduced in F_0 ($U = 53$, $P = 0.025$), F_1 [$t_{(25)} = 2.837$, $P = 0.009$], and F_2 [$t_{(25)} = 2.298$, $P = 0.030$] generations compared with their matched CTR lineage (Fig. 2*A*). There was no significant difference in the behavior of the F_3 from the HFL [$t_{(26)} = 1.430$, $P = 0.165$].

The females (*SI Appendix*, Fig. S1*B*) from the LFL group in F_0 [$t_{(27)} = 1.761$, $P = 0.090$], F_1 [$t_{(27)} = -0.447$, $P = 0.659$], and F_2 [$t_{(29)} = -0.623$, $P = 0.538$] did not show any alterations in their behaviors. The females from the HFL were also unaffected [F_0 ($t_{(26)} = 0.759$, $P = 0.455$); F_1 ($U = 88$, $P = 0.497$); F_2 ($t_{(27)} = 0.745$, $P = 0.463$); and F_3 ($U = 68$, $P = 0.071$)]. Only the F_3 [$t_{(31)} = -3.464$, $P = 0.002$] from the LFL exhibited a significant increase in the locomotor and exploratory behaviors compared with the CTR.

Blunted Cortisol Levels Are Responsible for the Reduced Locomotor and Exploratory Behaviors in FLX-Treated Males. The observed association between the FLX-induced decreases in cortisol and behaviors in males across several generations led us to the hypothesis that cortisol regulates locomotor and exploratory behaviors. To test this hypothesis, we assessed the behavioral responses of naïve male fish (from a clean population) to the novel-tank diving test following treatment with metyrapone, an 11β-hydroxylase inhibitor that blocks cortisol synthesis. A two-way ANOVA revealed that metyrapone significantly decreased whole-body cortisol levels of the naïve fish [$F_{(1,36)} = 20.724$, $P < 0.001$; Fig. 2*B*], but no effects were observed with the novel-tank diving test [$F_{(1,36)} = 3.300$, $P = 0.078$]. However, there was a statistically significant interaction between the effects of the novel-tank diving test and the metyrapone treatment on total cortisol levels [$F_{(1,36)} = 4.660$, $P = 0.038$; Fig. 2*B*]. Indeed, the locomotor and exploratory activities of the males treated with metyrapone were significantly decreased during the novel-tank diving test [$t_{(28)} = 3.389$, $P = 0.002$; Fig. 2*C*].

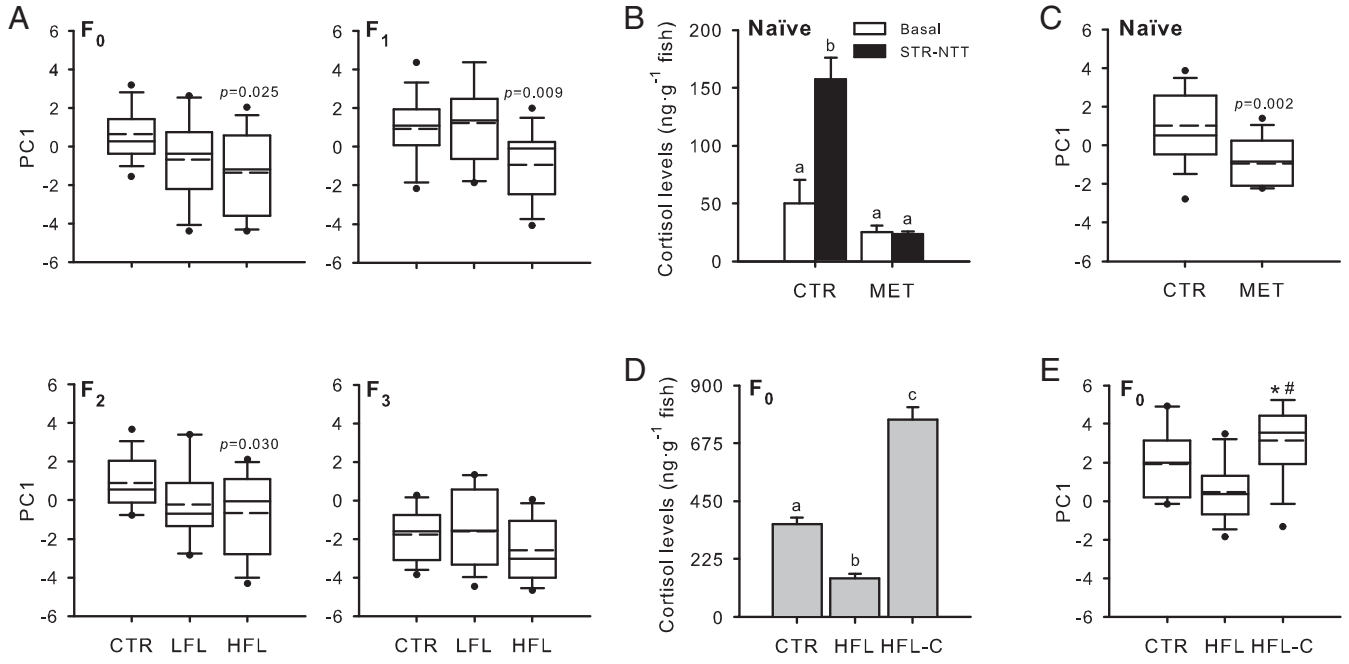

Fig. 2. The reduced cortisol levels in males from the HFL decreased their locomotor and exploratory behaviors. (*A*) PC1 scores representing the locomotor and exploratory behavioral responses to the novel-tank diving test (NTT) of the CTR and FLX lineages. PCA was used to reduce the dimensionality of the dataset composed of 10 behavioral metrics. PC1 accounted for 51% of the behavioral variance of which positive weights are associated with high exploratory and locomotor activities. $n = 13$–16 biological replicates per group in each generation. (*B*) Metyrapone (MET) exposure inhibited the total cortisol production in naïve adult males subjected to the NTT. STR-NTT, stressed levels following the NTT; $n = 4$ and 16 fish for basal and novel-tank diving test groups, respectively. Analyzed by two-way ANOVA on ranks. (*C*) Pharmacological cortisol reduction in naïve males with MET recapitulated the transgenerational inherited behavioral phenotype observed in the males from the HFL. $n = 15$ fish in each group. (*D*) Whole-body cortisol levels (stressed) of males from the F_0 following the NTT. $n = 8$–18 fish in each group. Analyzed by one-way ANOVA. (*E*) Cortisol (C) supplementation to the F_0 male adult fish from the HFL (HFL-C) rescued their locomotor and exploratory behaviors. $n = 13$–17 fish in each group. Data (*A*, *C*, and *E*) are presented in box plots showing the median (solid line), the mean (dashed line), the interquartile range (box), and the whiskers embracing data within the 10th and 90th percentiles; all data outside the range of the whiskers are presented as individual data points. The asterisk (*$P = 0035$) or *P* values shown above the bars represent significant differences within treatment compared with the CTR. The number sign ($^{\#}P < 0.001$) identifies statistical differences between HFL and HFL-C. Behavioral data were analyzed using Student's *t* test (or Mann–Whitney *U* test for *A*, HFL F_0 and LFL F_3). Whole-body cortisol levels (*B* and *D*) are expressed as mean ± SEM; $P < 0.05$.

We then examined whether cortisol supplementation to the males from the HFL F_0 could rescue their impaired locomotor and exploratory activities. Cortisol treatment of the HFL males in F_0 significantly increased their total cortisol levels [$F_{(2,33)} = 93.779$, $P < 0.001$; Fig. 2*D*] and their locomotor and exploratory behaviors [$t_{(28)} = -4.157$, $P < 0.001$] compared with the males from the untreated HFL treatment group (Fig. 2*E*). Cortisol exposure normalized the behavioral response of the HFL males since no significant difference was found between the HFL males supplemented with cortisol and the males from the CTR [$t_{(28)} = -1.788$, $P = 0.085$]. As previously shown (Fig. 2*A*), the behavioral phenotype of the males from the HFL without cortisol treatment was confirmed to be reduced compared with the CTR [$t_{(24)} = 2.235$, $P = 0.035$; Fig. 2*E*].

Transcriptomic Profiling of Male ZF Kidney Confirms Impaired Steroidogenesis. We next tested whether impaired cortisol production caused by early-life exposure to FLX was associated with shifts in steroidogenesis-related transcriptional profiles. In teleosts, steroidogenic interrenal cells homologous to the steroidogenic cells of the mammalian adrenal cortex are located mainly within the head kidney, interspersed and scattered among other cell types including chromaffin, hematopoietic, and epithelial cells (28). The entire kidney was used since the head kidney in ZF and many other teleosts is not a well-defined, compact organ as the adrenal is in mammalian species (28). The RNA sequencing (RNA-seq) analysis identified 17,098 and 16,864 unique transcripts expressed in the whole kidney in the F_0 and F_3, respectively. Of these, the expression of 596, 917, 1,424, and 3,544 genes with an average fold change of 9 (*SI Appendix*, Fig. S2) was significantly ($P < 0.05$) altered by FLX in F_0 LFL, F_0 HFL, F_3 LFL, and F_3 HFL, respectively. The number of unique and shared differentially expressed genes (DEGs) among the two treatments in the F_0 and F_3 generations is displayed as a Venn diagram (*SI Appendix*, Fig. S3).

Ingenuity Pathway Analysis (IPA) performed with each of the four DEGs lists revealed 42–275 significant ($P < 0.05$) canonical pathways altered by FLX. Of these, a total of 30 pathways was identified in common across at least three of the FLX-treated groups. The top five most significantly enriched canonical pathways in each group (*SI Appendix*, Table S3) were associated with immune functions [antiproliferative role of the transducer of ERBB2 (TOB) in T-cell signaling], cellular signaling [calcium signaling, ERK/MAPK signaling, integrin linked kinase (ILK) signaling, integrin signaling, pancreatic adenocarcinoma signaling, and paxillin signaling], molecular trafficking (caveolar-mediated endocytosis signaling), protein synthesis [eukaryotic initiation factor 2 (EIF2) signaling], cellular structure (epithelial adherens junction signaling and remodeling of epithelial adherens junctions), energy metabolism [mitochondrial dysfunction (Table 1) and sirtuin signaling pathway], and transcriptional activation [retinoic acid receptor (RAR) activation and thyroid receptor/retinoic X receptor (TR/RXR) activation]. Strikingly, alterations in genes associated with mitochondrial dysfunction observed in the FLX lineages of F_0 and F_3 are likely related to the cortisol disruption of the treated males since the primary site of cortisol biosynthesis is the mitochondria.

We further investigated the most prominent overrepresented canonical pathways centered on steroidogenesis (Table 1). Through the activation of Gαs and cAMP-mediated signaling, ACTH stimulates the synthesis of cortisol in the interrenal cells (29). These two cell signaling pathways were altered in the LFL F_0 and HFL F_3. IPA also revealed a significant alteration in the signaling of the glucocorticoid receptor (GR) in both FLX lineages of the F_3. Circadian rhythm signaling was another pathway disrupted by FLX, which was solely identified in both FLX lineages of the F_3. To strengthen biological interpretation of the data, enrichment analysis of the four DEGs listed was also performed using another leading commercial software, Pathway Studio. Similar enriched pathways were uncovered with Pathway Studio (*SI Appendix*, Table S4) compared with IPA. An interesting unique pathway revealed by this analysis was the biosynthesis of cholesterol, the universal precursor for steroid synthesis. This pathway was significantly down-regulated in the HFL from both generations and in the LFL from the F_3 generation (*SI Appendix*, Table S4).

To gain insights into the biological processes and functions enriched in the kidney of the FLX-treated fish in the generation F_0 and F_3, we performed functional clustering analysis with the IPA software on the DEGs. The enriched functional categories ($P < 0.05$) commonly affected by FLX in all four groups were mainly related to lipid metabolism, transport of molecules, carbohydrate metabolism, cellular function and maintenance, protein synthesis, inflammatory responses, and tissue morphology. Among these, we focused on the biological processes and functions associated with lipid metabolism and transport of molecules for their importance to cortisol production (Table 2). Many cholesterol-related processes, including its synthesis and metabolism, were affected by FLX. The synthesis of steroids and specifically glucocorticoids was also detected as a target of disruption by FLX. IPA also uncovered many metabolic enzymes to be disrupted (enzymopathy) in the LFL and HFL of the F_3.

We performed additional quantitative real-time PCR (qRT-PCR) analysis on a set of 10 genes (*SI Appendix*, Table S5) that showed significantly different expression levels in at least one of the four DEG lists. These genes were selected because they are key genes associated with either cholesterol or steroid synthesis. Comparison of expression levels of all of the 10 genes examined determined by RNA-seq and qRT-PCR revealed a 68% agreement in the direction and magnitude of change across all groups (LFL and HFL from F_0 and F_3). However, considering only conditions in which the genes were identified as differentially expressed (value of $P < 0.05$) by RNA-seq, there was 92% concordance between the RNA-seq and qRT-PCR results.

Table 1. *P* values of key cortisol-related canonical pathways in the kidney of males from the F_0 and F_3 FLX lineages

	F_0		F_3	
Canonical pathways	LFL	HFL	LFL	HFL
Biosynthesis of cholesterol*	NA	$<1.0 \times 10^{-3}$	1.1×10^{-2}	2.0×10^{-3}
cAMP-mediated signaling	1.9×10^{-2}	NA	NA	2.4×10^{-2}
Circadian rhythm signaling	NS	NS	3.7×10^{-2}	3.5×10^{-3}
Gαs signaling	2.3×10^{-2}	NA	NA	1.5×10^{-2}
Glucocorticoid receptor signaling	NS	NS	3.2×10^{-2}	1.3×10^{-7}
Mitochondrial dysfunction	3.9×10^{-3}	NS	5.0×10^{-11}	1.4×10^{-3}

NA, not available; pathway not detected. NS, not significant.
*This enriched pathway was obtained following gene set enrichment analysis in Pathway Studio.

Table 2. *P* values of significantly enriched key cortisol-related biological pathways and functions in the kidney of males from the F_0 and F_3 FLX lineages

	F_0		F_3	
Enriched pathways and functions	LFL	HFL	LFL	HFL
Concentration of cholesterol	NA	6.1×10^{-5} (−0.809)	NA	2.2×10^{-7} (0.561)
Enzymopathy	NA	NA	2.2×10^{-11}	7.0×10^{-10} (0.568)
Metabolism of cholesterol	NA	5.1×10^{-4} (−0.555)	NA	NA
Quantity of steroid	NA	2.4×10^{-5} (−0.805)	NA	4.6×10^{-7} (1.119)
Steroid metabolism	NA	1.7×10^{-5} (0.923)	4.4×10^{-5} (1.437)	NA
Synthesis of glucocorticoid	5.1×10^{-3}	NA	NA	NA
Synthesis of steroid	NA	NA	9.9×10^{-6} (3.107)	NA
Transport of molecule	NA	2.5×10^{-7} (1.412)	7.0×10^{-9} (1.637)	8.0×10^{-19} (−4.015)
Uptake of cholesterol	NA	2.4×10^{-4} (−1.095)	NA	NA

Z scores are presented in brackets to the right of the *P* values when available. A positive *Z* score indicates a predicted activation, whereas a negative *Z* score indicates a predicted inactivation of the enriched pathway. NA, not available; pathway not detected.

Discussion

Our findings revealed that exposure to human physiological and environmentally relevant doses of FLX during a critical period of brain development reduces both basal and stress-induced cortisol levels in adult ZF across generations. Strikingly, the magnitude of this attenuation is not consistent between the basal and stress conditions. The FLX-induced cortisol reduction is more pronounced following a stressor; hence FLX treatment also dampened the magnitude of stress axis reactivity. The stress response is critical to the organism as it is the driving force for its adaptation and survival to changes (30). Its disruption can trigger the alteration of a set of neural, behavioral, endocrine, and molecular responses. Disruption of the stress response strongly contributes to the development of diverse psychological and behavioral phenotypes (16, 31, 32). For instance, chronic blunted cortisol levels have been associated with long-term detrimental effects to human health including burnouts, chronic fatigue, fibromyalgia, immune disorders, and post-traumatic stress disorder, among others (33–37). Hyporeactivity of the HPA axis is also observed in children prenatally exposed to SSRIs through maternal treatment (19, 38).

Attenuation of the cortisol response was more severe in males than females from the FLX lineages. The HFL was the most affected with 30–52% reductions in cortisol across generations compared with CTR males, whereas ZF males from the LFL displayed 0.4–40% reductions across generations. Conversely, the females in the FLX lineages experienced a wider range of variation in their stress response, from 37% reduction to a 55% increase compared with the CTR females. In humans and rodents, sex differences in the efficacy and toxicity of pharmacological treatments have been extensively studied. Differences are generally attributable to sex-specific levels of sex steroids in addition to sex-based variability in pharmacokinetic parameters (39–42). These findings on the male-specific disruption of the stress axis are consistent with a previous study on rats where exposure of the mother to FLX throughout lactation during the neural development stage of the pups displayed a reduction in serum corticosterone levels (20).

Inadequate, excessive, or prolonged disruption of cortisol levels are all implicated in the alteration of many critical biological processes including behavioral responses in fish (43) and mammals (44, 45). Our assessment of the behavioral responses to novelty revealed a significant sex-specific decrease in the locomotor and exploratory activities in males from the HFL. The attenuation of these behaviors persisted to generation F_2 without diminution. Our findings of reduced locomotor and exploratory activities in ZF treated with FLX during a critical period of brain development coincide with studies performed on male rats (46, 47), suggesting that the effects of FLX on ZF could be cautiously extrapolated to mammals. The sexually dimorphic behavioral response to novelty observed in our FLX lineage was also displayed in rats prenatally exposed to the SSRI citalopram (48). Our results are also consistent with two human studies where prenatal exposure to SSRIs through maternal treatment impaired gross motor and adaptive responses in 10-mo-old infants (49), and increased internalizing and anxious behaviors in 3- and 6-y-old children (50).

The pharmacological inhibition of cortisol synthesis with metyrapone recapitulated the behavioral phenotype found in the males from the HFL group. Naïve male ZF exposed to metyrapone displayed reduced exploratory and locomotor activities. In support of this finding, supplementation of cortisol to the HFL F_0 males rescued their behavioral response to novelty. These results indicate that the impaired behavioral response observed in our F_0 to F_2 males is the result of the blunted cortisol levels elicited by the developmental FLX exposure to the F_0. These observations are in agreement with those on chronic hypocortisolism in humans, where associated psychological disorders are linked to low movement (chronic fatigue, burnouts, etc.) (33–37). Additionally, the examination of the cortisol levels in response to ACTH revealed a disruption in the capacity of the steroidogenic interrenal cells to synthesize cortisol in males from the FLX lineages, suggesting that the steroidogenic cells are one target of the transgenerational disruptive mechanisms induced by FLX.

To gain additional insights into the biological functions and canonical pathways transgenerationally disrupted by early-life FLX exposure in the F_0, we analyzed transcriptomic profiles of male kidneys of the CTR and the two FLX lineages from the F_0 and F_3 generations. The approximately fourfold increase in the number of DEGs observed in the F_3 compared with the F_0 from the HFL suggests that the F_3 underwent adaptive mechanistic responses to cope with the disruptive effects of FLX, as only 9% of the total detected unique transcripts were found to differ between these two generations. Such transcriptional responses are not well documented, but a few studies in invertebrate systems suggest that changes induced by environmental stressors can be both adaptive and maladaptive (51, 52). Interestingly, differential expression analysis of the FLX-treated kidneys revealed a down-regulation of key genes associated with DNA and histone modifications including DNA methyltransferases (*dnmt*) and histone deacetylases (*hdac*) (*SI Appendix*, Table S6). Since these DEGs are implicated in epigenetic modifications, their down-regulation may be associated with the underlying mechanism(s) of the observed transgenerational disruption of the stress axis from early FLX exposure to the F_0. Other important transcripts directly involved in steroidogenesis that were

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found to be disrupted in the FLX-treated tissue were *star* and *cyp11a1*. Additionally, the alteration of the transcriptional profiles in the kidney of the fish from the FLX lineages is associated with numerous pathways and functions (>400) that were disrupted by FLX. Altered processes were related to kidney function, immune response, hematopoietic and epithelial cell processes, and diverse adrenal pathways, among others (the top five canonical pathways from each treatment can be found in *SI Appendix*, Table S3).

Functional analysis of the DEGs confirmed the disruption of the steroidogenic process by FLX across the examined generations. It also revealed alterations in cholesterol-related pathways including its biosynthesis, uptake, and metabolism. Cholesterol availability is crucial for steroid synthesis as it is the metabolic precursor for these enzymatic reactions (53, 54). Additionally, enrichment analysis revealed that the Gαs and cAMP-mediated signaling pathways, the activation of which is involved in stimulating cortisol biosynthesis upon the binding of ACTH (29), were significantly affected by FLX. Circadian rhythm signaling was also impaired by FLX. Interrenal cells exhibit a circadian pattern of cortisol secretion (55, 56); therefore, disruption of this pattern may be shifting the basal cortisol levels that are subjected to robust daily variations (57). This may be associated with the blunted basal cortisol levels observed in the FLX lineages, since cortisol levels in each generation were measured at the same time of the day. Overall, the transcriptomic profile of the kidney uncovered key biological functions and pathways that are ultimately disrupted by early-life FLX exposure and that could explain the reduced ability of the interrenal cells to synthesize cortisol upon a stressor and ACTH induction.

Overstimulation of 5-HT signaling during a critical period of brain development has been shown to increase GR expression in the rat hippocampus (58). It is also well known that activation of GR in the hippocampus controls cortisol negative-feedback regulation (59–61), thus attenuating the HPA response to stress. Alteration of GR expression induced by 5-HT is mediated by epigenetic mechanisms (62, 63), and due to their stability, they are potentially transmitted to subsequent generations (64, 65). Therefore, we should consider the possibility of an integrated disruptive action by FLX on this transgenerational phenotype where both 5-HT–induced epigenetic modifications and the disruption of the steroidogenic cells are involved in the blunted cortisol levels observed in the treated males.

In conclusion, a 6-d FLX exposure during ZF brain development to a concentration within the lower range of that detected in the cord blood of FLX-treated pregnant women (HFL) leads to a male-specific impairment of cortisol synthesis for at least three consecutive generations. These findings on the impairment of cortisol levels are consistent with the disrupted biological pathways and functions linked to interrenal steroidogenesis that were altered by FLX. The impairment of the cortisol response in males elicited by FLX impairs the exploratory and locomotor activities in response to novelty for two subsequent generations. Given this evidence of the transgenerational effects of developmental FLX exposure on the stress axis and behavioral response, it would be appropriate to determine whether these effects occur in humans because FLX is generally the first line of pharmacological treatment in pregnant women suffering from affective disorders (6, 66–69). Given that levels of FLX detected in the aquatic environment (70) also reduced the stress response over the three generations in the LFL lineage, our findings also highlight potential risks to wild populations of fish.

Materials and Methods

Transgenerational Animals and in Vivo Exposure. All procedures conducted in this study were approved by the University of Ottawa Animal Care Protocol Review Committee and are in compliance with the guidelines of the Canadian Council on Animal Care for the use of animals in research. At 6 mo postfertilization (mpf), 15 pairs of adult ZF (AB strain) were used to produce a F_0 generation and each of the lineages from all subsequent generations, F_1 to F_3. Pairs in F_0 to F_3 that did not spawn at trial 1 were provided a second opportunity with a different mate randomly chosen from nonspawning individuals within the same lineage to eliminate the possibility of mate preference. Mating pairs were set up in the late afternoon in crossing cages with a plastic divider that separated the female from the male and left undisturbed until the following morning. The pairs were allowed to spawn for 1 h, 45 min between 0900 and 1100 h in fresh new water.

Eggs in each generation were immediately collected and counted. At 3 h postfertilization, embryos from the F_0 were randomly distributed to Petri dishes containing either embryo medium alone (CTR) or supplemented with one of two concentrations of FLX (Millipore Sigma; catalog #F132; purity, ≥98%) from 0 to 6 d postfertilization (dpf), and no further FLX exposures were conducted. The exposure solutions of 0.54 $\mu g \cdot L^{-1}$ (LFL) and 54 $\mu g \cdot L^{-1}$ (HFL) were prepared by serial dilutions of a 4 $mg \cdot mL^{-1}$ stock and quantified by nano–LC-MS/MS.

The F_1 to F_3 embryos from each lineage were distributed in plastic Petri dishes and labeled with the number assigned to their parents to monitor for any embryonic or larval developmental effects in a specific clutch. Embryos throughout the study were reared in Petri dishes until 6 dpf at a maximum density of 1 embryo per mL and maintained at 28 °C without feeding. The embryo media or FLX exposure solutions were renewed daily concomitantly with the removal of dead embryos.

Larvae and adult ZF were maintained under a 14-h light/10-h dark photoperiod in a temperature-controlled ZF facility and reared in tanks containing heated (28.5 ± 0.2 °C), aerated, dechloraminated City of Ottawa tap water (hereby referred to as system water). Fish from the same lineage in each generation were randomly mixed every month to avoid formation of social hierarchies and to reduce potential tank effects. Additional details are provided in *SI Appendix, SI Materials and Methods*.

Acute Stress Experiment and Sampling. Females and males (6 mpf) arbitrarily chosen from the same lineage were transferred to the experimental room and allowed to acclimate for 1 wk in 3-L tanks wrapped in black plastic and supplied with flow-through system water. The darkened tanks were used to prevent any unnecessary stress brought on by the presence of the experimenter while the animals were handled for the stress study. The lids of the tanks were not covered, so these fish still experienced the normal photoperiod. Fish were fed normally until 1 d before the experiment. At the end of the acclimation period, a group of fish was immediately killed (Basal group) and the rest underwent the standardized net stressor (71) before sampling (Stressed group). In all experiments, fish were killed by submerging them in ice-cold water to avoid any confounding effects of anesthetics (72). Fish were weighed, immediately snap-frozen in liquid nitrogen, and stored at −80 °C for further whole-body cortisol analysis. The acute stressor was performed in each generation between 0915 and 1030 h.

Intraperitoneal Injection of ACTH. Adult (6 mpf) males from the F_0 and F_3 generations were i.p. injected with a single dose of porcine ACTH (Millipore Sigma). The ACTH concentration of 0.0625 $IU \cdot g^{-1}$ body weight (BW) was estimated by conducting a prior dose–response experiment from which the minimal stimulatory ACTH concentration that triggered the maximum synthesis of cortisol was selected. Before the i.p. injection, fish were anesthetized in a 60 μM ethyl 3-aminobenzoate methane sulfonate salt (Millipore Sigma) solution (73), weighed, and immediately placed on a sponge saturated with cold water. The injection procedure was conducted in such a way as to guarantee that the animal did not spend more than 20 s out of water. The total injection volume of either ACTH or Ringer's solution [ZF saline (73)] was 10 $\mu L \cdot g^{-1}$ BW. After the injection, the animals were individually placed in a tank with system water, and 30 min following their recovery from the anesthesia, the fish were killed. All of the animals recovered in less than 1 min, and no mortalities occurred from the injection. Injections were performed between 0930 and 1230 h.

Whole-Body Cortisol Extraction and Quantification. Whole-body cortisol was extracted using a protocol adapted from Folch et al. (74). Briefly, individual fish were pulverized in liquid nitrogen using a mortar and pestle and homogenized in 15 mL of $CHCl_3$:MeOH [2:1 (vol/vol)]. After 15 min of incubation at room temperature, 5 mL of 2 M KCl buffered with 5 mM EDTA was added to the homogenate, vortexed, and incubated for an additional 20 min. The organic phase was then transferred to a clean glass tube and evaporated to dryness under a stream of nitrogen while the tubes were heated at 45 °C; the lipid extract was reconstituted in ethylene glycol monomethyl ether. The whole-body cortisol extraction efficiency was

determined to be 87%; values were not corrected for individual extraction efficiencies. Total cortisol concentrations were assessed by using a ^{125}I RIA kit (MP Biomedicals) according to the manufacturer's protocol followed by the estimate of the radioactive counts with a Wizard gamma counter (PerkinElmer). The intraassay and the interassay coefficients of variation were calculated to be 4–8% and 7–15%, respectively.

Metyrapone Exposure. Naïve male ZF (6–8 mpf) of the AB strain bred in-house were exposed in system water to either 325 μM metyrapone (Adooq Bioscience) or the DMSO vehicle [0.02% (vol/vol)] for 1 wk. Effective metyrapone dose and treatment period were estimated using a pilot study to determine levels that inhibit cortisol production in ZF. Fish were placed in 5-L glass tanks at a density of 5 fish per L and were provided with adequate aeration. The experiment was designed as static renewal, where 100% of the water was replaced daily 1 h after feeding. Ammonia, nitrate, and nitrites were measured; however, no differences were found between treatments. At the end of the 1-wk exposure, five fish from each treatment were immediately killed to assess unstressed cortisol levels. The remaining fish from both treatments were subjected to the novel-tank diving test before being terminally anesthetized, weighed, snap-frozen in liquid nitrogen, and stored at −80 °C for further whole-body cortisol assessment.

Cortisol Supplementation Experiment. Male adults (5 mpf) from the HFL group in the F_0 were exposed in system water to either 50 mg·L^{-1} cortisol (hydrocortisone; Millipore Sigma) or the DMSO vehicle [0.0625% (vol/vol)] using the same experimental design as the metyrapone exposure. The cortisol exposure was performed over a period of 4 d to allow the fish to physiologically adjust to the new cortisol levels. The CTR males from the F_0 were only exposed to DMSO. Before the experiment, new F_0 fish were generated and their cortisol levels and behaviors were examined to ensure both F_0 displayed the same effects following FLX treatment as embryos. An initial pilot study revealed the highest accumulation of cortisol in ZF occurred after 3 h of in tank exposure, and therefore 3 h into the fourth exposure day to cortisol, the fish underwent the novel-tank diving test. Fish were subsequently killed, weighed, snap-frozen in liquid nitrogen, and stored at −80 °C for further cortisol measurements.

Behavioral Experiments and Analyses. Adult females and males (6 mpf) were subjected to the novel-tank diving test adapted from Levin et al. (27). Briefly, the fish was individually placed in the trapezoid-shaped test tank filled with system water and its behavioral activity was recorded from the front for 6 min in the absence of the camera operator. All behavioral testing was performed over a 3-d period, between 0930 and 1430 h. Videos were analyzed using an AT Python script. Additional details are provided in *SI Appendix, SI Materials and Methods*.

Transcriptomics and Pathway Analyses. RNA-seq was performed on individual adult male kidney of the F_0 and F_3 generations. Total RNA was extracted using the RNeasy Plus Micro kit (Qiagen). Illumina cDNA libraries were prepared from 50 ng of total RNA using the TruSeq Stranded mRNA Library Prep Kit according to the manufacturer's instructions. Enrichment pathway analyses were performed using IPA and Pathway Studio with the four lists of DEGs obtained from the RNA-seq analysis. Additional details are provided in *SI Appendix, SI Materials and Methods*.

Gene Expression Analysis by qRT-PCR. The relative expression levels of 10 genes were analyzed by qRT-PCR. cDNA was synthesized using the QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer's protocol. qRT-PCR was performed using the Rotor-Gene SYBR Green PCR kit (Qiagen) and the thermal cycling conducted in a Rotor-Gene Q real-time PCR cycler (Qiagen). Primers (*SI Appendix*, Table S7) were designed using the online software Primer-BLAST (75) and validated by sequencing their amplicon to verify target specificity and by determining their efficiency ($100 \pm 10\%$, $R^2 > 0.98$). Melting-curve analysis was conducted at the end of the qRT-PCR protocol between 60 and 95 °C with an interval of 0.5 °C. Samples were run in technical triplicates, and their absolute abundance was calculated based on standard curves using Rotor-Gene Q Series Software 2.0.3 (Qiagen). The mRNA absolute abundance was normalized using the NORMA-GENE algorithm (76).

Statistical Analyses. Statistical analyses were conducted using SigmaPlot 11.0 (Systat Software). For ANOVAs, datasets were examined for normality and homogeneity of variance using the Shapiro–Wilk test and Levene median test, respectively. Cortisol data were analyzed using two-way ANOVA, except for Fig. 2*D* where the analysis was conducted with a one-way ANOVA. Box–Cox (77) transformations were applied when the cortisol data were not normally distributed. Alternatively, ANOVA on ranks indicated in each graph, where applicable, was used when the distribution of the data were non-Gaussian even after undergoing transformations. The behavioral dataset (PC1 scores) was analyzed using the Student *t* test or Mann–Whitney *U* test (for nonnormally distributed data). Significance was set at $P < 0.05$. To compare significance within the groups, the analysis was followed by Tukey's post hoc test.

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