Research Article

Bisphenol A and 17 α -ethinylestradiol-induced transgenerational gene expression differences in the brain–pituitary–testis axis of medaka, *Oryzias latipes*[†]

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Abstract

Endocrine disrupting chemicals (EDCs), such as bisphenol A (BPA) and 17 α -ethinylestradiol (EE2), can have far reaching health effects, including transgenerational abnormalities in offspring that never directly contacted either chemical. We previously reported reduced fertilization rates and embryo survival at F2 and F3 generations caused by 7-day embryonic exposure (F0) to 100 μ g/L BPA or 0.05 μ g/L EE2 in medaka. Crossbreeding of fish in F2 generation indicated subfertility in males. To further understand the mechanisms underlying BPA or EE2-induced adult onset and transgenerational reproductive defects in males, the present study examined the expression of genes regulating the brain–pituitary–testis (BPT) axis in the same F0 and F2 generation male medaka. Embryonic exposure to BPA or EE2 led to hyperactivation of brain and pituitary genes, which are actively involved in reproduction in adulthood of the F0 generation male fish, and some of these F0 effects continued to the F2 generation (transgenerational effects). Particularly, the F2 generation inherited the hyperactivated state of expression for kisspeptin (*kiss1* and *kiss2*) and their receptors (*kiss1r* and *kiss2r*), and *gnrh* and *gnrh* receptors. At F2 generation, expression of DNA methyltransferase 1 (*dnmt1*) decreased in brain of the BPA treatment lineage, while EE2 treatment lineage showed increased *dnmt3bb* expression. Global hypomethylation pattern was observed in the testis of both F0 and F2 generation fish. Taken together, these results demonstrated that BPA or EE2-induced transgenerational reproductive impairment in the F2 generation was associated with alterations of reproductive gene expression in brain and testis and global DNA methylation in testis.

Summary Sentence

Ancestral BPA or EE2 exposure during the first phase of primordial germ cell reprogramming in medaka fish leads to fertilization defects in male grandchildren and transcriptional alterations in male reproductive tissues.

Graphical Abstract

Key words: DNA methylation, developmental origins of health and disease, endocrine disruptors, epigenetics, fertilization, fish reproduction, gene expression.

Introduction

Some endocrine-disrupting chemicals (EDCs) bind to hormone receptors thereby interfering with hormone action. By directly binding to hormone receptors, EDCs can alter expression of genes that are essential for reproductive and immune functions [1, 2]. Bisphenol A (BPA) is one of the most widely manufactured chemicals that is used in numerous products and applications, including polycarbonate plastic, resins used in the lining of metal food cans, some dental sealants, thermal receipt papers, food storage vessels, and many others [3, 4]. Due to its widespread use, measurable concentrations of BPA have been found in the environment among surface and ground water samples around the world [5–8], and exposure to BPA has been almost unavoidable [9, 10]. In humans, BPA has been found in blood, urine, fetal plasma, placenta, and breast milk samples from populations around the world [11–19]. 17 α -ethinylestradiol (EE2) is a synthetic active estrogen used in human birth control pills [20]. Approximately 16–68% of EE2 is excreted through urine and feces [21], making it a frequently detected EDC in waste water sources [5, 22]. EE2 exposure has widespread effects in aquatic species, including reproductive impairment, intersex development, and transgenerational reproductive and neurobehavioral effects in subsequent generations of fish [23, 24].

Normal development of the reproductive system and programming of later adult behavioral and cognitive traits are dependent upon the correct concentration and timing of exposure of the developing organs to steroid hormones, in particular estrogen and testosterone [25–29]. Moreover, sex steroid hormones also regulate maturational processes of male and female reproductive organs, as well as their maintenance. EDCs, including BPA and EE2, can interfere with these processes at various levels of biological organization, including epigenetic levels. Brain–pituitary–testis (BPT) axis is central for testicular development, maintenance of male sexual characteristics, and overall male reproduction. The BPT axis depends heavily on various signaling molecules: autocrine, paracrine, and endocrine. These include peptide hormones such as kisspeptin (KISS) and gonadotrophin-releasing hormone (GnRH) from the hypothalamus and luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary [10]. Also, multiple testicular

proteins (including LHR, FSHR, CYP11B1, AR α , ESR1, and STAR) are directly involved in gonadal steroidogenesis and serve as contributors to gametogenesis [10, 30–32].

The release of GnRH and pubertal onset includes regulation by kisspeptins (products of *kiss1* gene) and their G protein-coupled receptor 54 (GPR54, also known as KISS1R or KISS2R) [10, 33, 34]. Kisspeptin is a neuropeptide synthesized and secreted by the neurons in the hypothalamic arcuate, periventricular, and the anteroventral periventricular nuclei [35]. This peptide hormone stimulates GnRH secretion, and consequently, LH and FSH secretion. The activation of GnRH is mediated by GPR54 receptor [35]. GnRH mediates the reproductive axis by a cascade of events involving pituitary GnRH receptors, release of gonadotropins by the pituitary, and secretion of sex steroids by gonads [36]. Direct adult exposure to environmental estrogens suppresses male sexual behavior and causes downregulation of brain *gnrhr1*, *ara*, and testicular *cyp17* expression in medaka [37]. BPA exposure leads to increased expression of *kiss1*, *kiss2*, their receptor genes, and *gnrh3* in the brain of developing zebrafish [38] and adult *Catla catla* [39], while EE2 exposure suppresses expression of *kiss1*, *kiss2*, and *gnrh* genes in adult rare minnow [40], suggesting that BPA and EE2 affect the reproductive neuroendocrine system. However, it is unknown if embryonic exposure effects on the BPT axis are inherited by subsequent generations.

Environmentally induced transgenerational inheritance is the process that involves transmission of environmentally induced molecular changes in germ cells at one generation to the offspring in subsequent generations whose cells are not directly exposed to the stressor [41]. In mammals, transgenerational phenotypes can be observed at F3 generation with exposure via the mother (F0) [42]; whereas in egg laying species, transgenerational phenotypes can be observed in individuals at F2 generation, irrespective of direct embryonic or parental exposure [43]. In our previous study, developmental exposure to nominal water concentrations of BPA (100 μ g/L) or EE2 (0.05 μ g/L) during the first 7 days of early embryonic development resulted in no reproductive abnormalities in the exposed F0 males but caused a significant reduction in fertilization rate in fish in the F2 and F3 generations [23]. There was also a significant increase in embryo mortality in the F3 and F4

generations. To further understand potential disruptions in signaling pathways associated with these transgenerational reproductive phenotypes, F0 and F2 generation fish from the previous study [23] were examined for transgenerational molecular alterations within the BPT-axis.

Results

The present study was designed to examine transgenerational gene expression differences in the fish that developed reproductive phenotypes at F2 generation, particularly the reduced rate of egg fertilization [23]. Gene expression changes of F0 fish were found in the adult stage due to exposure during the first 7 days of embryonic life, typically suggesting developmental origins of adult health and disease (the DOHaD hypothesis) in fish, and in F2 generation fish suggesting transgenerational effects of ancestral exposure. We compared gene expression between control (ethanol) groups from F0 and F2 generations and did not find significant alterations. No significant differences were found in morphometric parameters—body length, body weight, gonadosomatic index (GSI), and liver somatic index (Supplementary Figures S2 and S3).

Egg production and fertilization efficiency

The present study did not find significant alterations in egg production in any exposure groups in any generations examined (F0 and F2) [23]. During the crossbreeding test that was performed for 17 consecutive days, no significant differences in total egg production were found (Figure 1). Crossbreeding of males from the F2 control lineage with females from the F2 exposed lineage did not show significant differences; whereas crossbreeding of females from the F2 control lineage and males from the F2 treated lineage showed a significant reduction in fertilization efficiency, when compared with mating of control females with control males (Figure 1, $P < 0.001$). These observations suggested that the males from exposed lineages had fertilization defects.

Expression of kisspeptin and their receptor genes in the brain

The present study measured the mRNA expression levels of the genes encoding kisspeptin (*Kiss1* & *Kiss2*) and their receptors (*Kiss1r* & *Kiss2r*; also called Gpr 54-1, Gpr 54-2, respectively) in the brain of F0 and F2 generation male medaka (Figure 2). In the brains of F0 generation fish, *kiss1* mRNA expression was significantly increased by EE2 exposure relative to F0 controls ($P < 0.05$), while *kiss1r* mRNA levels did not change. In contrast, *kiss1* and *kiss1r* mRNA levels were increased 15- and 50-fold compared with controls in the F2 generation ($P < 0.01$ and $P < 0.001$), respectively. The *kiss2* mRNA expression was increased by BPA exposure compared with controls within the F0 generation ($P < 0.01$). Both *kiss2* and *kiss2r* mRNA levels were significantly increased in the F2 generation fish of lineages from both the BPA and EE2 exposure groups (Figure 2, $P < 0.01$ and $P < 0.001$). Overall, the expression levels of *kiss1*, *kiss2*, *kiss1r*, and *kiss2r* mRNAs in both BPA and EE2 lineage fish were significantly increased in the F2 generation compared with the F0 generation for the same treatment.

Expression changes of GnRH and their receptors genes

The mRNA expression levels for the genes encoding GnRH (*Gnrh1* & *Gnrh2*) and their receptors (*Gnrhr1* & *Gnrhr2*) were examined

Figure 1. Transgenerational differences in egg production (A) and fertilization defects (B) in adult medaka in the F2 generation caused by BPA or EE2 exposure of F0 embryos. Egg production has been presented as a total number of eggs per tank per day (six pairs/tank), whereas the fertilization efficiency of males from each treatment has been presented as a percentage of eggs fertilized per tank per day. Each tank contained six males and six females for mating and served as a biological replicate. Total of three biological replicate tanks were used per exposure group. Crossbreeding was performed by placing males from the control lineage with females from the treated lineage in a tank or females from the control lineage with males from the treated lineage. The fecundity graph (A) represents the average number of eggs collected from each tank (six pairs of fish) per day, whereas the fertilization efficiency graph (B) represents the average number of eggs fertilized per tank per day ($\pm$ standard error of the mean). The data were derived from three biological replicate tanks for each exposure, and egg collection and fertilization efficiency data were collected for 17 days. Asterisks indicate statistical significance (***) $P < 0.001$ when compared with control. Letters indicate statistical significance when mating combinations were compared with each other at $P < 0.001$. The overall transgenerational fecundity and fertilization phenotypes have been previously reported, in which the contribution of male or female to fertilization defects was not determined [23].

in the brain and pituitary of both F0 and F2 generation males, respectively (Figure 3). The expression levels of *gnrh1* mRNA were significantly increased by EE2 exposure within the F0 generation relative to F0 controls ($P < 0.01$), while significantly decreased in the F2 generation relative to F2 controls ($P < 0.05$). There was thus a significant transgenerational decrease in *gnrh1* expression in F2 generation males compared with F0 males from EE2 lineage ($P < 0.01$). The levels of mRNAs for GnRH1 receptor (*gnrhr1*) were significantly decreased in BPA exposure group at F0 generation ($P < 0.001$), while no significant changes were observed in either BPA or EE2 exposure group lineages within the F2 generation. The *gnrh2* mRNA levels tended to increase in the BPA exposure fish ($P < 0.08$) from F0 generation relative to the F0 controls, and this increase was maintained in the F2 generation relative to F2 fish from control lineage ($P < 0.01$). *gnrhr2* mRNA levels were significantly increased in both BPA and EE2 treatment groups in the F0 generation (Figure 2, $P < 0.05$), and similar to *gnrhr1*, a tendency to increase ($P < 0.07$) was observed in *gnrhr2* within the F2 generation of the BPA exposure lineage group relative to F2 controls (Figure 3). No significant differences were found between F0 and F2 generations for *gnrh2*, *gnrhr1*, or *gnrhr2* when compared for specific exposure lineage.

Figure 2. Relative expression of kisspeptin and their receptor genes in the whole brain of the male medaka fish in F0 and F2 (transgenerational) after embryonic BPA or EE2 exposure of F0. The results are presented as mean $\pm$ SEM. Asterisks indicate statistical significance (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Expression changes of gonadotropin genes and their receptors

The mRNA levels for the pituitary genes encoding gonadotropins (*Fsh β* and *Lh β*) and their testicular receptors (*Fshr* & *Lhr*) were examined for both F0 and F2 generation (Figure 4). In the pituitary, the expression of *fsh β* was significantly increased in the F0 BPA-exposed fish relative to F0 controls ($P < 0.001$). Within the F2 generation, no significant differences were observed. When F2 generation was compared with F0 generation, the relative *fsh β* expression levels were significantly decreased in the BPA lineage males ($P < 0.01$). In testes, expression of *fshr* was significantly decreased in both BPA and EE2 exposure groups relative to controls in the F0 generation ($P < 0.001$ and $P < 0.05$ in BPA and EE2 exposure group, respectively); however, these changes were not retained in the F2 generation. Gene expression levels for *fshr* were significantly greater in F2 generation BPA and EE2 lineage males when compared with F0 generation males. The expression of pituitary *lh β* in EE2 exposure fish tended to increase relative to controls in the F0 generation ($P < 0.08$), and while in the F2 generation *lh β* was significant increased by EE2 compared with F2 controls ($P < 0.01$). The expression of *lhr* in the testis remained high with high error bars in the F0 generation, while it tended to be elevated ($P < 0.06$) in the EE2 treatment fish relative to controls in the F2 generation. When compared between generations, *lhr* expression tended to be decreased in the F2 generation EE2 lineage fish ($P < 0.06$).

Expression of reproduction-related genes

The mRNA levels for the gene encoding steroidogenic acute regulatory protein (*Star*), steroidogenic enzyme aromatase (*Cyp19a1a*), and sex steroid hormone receptors (*Era* and *Ara*) were measured in the testis of both F0 and F2 generation males (Figure 5). None of the genes examined were altered significantly in the F0 generation due to embryonic exposure to either BPA or EE2. There was an increased expression of *star* ($P < 0.05$) and decreased expression of *era* ($P < 0.001$) within fish from the F2 generation of the EE2 exposure lineage. No significant differences were observed between the two generations.

Expression of DNA methyltransferase genes and global genome methylation changes

The expression patterns for the genes encoding DNA methyltransferases (*Dnmt3aa*, *Dnmt3bb*, & *Dnmt1*) were measured in the brain and testis at both F0 and F2 generation males (Figures 6 and 7). In the brain, *dnmt3aa* expression did not change in either F0 or F2 generations under BPA or EE2 treatment (Figure 6). In the F0 generation, *dnmt3bb* expression was significantly decreased in the brains of EE2 exposure lineage relative to F0 controls (Figure 6, $P < 0.001$). The F2 generation of the EE2 treatment lineage fish expressed elevated *dnmt3bb* relative to F2 controls as well as relative to F0 EE2-exposed fish (Figure 6, $P < 0.01$). *dnmt1* gene expression

Figure 3. Relative expression of gonadotropin in the brain and their receptor genes in the pituitary of male medaka fish in F0 and F2 (transgenerational) after embryonic BPA or EE2 exposure of F0. The results are presented as mean $\pm$ SEM. Asterisks indicate statistical significance (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

was significantly increased in brains of fish from the BPA exposure lineage in the F0 generation relative to F0 controls (Figure 6, $P < 0.05$) and significantly decreased in the F2 generation of that same BPA treatment lineage relative to F2 controls as well as relative to F0 BPA-exposed fish (Figure 6, $P < 0.001$).

In the testis, mRNA levels for *dnmt3aa* were increased in the F0 generation for the EE2 exposure treatment group (Figure 7, $P < 0.05$), while no differences in *dnmt3aa* expression between control and exposed lineage fish were found in the F2 generation. No significant increases in the expression of *dnmt3bb* or *dnmt1* were observed in testes of fish from either treatment within the F0 generation (Figure 7). In contrast, the expression of *dnmt3bb* (Figure 7, $P < 0.01$) and *dnmt1* (Figure 7, $P < 0.05$) increased in the testes of fish from the F2 generation of both BPA and EE2 exposure lineages relative to F2 controls.

The global genomic DNA methylation levels in the brain were significantly increased in the BPA exposure group of the F0 generation (Figure 8, $P < 0.05$). In the testes, the global genomic DNA methylation of the EE2 exposure group showed significant decreases in both F0 and the F2 generations relative to same-generation controls (Figure 8, $P < 0.05$).

Discussion

The ability of environmental BPA and EE2 to induce transgenerational reproductive health effects in medaka has been previously

demonstrated [23]. Here, we examined phenotype-associated molecular endpoints along the reproductive axis of the same male fish previously reported to show transgenerational fertilization efficiency impairments [23]. The crossbreeding experiment clearly demonstrated that the males from the exposed lineage in the F2 generation were sub-fertile. We observed BPA- and EE2-induced alterations in molecular pathways along the BPT axis of male medaka in two separate generations; one in the adult males exposed directly as embryos (F0), and the other in adult males two generations after the brief embryonic exposure of only the F0 generation. Observations suggest that BPA and EE2 exposure leads to subfertility in males and significant alterations in expression of genes encoding kisspeptin and their receptors in the subsequent generations.

Embryonic BPA exposure caused a significant increase in F2 males in expression of genes encoding kisspeptin, their respective receptors, *gnrh2* in the brain, and DNA methyltransferases in the testis, suggesting that the molecular networks embryonically programmed by BPA can result in transgenerational alterations in their expression pattern in subsequent generations. Alterations in gene expression profiles in the BPT axis in a comparison of F0 and F2 generation male medaka fish following embryo (F0) exposure provides insights into understanding the reproductive impairment we previously reported in these same fish [23].

Sex steroid treatments have been shown to increase expression of *kiss* genes and their receptors in the brains of fishes [44–46], although, only a few such studies have been published on endocrine

Figure 4. Relative expression of gonadotropin subunit (*fshβ* and *lhβ*) in the pituitary and their receptor (*fshr* and *lhr*) genes in the testis of male medaka fish in F0 and F2 (transgenerational) after embryonic BPA or EE2 exposure of F0. Asterisks indicate statistical significance (∇ $P < 0.08$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

disruptor effects on kisspeptin system in fish. In the present study, the expression of *kiss2* and *kiss1r* were significantly upregulated by BPA exposure in F0 generation fish, while the expression of *kiss1* was significantly upregulated by EE2 exposure in F0 generation fish, indicating that the kisspeptin system in fish is sensitive to environmental estrogens, although as is often the case, the activity of chemicals such as BPA, which is a selective estrogen receptor modulator, do not match the results of drugs such as EE2. In zebrafish, BPA exposure significantly upregulated the expression of *kiss1* and *kiss1r*, but not *kiss2* and *kiss2r* of the F0 generation [38]. In *Catla catla*, *kiss1*, *kiss2*, and *kiss1r* were upregulated by direct exposure to BPA [39]. Our data, together with that of others in the literature, suggest that direct exposure to BPA results in disruption of *kiss* and *kiss* receptor expression in these species of fish. In response to EE2 exposure, *kiss1* mRNA expression in rare minnow, *Gobiocypris rarus*, showed a complex pattern with 3-day exposure up-regulating expression while 6-day exposure downregulated it [40]. The studies in zebrafish, *Catla catla*, and the rare minnow were focused on exposure effects caused by chronic exposure for a specific time period. In contrast, the present study was designed in such a way that effects were examined in adulthood in medaka which were exposed chronically only during the early embryonic period, indicating that effects seen on expression patterns of *kiss* genes and their receptors in adulthood are probably due to alterations in developmental programming of these genes.

Kisspeptin protein is widely distributed in the vertebrate brain and can be detected not only in the hypothalamus but also in the extrahypothalamic areas and in gonads [47]. Kisspeptin has

been tagged as a gatekeeper of puberty onset in mammals, since a mutation in the *Kiss1r* gene correlated with an absence of puberty onset and hypogonadotropic hypogonadism [47–49]. Slightly different results were found in zebrafish mutants for *kiss* and *kiss* receptors. *Kiss/kiss* receptor mutation resulted in no change in spermatogenesis, folliculogenesis, and reproductive capacity of zebrafish [50], suggesting a nonessential role of *kiss/kiss* receptor systems in zebrafish reproduction. Thus, the role of the kisspeptin-GnRH neuronal system that studies suggest is the central integrating unit may not apply universally to teleosts, as in mammals [51]. In the present study, embryonic BPA or EE2 exposure upregulated the expression of the *kiss* and *kiss* receptors which are potent upstream regulators of GnRH neurons. In mice, deletion of *Kiss1* from GnRH neurons resulted in infertility, demonstrating that GnRH neurons are key sites of *kisspeptin-Kissr* signaling for fertility [52]. In the medaka brain, *kissr* expressing cells have been found adjacent to GnRH1 neurons, but not GnRH2 or GnRH3 neurons [53]. In the present study, a similar trend of upregulation in *kiss* and *kiss* receptors and *gnrh2* expression was observed in response to developmental exposure to BPA and EE2, suggesting an interaction between *kiss/kissr* system and GnRH neurons.

In the medaka brain, *kiss* receptors are co-expressed in isotocin and vasotocin expressing cells [53], indicating that medaka kisspeptin neurons may function via isotocin/vasotocin neurons as well [53]. GnRH regulates reproduction by binding and activating GnRH receptors of pituitary gonadotroph cells, which synthesize and secrete the gonadotropins, LH and FSH [54]. In the rat, direct

Figure 5. Relative expression of reproductive genes (*star*, *cyp11b*, *era*, and *ara*) in the testis of male medaka fish in F0 and F2 (transgenerational) after embryonic BPA or EE2 exposure of F0. The results are presented as mean $\pm$ SEM. Asterisks indicate statistical significance (* P < 0.05; *** P < 0.001).

Figure 6. Relative expression of DNA methyltransferase genes (*dnmt1*, *dnmt3aa*, and *dnmt3bb*) in the whole brain of male medaka fish in F0 and F2 (transgenerational) after embryonic BPA or EE2 exposure of F0. The results are presented as mean $\pm$ SEM. Asterisks indicate statistical significance (* P < 0.05; ** P < 0.01, *** P < 0.001).

exposure to BPA significantly increased the expression of *Gnrhr*, *Lhb*, and *Fshb* [55]. Our results of developmental BPA exposure in medaka supported the expression patterns of *gnrhr2*, *lhb*, and *fshb* observed in other organisms, except for *gnrhr1* which was opposite in the F0 generation fish. Surprisingly, developmental exposure to BPA or EE2 only downregulated expression of *fshr* among those genes examined in testes in the F0 generation. In the mouse testis, *Fshr* is expressed in spermatogonial stem cells and can be stimulated by *FSH* [56]. As *FSH* acts exclusively through *FSHR*, downregulation of *Fshr* expression in the testis may neutralize the effects of upregulation of *Fshb* in pituitary [57]. These results may partially

explain why we did not observe significant reproductive defects in fish in the F0 generation.

The transgenerational reproductive effects from early developmental exposures to BPA and EE2 have been previously demonstrated [23]. These reproductive defects were mediated through the male germ line. In the same male fish that demonstrated fertilization defects, the expression patterns of *kiss* and *kissr* were altered by both BPA and EE2 exposure. Fish in the BPA and EE2 exposure lineages expressed *kiss* and *kissr* at greater levels than fish from control lineages, and these multifold increases in expression of all *kiss* and their receptor genes in the brain of F2 generation fish were

Figure 7. Relative expression of DNA methyltransferase genes (*dnmt1*, *dnmt3aa*, and *dnmt3bb*) in the whole testis of male medaka fish in F0 and F2 (transgenerational) after embryonic BPA or EE2 exposure of F0. The results are presented as mean $\pm$ SEM. Asterisks indicate statistical significance (▽ $P < 0.08$; * $P < 0.05$; ** $P < 0.01$).

Figure 8. Differences in global genome DNA methylation (5-mC) levels in the brain and testis of adult male medaka fish in F0 and F2 (transgenerational) after embryonic BPA or EE2 exposure of F0. Global DNA methylation (5-mC) assay was validated for medaka with bisulfite sequencing of DNA [75]. The results are presented as mean $\pm$ SEM (% of whole genome). Asterisks indicate statistical significance (* $P < 0.05$) when compared with control in the same generation.

a surprise to us. This magnitude of increase was not observed for either *gnrh* or *gnrhr* expression, which could be due to the fact that *kiss/kissr* are not co-expressed in GnRH neurons in medaka [58]. Also, kisspeptin may be dispensable in fish as recently reported for zebrafish [50]. Tang et al systematically generated the zebrafish *kiss1*^(-/-), *kiss2*^(-/-), and *kiss1*^(-/-)/*kiss2*^(-/-) mutant lines as well as the *kissr1*^(-/-), *kissr2*^(-/-), and *kissr1*^(-/-)/*kissr2*^(-/-) mutant lines using transcription activator-like effector nucleases [50]. Interestingly, no impairment in spermatogenesis and folliculogenesis as well as reproductive capability was observed in any of these six mutant lines [50]. On other hand, in the present study, BPA exposure led to a decreased expression of *gnrh1*, and EE2 exposure led to the increased expression of *gnrh2* in F2 generation lineages. The GnRH–GnRHr–GTH axis is integral to reproduction in all vertebrates and their gene expression events are tightly coordinated [59, 60]. Despite massive expression of kisspeptin and their receptors in the brain of F2 generation BPA and EE2 lineage fish, we did not observe any significant changes on the expression of *gnrh* receptors in pituitary, suggesting that the observed BPA or EE2-induced transgenerational reproductive defects in medaka might not be mediated by the putative brain–pituitary–reproductive axis. Possibilities for altered behaviors cannot be ignored as environmental estrogens have been

found to affect transgenerational male behaviors in other animal models [61–63].

Transgenerational effects on the expression of reproductive genes were observed in the EE2 treatment lineage in the F2 generation, especially *lhβ* in pituitary, and *star* and *esr1* in testicular tissues. STAR is an enzyme that catalyzes the production of pregnenolone from cholesterol, the initial step of sex steroid biosynthesis [64]. Alteration in expression of *star* by EE2 exposure suggests potential effects on steroidogenesis in the F2 generation. BPA is as an estrogenic chemical and acts via estrogen receptors [65]. A recent study in zebrafish demonstrates that BPA-induced effects are mediated by nuclear estrogen receptors, mainly *erb2* [66]. It is important to determine if the developmental BPA exposure-induced transgenerational reproductive phenotypes are mediated by *erb2*. In the present study, within all the genes examined, only those expressed in brain were significantly altered in the BPA treatment lineage fish at F2 generation. Given the reproductive defects observed in the F2 generation of these lineages, there were no significant changes in genes expressed in testis and pituitary, indicating that defects in the brain may have contributed to the reproductive defects observed in the F2 generation fish of the BPA exposure group.

Analysis of reproductive genes in the brain–pituitary–gonad axis revealed a significant increase in expression of kisspeptin and their receptor genes in the brains of the F2 generation fish of both the BPA and EE2 exposure lineage groups. These fish were not directly exposed to BPA or EE2. How these effects were inherited by offspring is unknown. Both BPA and EE2 had similar patterns of transgenerational inheritance related to the expression of brain kisspeptin and its receptors in F2 generation fish, indicating the possibility for a conserved pathway in transgenerational inheritance for these reproduction defects. To address this question, we investigated the expression of DNA methylation related genes and global DNA methylation levels in brain. In the brain, the expression of *dnmt1* was significantly increased among F0 generation fish from the BPA treatment group but was significantly decreased in the F2 generation fish. Consistent with the changes of *dnmt1* expression, the global DNA methylation levels were significantly increased in the F0 generation and decreased in F2 generation of the BPA exposure group, suggesting epigenetic regulation of transgenerational inheritance of BPA-induced somatic gene expression patterns in the brain. In contrast, there were no significant changes of *dnmt1* expression or global DNA methylation in EE2 treatment group in either generation, indicating that mechanisms by which BPA and EE2 act on transgenerational inheritance of somatic gene expression changes may be different. The literature suggests that several epigenetic mechanisms act in concert to regulate gene transcription and that DNA methylation contributes only a part of the regulation of gene expression [67, 68]. The observed expression of DNA methyltransferase genes in somatic tissues and alterations in transgenerational global DNA methylation levels present a global picture of transgenerational epigenetic alterations in the male reproductive axis. Global DNA methylation changes generated by 5mC-ELISA, however, cannot be correlated with transcription of individual target genes because of the lack of base-pair resolution picture of the promoter region. Future studies will address this issue by sequencing the whole epigenome of the brain and testicular somatic cells affected by ancestral BPA and EE2 exposure. In inland silverside, *Menidia beryllina*, whole body epigenome and transcriptome analyses revealed significant differences in the promoter and/or gene body methylation in EE2-exposed fish relative to controls and all generations (F0–F2), indicating an association of inheritance of DNA methylation alterations with both multigenerational (F1) and transgenerational (F2) effects that were observed [69]. In zebrafish, epigenetic alterations are associated with gonad homeostasis [70] and sexual development [71]. Embryonic exposure to a high concentration of BPA (4000 µg/L) did not alter global DNA methylation in sperm but reduced H3K27ac levels in zebrafish, suggesting alterations in histone acetylation profile [72]. Epigenetic mechanisms work in concert with each other to control gene expression. Hence, it is imperative to examine all epigenetic modifications associated with BPA or EE2-induced adverse health phenotypes using appropriate methodologies.

Conclusions

A conserved pattern of expression of key genes in the reproductive axis, especially upregulation of kisspeptin and their receptors was observed, suggesting that transgenerational impacts of ancestral BPA or EE2 exposure occurred not only in gonads, but can also be found in the brain. Taken together, the present results demonstrated BPA- and EE2-induced transgenerational alterations in male fertility and expression of reproductive genes in brain and testis and global DNA methylation in the testis.

Materials and methods

Ethics statement

All animal work was performed according to the guidelines stipulated by institutional animal care and use committee (IACUC) of the University of North Carolina Greensboro (UNCG) protocol number 16–003 and according to the procedures described by American Institute of Fishery Research Biologists (AIFRB) Guidelines for Use of Fishes in Field Research, and with all US Geological Survey CERC guidelines for the humane treatment of test organisms during culture and experimentation. Experimental protocols and the study plan was approved by USGS-CERC and UNCG Institutional Animal Care and Use Committee.

Animals

The animals and chemical exposure procedure have been previously described [23, 73]. In brief, Hd-rR strain of medaka (*Oryzias latipes*) was maintained at the USGS Columbia Environmental Research Center (CERC) under standard conditions. Water temperature was maintained at 26 ± 0.5 °C throughout the study. All animal procedures were conducted in accordance with the procedures described by the American Institute of Fishery Research Biologists (AIFRB), “Guidelines for Use of Fishes in Field Research”; and with all US Geological Survey CERC guidelines for the humane treatment of test organisms during culture and experimentation. Experimental protocols and the study plan were approved by USGS-CERC Institutional Animal Care and Use Committee.

Chemical exposure

Chemical exposure procedures were described previously [23, 73]. Bisphenol A (BPA, CAS no. 80-05-7, purity $\geq 99\%$) was purchased from Sigma-Aldrich (Cat. 239658). Fertilized eggs (F0 generation) were collected from 10 breeding pairs, pooled, sorted, and assigned to nine glass petri dishes. Each petri dish contained 50 fertilized eggs in 50 mL well water. Embryos were kept in glass petri dishes for another 8 h to grow to blastula stage without exposure. During this stage [74], medaka embryos complete the event of epigenetic reprogramming [75], so the embryo reprogramming was not affected by exposure. For exposure, three petri dishes were randomly selected for each treatment group (control, BPA, or EE2), so each exposure group had three biological replicates (three petri dishes per treatment group, each petri dish containing 50 embryos, see [Supplementary Figure S1](#)). Eight hours after fertilization, water in the petri dishes was replaced with exposure solution containing BPA at a concentration of 100 µg/L, or EE2 µg/L at a concentration of 0.05 µg/L in 0.001% ethanol (solvent). Measured concentrations of test chemicals and their uptake by embryos has been published elsewhere, i.e., the total uptake of BPA was 178 pg/mg egg and EE2 was 4.0 pg/mg egg after 7 days of exposure [23]. The control water and test chemical solutions were replaced daily until 7 days post fertilization (dpf). There were no further chemical exposures for these fish (F0) or in any subsequent generations. Specifically, F0 fish were either unexposed (controls) or directly exposed as embryos (7 days) to the test chemical, whereas F1 fish were exposed only as primordial germ cells while in F0 embryo gonads. Therefore, the first transgenerational phenotype was examined in offspring at the F2 generation. No analysis of F1 gene expression was conducted in this study.

In each generation, a total of 6 adult pairs (12 fish, 6 males and 6 females) from the same exposure group lineage were randomly selected and mated on the 150 dpf stage, overlapping the second

spawning cycle, to produce offspring for each subsequent generation. Each exposure group contained three biological replicate tanks (lineages) at all times.

Crossbreeding of fish and morphometric data collection

To determine whether males or females contributed to a reduced fertilization rate in the F2 generation, 6 males from the control lineage were paired with 6 females from the treated lineage and 6 females from the control lineage were mated with 6 males from the treated lineage for each biological replicate. Total egg production/per day was recorded, and fertilization rate was determined per tank per day. The data were collected continuously for 17 days. On the day of sample collection, morphometric measurements of body (weight and total length) gonads and liver were taken. Fish were dried with a paper towel to remove water from body before wet weight measurement. Total length was measured from tip of the snout to the end of the tail. GSI was calculated as [(gonad weight/body weight) × 100] and hepatosomatic index was calculated as [(liver weight/body weight) × 100].

Sample collection and DNA/RNA extraction

In each generation examined (F0 and F2), brain, pituitary, and testes tissues were collected from three adult male fish of each biological replicate and frozen in liquid nitrogen, then stored in a −80 °C freezer at CERC until analysis. Each exposure group had three biological replicates, so total of nine tissue samples were collected from each exposure treatment group for downstream analysis. Tissue samples were transferred to the Bhandari laboratory at the University of North Carolina Greensboro for molecular analysis. DNA/RNA extraction was performed using the procedure as described previously [23, 73]. The tissue samples were homogenized using a tissue homogenizer, genomic DNA and total RNA were extracted using Qiagen All Prep DNA/RNA/miRNA extraction kit according to the manufacturer's instructions. DNA and RNA were quantified by nanodrop 2000 (ThermoFisher Scientific, USA) and stored at −80°C until further analysis.

Real-time quantitative RT-PCR

cDNAs were synthesized using both Oligo(dT) and random primers with M-MLV reverse transcriptase enzyme system (ThermoFisher Scientific, USA) as described previously [23, 73]. Real time qPCR was conducted using PowerUp SYBR Green PCR Master Mix (Applied Biosystems, USA) with an Applied Biosystems StepOne Plus real-time PCR system (Applied Biosystems, USA) with the following temperature regimes: 2 min at 50 °C and 10 min at 95 °C for pre-incubation, 40 cycles at 95 °C (15 s), and 60 °C (1 min). A total of 4 ng cDNA was used per reaction, and two technical replicates per reaction were used. Five different internal control genes were verified for their consistency in expression pattern. The *ef1a* gene was selected as an internal control for qPCR assay of brain samples, and 18S rRNA was used for pituitary and testes samples. A list of primers is provided in the [Supplementary Table S1](#). Some of these primers have previously been validated [37]. Primer sequences spanned at least one junction of exon and intron to avoid amplification of genomic DNA. Each pair of primers was examined to make sure only a single product was amplified and verified by melting curve and agarose gel electrophoresis [76, 77]. The efficiency of qPCR has been presented in [Supplementary Table S1](#) and [Supplementary Figure S4](#).

Global DNA methylation quantification assay

Global DNA methylation was measured using 5-mC DNA ELISA Kit (ZymoResearch, USA) according to the manufacturer's instructions, as described previously [75]. Total of 100 ng genome DNA was used for each technical replicate, and two technical replicates were used for each biological replicate. Absorbance was measured at 405 nm using BioTek Synergy2 plate reader. To quantify the percentage of 5-mC, the negative control (100 ng/μL) and positive control (100 ng/μL) were premixed to generate a set of standards with known 5-mC percentage and assayed in parallel with the samples. A logarithmic second-order regression was calculated to determine the relation of 5-mC percentage and absorbance, using the equation below:

$$\%5 - mC = e^{\left\{ \frac{(\text{Absorbance} - y_{\text{intercept}})}{\text{Slope}} \right\}}$$

Then, the 5-mC percentage was corrected with the medaka CpG density. According to the manufacturer, the *Escherichia coli* CpG density/genome length is 0.075, and medaka CpG density/genome length is 0.019 (calculated according Ensembl Genome Release, version 89). The fold difference between *E. coli* and medaka CpG density is 3.94, and all genome DNA methylation ratios were corrected accordingly using the multiplication factor of 3.94.

Statistical analysis

Target gene expression was analyzed by the $2^{-\Delta\Delta Ct}$ method [78]. Expression of each gene has been expressed as relative quantification, fold change against control. Data represent mean ± SEM. Significant differences between the means of three exposure groups in each generation was determined by one-way ANOVA followed by Tukey multiple comparison test (MCT). Differences with $P < 0.05$ were considered significant. Asterisks indicate statistical significance (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Egg production data were calculated using a repeated measure design on Graph pad Prism using *t*-test for the targeted comparison between control and each exposed group, whereas one-way ANOVA was used to analyze effects among all groups. For F2 mating trials, two-way ANOVA was used (sex vs. chemical) followed by Tukey MCT.

Supplementary material

[Supplementary material](#) is available at *BIOLRE* online.

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Conflicts of interest

All authors have no conflicts of interests.

Disclosures

All authors have no competing financial interests.

Disclaimer

Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Author contributions

Conceived experiments: RKB, DET, FVS; provided space and reagent: DET, FVS, RKB; performed experiment: RKB, PB, AJT, XW; data analysis: RKB, AJT, XW; wrote manuscript and prepared figures: AJT, RKB, XW; proofread and improved manuscript: RKB, DET, FVS, XW.

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