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SHORT COMMUNICATION

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Optogenetic induction of chronic glucocorticoid exposure in early-life leads to blunted stress-response in larval zebrafish

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Abstract

Early life stress (ELS) exposure alters stress susceptibility in later life and affects vulnerability to stress-related disorders, but how ELS changes the long-lasting responsiveness of the stress system is not well understood. Zebrafish provides an opportunity to study conserved mechanisms underlying the development and function of the stress response that is regulated largely by the neuroendocrine hypothalamus–pituitary–adrenal/interrenal (HPA/I) axis, with glucocorticoids (GC) as the final effector. In this study, we established a method to chronically elevate endogenous GC levels during early life in larval zebrafish. To this end, we employed an optogenetic actuator, *beggiatoa* photoactivated adenylyl cyclase, specifically expressed in the interrenal cells of zebrafish and demonstrate that its chronic activation leads to hypercortisolaemia and dampens the acute-stress evoked cortisol levels, across a variety of stressor modalities during early life. This blunting of stress–response was conserved in ontogeny at a later developmental stage. Furthermore, we observe a strong reduction of *proopiomelanocortin* (*pomc*)-expression in the pituitary as well as upregulation of *fkbp5* gene expression. Going forward, we propose that this model can be leveraged to tease apart the mechanisms underlying developmental programming of the HPA/I axis by early-life GC exposure and its implications for vulnerability and resilience to stress in adulthood.

KEYWORDS
cortisol, early-life stress, glucocorticoids, hypothalamus-pituitary–adrenal Axis, optogenetics, zebrafish

Abbreviations: ACTH, Adrenocorticotropin hormone; bPAC, Beggiatoa Photoactivated Adenylate Cyclase; CRH, Corticotropin-Releasing Hormone; DPF, Days Post Fertilization; ELS, Early Life Stress; FKBP5, FK506-binding protein 51; GC, Glucocorticoid; GR, Glucocorticoid receptor; HPA/I, Hypothalamus-Pituitary–Adrenal/Interrenal Axis; ISH, In-Situ Hybridization; POMC, Proopiomelanocortin; PTSD, Post-Traumatic Stress Disorder; StAR, Steroidogenic Acute Regulatory Protein.

1 | INTRODUCTION

Organisms respond to stressors by mounting an integrated physiological and behavioural adaptive stress response, mediated by the neuroendocrine Hypothalamus–Pituitary–Adrenal (HPA) axis (Chrousos, 2009). This allostatic maintenance can be challenged by cumulative stressor exposure, especially in the critical period of early life, leading to compromised adaptive brain–body responses and hence vulnerability to stress-related disorders including depression, anxiety and post-traumatic stress disorder (Agorastos & Chrousos, 2022; Lupien et al., 2009). The HPA axis is a cascade of physiological events culminating in the release of glucocorticoid (GC), a pleiotropic effector of the stress response (de Kloet et al., 2005). GC regulates its own release by negative feedback on the secretion of corticotropin-releasing hormone (CRH) and adrenocorticotropin hormone (ACTH) from the hypothalamus and proopiomelanocortin *(pomc)*-positive pituitary corticotrophs, respectively, acting via the GC receptor (GR) (Charmandari et al., 2005). The stress response is also shaped by the co-chaperone FK506-binding protein 51 (FKBP5/FKBP51). GR activation leads to the induction of FKBP5 expression, and with the inhibitory effect of FKBP5 on GR activity, it generates an ultra-short, negative feedback loop (Zannas et al., 2016). Additionally, polymorphisms in the FKBP5 gene have been associated with stress-related disorders in humans and rodents (Matosin et al., 2018).

Early-life dysregulation of the HPA-axis can cause maladaptive developmental and functional maturation of stress response machinery, which may provide a mechanistic basis for altered stress susceptibility in later life (Chen & Baram, 2016; Maccari et al., 2014; van Bodegom et al., 2017). To date, there are many studies that investigate the effects of ELS on the structure and function of the HPA-axis and associated regulatory regions (amygdala, hippocampus and prefrontal cortex), particularly addressing neuroendocrine consequences (Agorastos et al., 2019; van Bodegom et al., 2017). These alterations, primarily studied in rodent models, are dependent on the developmental time window affected, the sex of the individual and the developmental stage at which effects are assessed. Furthermore, HPA axis dysfunction is a hallmark in a variety of neuropsychiatric diseases (e.g. depression and PTSD) (Daskalakis et al., 2016; Pariante & Lightman, 2008). Many studies, encompassing rodent and human subjects exposed to ELS or chronic stress, have reported a blunted HPA axis response as a defining feature underlying the pathophysiology (Albeck et al., 1997; Bunea et al., 2017; Burke et al., 2005; Carpenter et al., 2007; Carroll et al., 2017; Cohen et al., 2006; Grimm et al., 2014; Heim et al., 2001; Lam et al., 2019; Ouellet-Morin et al., 2011; Tomiyama et al., 2011; Xin et al., 2020). Also, exogenous treatment with GC or GR agonist has been shown to result in blunting of the endogenous stress response (Felszeghy et al., 2000; Kinlein et al., 2015). However, although most findings support a causal relationship between early-life HPA axis dysfunction and maladjustment in later life, precise neurodevelopmental trajectories remain to be elucidated.

Larval zebrafish with external development, excellent genetic and developmental analysis tools and conserved stress-response machinery present an ideal complement to rodents to study the effects of stress on neurodevelopment and behaviour (de Abreu et al., 2021; Eachus et al., 2021; Gerlai, 2020). Importantly, the hypothalamus–pituitary-interrenal (HPI) axis in zebrafish is homologous to the HPA axis in mammals (Flik et al., 2006; Wendelaar Bonga, 1997) and larval zebrafish respond to stressors with increased cortisol (main GC in fish as in humans), indicative of a stress response that matures early in development (Alderman & Bernier, 2009; Alsop & Vijayan, 2008; Fuzzen et al., 2010; Steenbergen et al., 2011). Furthermore, comparative anatomical approaches have identified evolutionarily conserved chemo-architecture of stress-relevant hypothalamic regions (Gutierrez-Triana et al., 2014; Herget et al., 2014; Nagpal et al., 2019) and methods have been established to manipulate the endogenous level of GCs using optogenetic tools thereby allowing controlled GC exposure with unprecedented specificity and temporal resolution (De Marco et al., 2013, 2016; Gutierrez-Triana et al., 2015). Indeed, transgenic zebrafish with targeted expression of *beggiatoa* photoactivated adenylyl cyclase (bPAC) (Ryu et al., 2010; Stierl et al., 2011) both in pituitary corticotrophs, which produce ACTH (De Marco et al., 2013) and in steroidogenic cells that produce GCs within interrenal gland (Gutierrez-Triana et al., 2015) have been generated. Using this optogenetic approach, endogenous GC level was increased acutely upon blue light stimulation, and it was demonstrated that the pituitary–adrenal system exerts a rapid organizing effect on behaviour in larval zebrafish (De Marco et al., 2016).

Here, we leveraged optogenetic GC induction in larval zebrafish (Gutierrez-Triana et al., 2015) to achieve chronic elevation of cortisol during early life. We then investigated the effects of early-life GC exposure on basal and stress-induced cortisol changes. We further characterized the effects of endogenous GC elevation on *pomc* and *fkbp5* expression in this model.

2 | MATERIALS AND METHODS

2.1 | Zebrafish husbandry and maintenance

Zebrafish breeding and maintenance were performed under standard conditions on a 12:12 light/dark cycle (Westerfield, 2000). *Tg(2kbStAR:bPAC-2A-tdTomato)hd19* (Gutierrez-Triana et al., 2015) were bred with wild-type AB/TL zebrafish strain and the embryos were raised in egg water (3 g sea-salt/10 L ultrapure water) at 28°C inside an incubator (RuMed 3101, Rubarth Apparate Germany). Transgenic and non-transgenic siblings were screened at 3 days post fertilization (dpf) on a fluorescent stereomicroscope and transferred to petri dishes with egg water for experiments at later time points. bPAC is activated strongly at UV-blue wavelengths of the spectrum (370–500 nm) with activation light intensities in the μW ranges (De Marco et al., 2013, 2016; Ryu et al., 2010; Stierl et al., 2011), which is amply provided by the white light illumination in the incubator. For experiments requiring avoiding activation of bPAC, *Tg(2kbStAR:bPAC-2A-tdTomato)* embryos were raised in custom-made containers covered by 550 nm long-pass filters (Thor- labs). Larvae older than 6 dpf were transferred to plastic tanks with 400 ml of egg water in groups of 30 and fed with paramecia daily. Zebrafish experimental procedures were carried out in compliance with the ethical guidelines of the German animal welfare law and approved by the local government (Landesuntersuchugsamt Rheinland-Pfalz, Germany – 23 177-07/G20-1-033 and UK Home Office PPL number PEF291C4D).

2.2 | Stressor treatment

2.2.1 | Osmotic and pH stressor

A hyperosmotic solution of 100 mM NaCl and a low pH solution of 1 mM HCl were employed as stressors (Castillo-Ramírez et al., 2019; De Marco et al., 2013; Gutierrez-Triana et al., 2015). Stock solutions of NaCl (from Sigma) and HCl (from Sigma) prepared in egg water were added to the petri dishes with zebrafish larvae (raised at fixed density for experiments) to achieve final concentrations of 100 mM NaCl and 1 mM HCl, which constituted the osmotic and pH stress, respectively. Samples were collected 10 minutes after the administration of stock solutions.

2.2.2 | Mechanical stressor

A previously established mechanical stress protocol (Castillo-Ramírez et al., 2019) that generated perturbation of the medium was employed. Petri dishes (inner diameter: 3.5 cm) containing 30 larvae and a plastic-covered stirrer (magnetic stir bar, micro PTFE 6 mm × 3 mm; Fisher Scientific) were placed on a magnetic stir plate (Variomag Poly 15 stirrer plate; Thermo Scientific) and kept at 28°C inside an incubator. The mechanical stress protocol was performed for 3 minutes at 330 rpm. Samples were collected 10 minutes after the onset of stimulation. Control larvae were collected after equal handling, omitting exposure to vortex flows (i.e., stir bars inside the Petri dishes were absent).

2.3 | Whole body cortisol measurement

The procedures for cortisol extraction were as previously described (De Marco et al., 2016; Yeh et al., 2013). Unexposed larvae (control samples) were collected after equal handling, omitting stressor exposure. Larvae were immobilized using ice-cold water, frozen in an ethanol/dry ice bath and stored at −20°C for subsequent extraction. Each replicate consisted of 15 larvae and stimulation and sample collection were carried out between 10:30 am and 13:00 pm. The cortisol ELISA assay was performed as per manufacturer's instructions using the Cisbio HTRF cortisol ELISA kit (62CRTPEG, Cisbio, Perkin Elmer) and measurements were performed on Tecan Infinite M1000 Pro multi-plate reader.

2.4 | Fluorescent in situ hybridization

Fluorescent in situ hybridization (FISH) for *pomc* was performed as described (Herget et al., 2014), using a *pomca* in situ probe (Herzog et al., 2003). Larvae were imaged in 80% glycerol in PBS using a 20x objective on a Leica SPE confocal microscope. Subsequent image processing and evaluation were performed on ImageJ as described (Herget et al., 2014). *fkbp5* ISH was performed using an *fkbp5* mRNA probe (Eachus, Oberski, et al., 2023) and whole brains were imaged on a Zeiss LSM 880 airyscan confocal microscope using a 10X objective. Fluorescent intensity of *fkbp5* mRNA expression was analysed from z-stacks of the whole brain using ImageJ; with hoechst staining to identify regions as previously described (Eachus, Choi, et al., 2023).

2.5 | RT-qPCR

Larvae were snap-frozen in dry ice, with each independent replicate comprising 15 larvae. RNA was extracted using the Quick-RNA Microprep Kit (Zymo Research) after grinding the tissue with an electric homogenizer (Kimble, VWR). RNA concentration was measured using a Nanodrop ND2000. cDNA was prepared with the High-Capacity RNA-to-cDNA Kit (Applied Biosystems) using between 1.5 µg and 2 µg of total RNA per 20 µl-reaction. qPCR was performed in a 96-well plate (Sapphire Microplatte, Greiner) with PowerUp SYBR Green Mastermix (Applied Biosystems) in the CFX BioRad Real-Time PCR Detection System following its recommended protocols. Primers (*fkbp5* F: 5′ GTGTTCGTCCACTACACC R: 5′ TCTCCTCACGATCCCACC; *rpl13a* F: 5′ TGGTGAGGTGTGAGGGTATCAAC R: 5′ AATTTG CGTGTGGGTTTCAGAC; *sep15* F: 5′ TATTGTTGATTGTTGCTGAGGG R: 5′ ACGCTGAGAGATGTACACAGGA (Xu et al., 2016); *pomc* F: 5' GAAGAGGAATCCGCCGAAA R: 5' CCAGTGGGTTTAAAGGCATCTC; *loopern4* F: 5′ TGAGCTGAAACTTTACAGACACAT R: 5' AGACTTTGGTGTCTCCA GAATG; *dna15ta1* F: 5' TACTGTGCTCAAATTGCTTCA and R 5' AATGAGTACTGTGAACTTAATCCAT (Vanhauwaert et al., 2014). For analysis, the $2^{-\Delta\Delta Ct}$ method was applied using the *rpl13a* and *sep15*, and *loopern4* and *dna15ta1* as housekeeping genes for two independent experiments for *fkbp5* and *pomc*, respectively.

2.6 | Statistics

All data are shown as bars or single measurement points, mean and standard error of the mean (S.E.M.). ANOVA was used for multiple group comparisons followed by Tukey's post hoc tests. Analyses were carried out using MS Excel (Microsoft) and Prism 5 (Graphpad Software Inc.).

3 | RESULTS

3.1 | Non-invasive elevation of GC level using optogenetics

To achieve endogenous GC increase in a non-invasive fashion, we utilized our previously reported (Gutierrez-Triana et al., 2015) transgenic line expressing the light-activated *beggiatoa* photoactivated adenylate cyclase (bPAC) (Ryu et al., 2010; Stierl et al., 2011) specifically in the steroidogenic interrenal cells, the fish counterpart of the adrenal gland (Figure 1). This transgenic line, *Tg (2kbStAR:bPAC-2A-tdTomato)*, employs a 2 kb promoter element of the steroidogenic acute regulatory protein (StAR), a rate-limiting mediator of steroid hormone biosynthesis (Stocco, 2000), to drive the expression of bPAC coupled to the fluorescent protein tdTomato exclusively in larval steroidogenic interrenal cells. Upon binding to its receptor, ACTH leads to cAMP production and Ca^{2+} influx in adrenocortical cells, which triggers the synthesis and release of GC (Gallo-Payet & Payet, 2003). Acute blue-light stimulation of the transgenic larvae has been shown to elicit transient hypercortisolemia (Gutierrez-Triana et al., 2015). bPAC, though with its peak activation in the blue wavelength range, has a broad activation spectrum with high light sensitivity (in µW range) (Ryu et al., 2010; Stierl et al., 2011). We reasoned, that to achieve chronic elevation of endogenous GC levels, the white light from the raising incubator in early-life (0 till 6 dpf) could be used to activate bPAC. Thus, the fish larvae were raised under a standard 12:12 light/dark cycle being exposed to white light for 12 hours per day from 0 dpf until the post-hatching stage of 6 dpf.

3.2 | Chronic early-life elevation of glucocorticoids leads to elevated basal cortisol levels and blunted stress response at 6 dpf and 12 dpf

As a function of chronic early-life GC elevation, we measured the basal and acute stress-evoked whole-body cortisol levels of *Tg(2kbStARp:bPAC-2A-tdTomato)* larvae and their non-transgenic siblings at 6 dpf. The basal cortisol levels were significantly elevated in transgenic fish as compared to the non-transgenic siblings, indicating a state of basal hypercortisolemia (two-way ANOVA, stressor: $F(1, 61) = 64.29$, $p < 0.0001$; genotype: $F(1, 61) = 17.68$, $p < 0.0001$; stressor X genotype: $F(1,61) = 83.41$, $p < 0.0001$; followed by Tukey's post-tests for within stressor-genotype pair comparisons, $p < 0.01$) (Figure 2A). The osmotic stress exposure strongly elevated the cortisol levels in non-transgenic siblings indicating a physiological stress-response (two-way ANOVA followed by Tukey's post hoc tests, $p < 0.0001$). However, there was no significant increase in cortisol levels for the transgenic larvae (Figure 2A), demonstrating a blunted stress response. A similar phenotype of basal hypercortisolemia (two-way ANOVA, stressor: $F(1, 26) = 24.93$, $p < 0.0001$; genotype: $F(1, 26) = 56.94$, $p < 0.0001$; stressor X genotype: $F(1,26) = 38.89$, $p < 0.0001$; followed by Tukey's post-tests for within stressor-genotype pair comparisons, $p < 0.0001$) and blunted cortisol response to osmotic stress was conserved in ontogeny at 12 dpf in transgenic fish (Figure 2B).

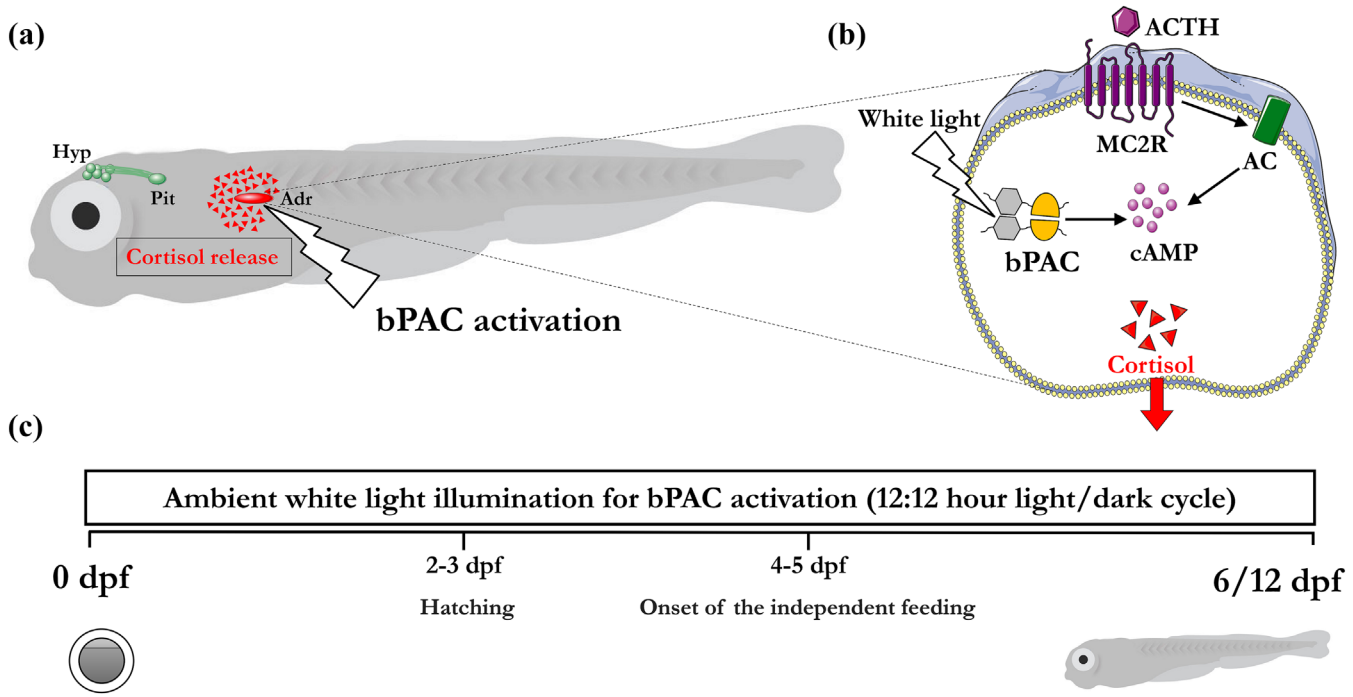

FIGURE 1 **A.** Activation of the hypothalamo-pituitary complex (green) by a stressor leads to the release of ACTH, which further acts on adrenal (interrenal in zebrafish) cells (red) to induce the release of cortisol (red triangles). Illumination with white light activates bPAC expressed in interrenal cells of *Tg(2kbStAR:bPAC-2A-tdTomato)*, thereby increasing cortisol output in transgenic animals. **B.** Schematic representation of the intracellular signal transduction in the interrenal cells and their optogenetic activation using photoactivatable adenylyl cyclase bPAC, increasing cortisol release (red triangles). **C.** Pictorial representation of the experimental design showing white light illumination (12:12 hour light/dark cycle) from 0 till 6 dpf or 12 dpf to induce early-life GC induction in zebrafish. Abbreviations: Hyp hypothalamus, pit pituitary, Adr adrenal (Interrenal) MC2R Melanocortin 2 receptor (ACTH) receptor, AC adenylyl cyclase, bPAC Beggiatoa photo-activated adenylyl cyclase.

We next investigated whether the blunted stress response phenotype was dependent on stressor modality. To this end, we exposed the *Tg(2kbStARp:bPAC-2A-tdTomato)* transgenic larvae and non-transgenic siblings at 6 dpf to different stressors: pH stress and mechanical stress (Figure 2C, D). pH Stress was induced by a sudden pH drop in the medium by the addition of HCl. For mechanical stress, we used a previously established paradigm of inducing perturbation of the medium by magnetic bead stirring. For mechanical stress, embryos were raised in smaller petri dishes which can be accommodated on the magnetic stir plate, which led to different basal cortisol levels. Significant basal hypercortisolemia was observed in transgenic larvae, and importantly, the levels of cortisol were not elevated upon exposure to either of the stressors for transgenic larvae as compared to non-transgenic siblings (pH Stress: two-way ANOVA, stressor: $F(1, 40) = 158.0$, $p < 0.0001$; genotype: $F(1, 40) = 18.64$, $p = 0.0001$; stressor X genotype: $F(1,40) = 84.62$, $p < 0.0001$; followed by Tukey's post-tests for within stressor-genotype pair comparison. Mechanical stress: two-way ANOVA, stressor: $F(1, 39) = 11.84$, $p = 0.0014$; genotype: $F(1, 39) = 0.96$, $p = 0.33$; stressor X genotype: $F(1,39) = 28.32$, $p < 0.0001$; followed by Tukey's post-tests for within stressor-genotype pair comparison). This indicated that the blunting of the cortisol response to acute stress in transgenic larvae was also observed using different stressor modalities.

In order to ascertain that these response dynamics were causally linked with optogenetic activation of bPAC, we raised the *Tg(2kbStARp:bPAC-2A-tdTomato)* transgenic larvae under conditions that restrict the activation light for bPAC (using a 550 nm long pass filter) and thus would inhibit the bPAC-mediated optogenetic activation of interrenal cells (Figure 2E). We observed that the basal cortisol levels of filter-raised larvae at 6 dpf were significantly lower as compared to those larvae that had been raised under activating white light (two-way ANOVA, stressor: $F(1, 12) = 8.773$, $p = 0.011$; filter: $F(1, 12) = 6.148$, $p = 0.029$; stressor X filter: $F(1,12) = 23.70$, $p = 0.004$; followed by Tukey's post-tests for within stressor-genotype pair comparison). Additionally, there was no blunting of the stress response observed as there was a significant elevation of cortisol upon 100 mM NaCl exposure in filter-raised larvae (two-way ANOVA followed by Tukey's post hoc tests, $p < 0.001$). These

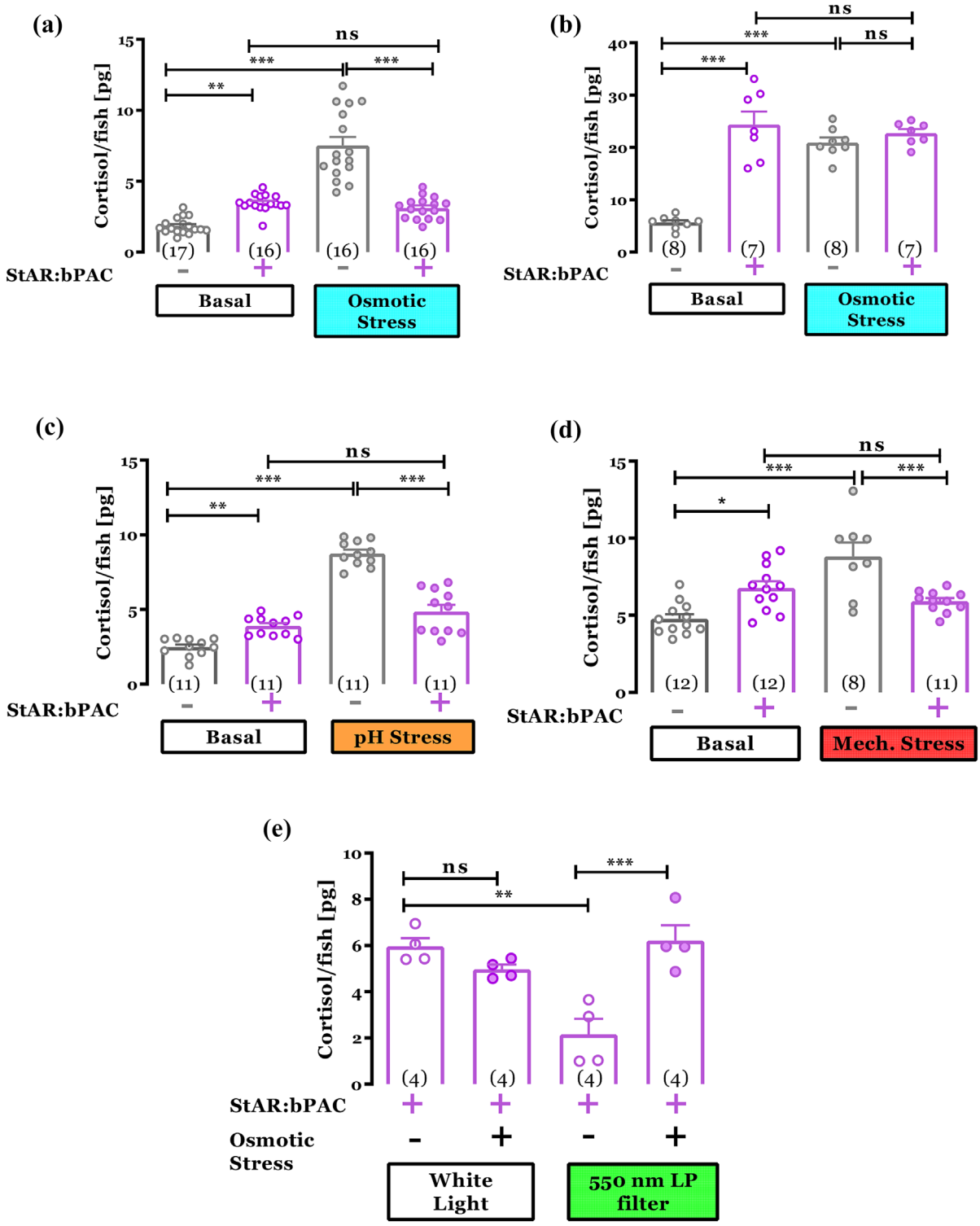

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FIGURE 2 Chronic optogenetic induction of glucocorticoids leads to a state of basal hypercortisolemia and blunted cortisol response to stressors in *Tg(2kbStARp:bPAC-2A-tdTomato)* larvae relative to non-transgenic siblings. **A.** Basal (open circles) and 100 mM NaCl osmotic stress (filled circles) evoked cortisol concentrations in bPAC-negative (−) (gray) and bPAC-positive (+) (purple) 6-dpf progenies of the *Tg (2kbStARp:bPAC-2A-tdTomato)* line. **B.** Basal and 100 mM NaCl osmotic stress evoked cortisol concentrations in bPAC-negative (−) (gray) and bPAC-positive (+) (purple) 12-dpf progenies of the *Tg(2kbStARp:bPAC-2A-tdTomato)* line. **C.** Basal and 1 mM HCl pH stress evoked cortisol concentrations in bPAC-negative (−) (gray) and bPAC-positive (+) (purple) 6-dpf progenies of the *Tg(2kbStARp:bPAC-2A-tdTomato)* line **D.** Basal and mechanical stress evoked cortisol concentrations in bPAC-negative (−) (gray) and bPAC-positive (+) (purple) 6-dpf progenies of the *Tg(2kbStARp:bPAC-2A-tdTomato)* line. **E.** Basal and 100 mM NaCl osmotic stress evoked cortisol concentrations in bPAC-positive (+) (purple) 6-dpf progenies of the *Tg(2kbStARp:bPAC-2A-tdTomato)* line, raised under activating white light and non-activating 550 nm long-pass (LP) filter. The number of samples (one sample comprising of 15 larvae) are indicated in the parenthesis in individual bar plots for all conditions. The individual values are depicted in circles, and the bar represents mean with standard error of the mean. Statistical analyses involved two-way ANOVA followed by Tukey's posthoc test: ns $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

results indicate that the blunted response is due to the hypercortisolism state induced by the activation of the optogenetic protein bPAC.

3.3 | Effects of chronic GC induction on *pomc* and *fkbp5* gene expression

Having ascertained the effects on cortisol levels, we next tested whether optogenetic elevation of GC leads to the regulation of two well-known GC target genes– *pomc* and *fkbp5* (Liu et al., 2003; Zannas et al., 2016). To evaluate the effect of chronic GC exposure on *pomc* and *fkbp5* gene expression, we performed fluorescent in situ hybridization and real-time quantitative PCR experiments. At 6 dpf, we observed strong suppression of *pomc* expression in the pituitary corticotrophs present in the rostral *pars distalis* region of the pituitary of *Tg(2kbStARp:bPAC-2A-tdTomato)* larvae relative to non-transgenic siblings at 6 dpf (unpaired two-tailed t-test, $p < 0.001$), while other *pomc*-expressing cells in the hypothalamus and in *pars intermedia* region of the pituitary appear less affected (Figure 3A,B). *fkbp5* expression was found to be increased in the hypothalamus and pituitary of transgenic fish in the in situ hybridization (unpaired two-tailed t-test, $p < 0.01$ for hypothalamus and $p < 0.001$ for pituitary) (Figure 3D, E). RT-qPCR was employed to quantitatively evaluate the effects of chronic early-life GC exposure on whole-body gene expression of *fkbp5 and pomc*. The gene expression for *fkbp5* was strongly upregulated in *Tg(2kbStARp:bPAC-2A-tdTomato)* transgenic larvae compared to non-transgenic siblings raised in white light at 6 dpf (unpaired two-tailed t-test, $p = 0.0011$), while *pomc* expression was decreased in transgenic larvae (unpaired two-tailed t-test, $p = 0.11$) (Figure 3C,F). These results indicate that optogenetic elevation of GC in larval zebrafish efficiently activates GC-induced regulatory mechanisms.

4 | DISCUSSION

Here, we utilized an optogenetic approach to develop a method for chronic GC elevation in early life in larval zebrafish. Specifically, we leveraged the broad activation spectrum (350-500 nm) and high light sensitivity (μW range) of bPAC (Ryu et al., 2010; Stierl et al., 2011) to induce its activation in *Tg(2kbStARp:bPAC-2A-tdTomato)* transgenic fish (Gutierrez-Triana et al., 2015) via the standard white light conditions used for raising the fish from 0 to 6 dpf or 12 dpf. As a result of this bPAC-led optogenetic chronic activation of GC-producing interrenal cells, we observed significantly elevated levels of basal cortisol at 6 dpf, a developmental stage where the HPI axis has matured in larval zebrafish and the larvae respond robustly to stressors with an increase in cortisol (Alsop & Vijayan, 2008; De Marco et al., 2013). Basal hypercortisolemia is observed in human conditions such as adrenal carcinoma or hyperplasia, Cushing's syndrome and neuropsychiatric conditions (Dienes et al., 2013; Pariante & Lightman, 2008; Vreeburg et al., 2009).

The other robust phenotype observed in our transgenic model across three different stressors (osmotic, pH, mechanical) is the severe blunting of the cortisol response to stress. This phenocopies the effects on the cortisol response observed in multiple studies where subjects (rodents and humans) were exposed to ELS or a GR agonist and their stress-responsivity was evaluated at a later time point (Carpenter et al., 2007; Elzinga et al., 2008; Felszeghy et al., 2000; Heim et al., 2001; Kinlein et al., 2015, 2019; Macmillan et al., 2008; Ouellet-Morin et al., 2011). Both phenotypes of basal hypercortisolaemia and blunted cortisol response were not observed in bPAC-restrictive illumination conditions (raising the larvae under 550 nm long pass filter), providing evidence of the optogenetic interrenal activation as the underlying cause for the observed cortisol phenotypes. Although the focus was indeed on the cortisol phenotype, bPAC

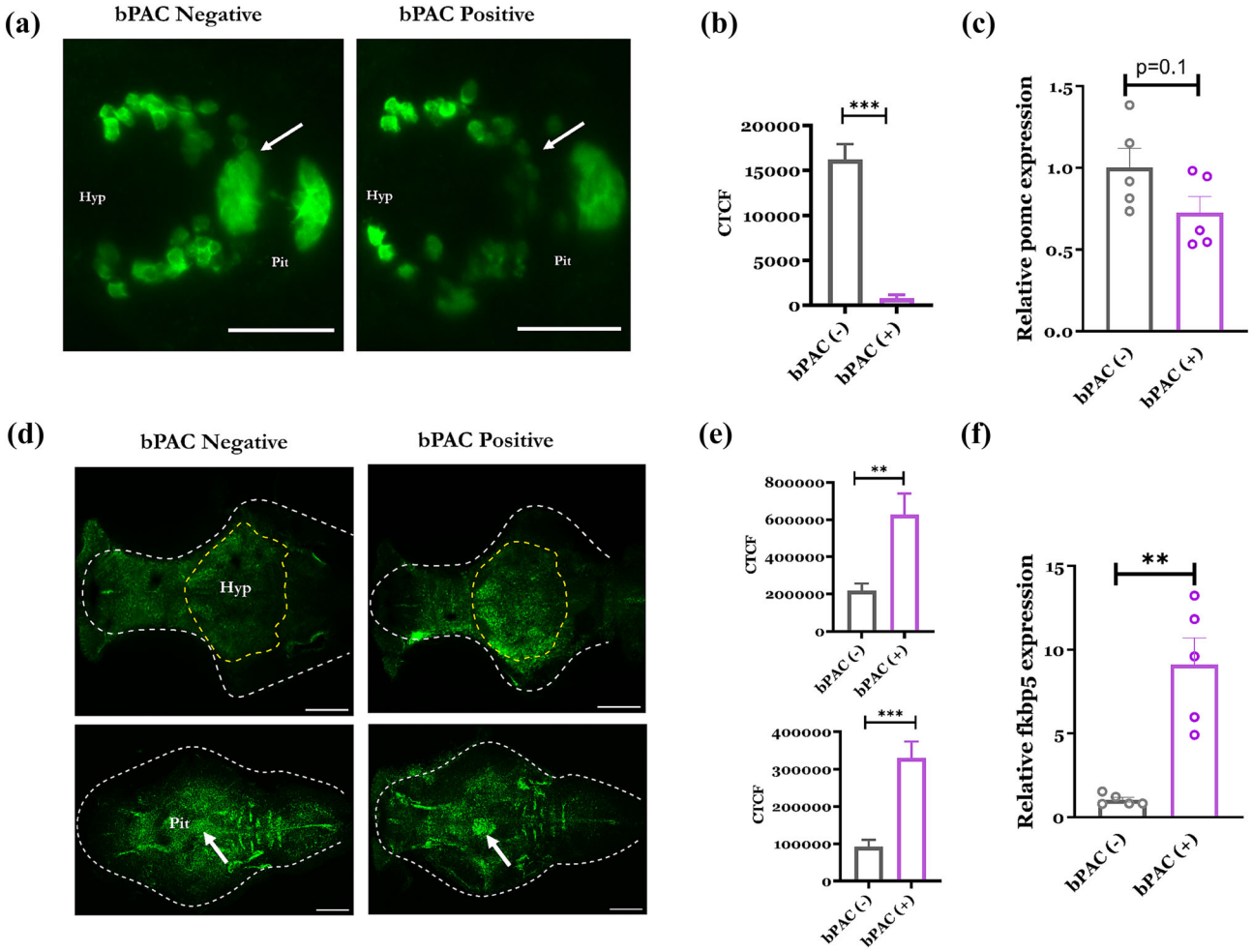

FIGURE 3 Effects of chronic early-life GC exposure on *pomc* and *fkbp5* gene expression. **A**. *Maximum* intensity projection of confocal stacks show suppression of *pomc* (ISH staining) expression in pituitary corticotrophs of the rostral *pars distalis* (marked with an arrowhead) in *Tg(2kbStARp:bPAC-2A-tdTomato)* transgenic larvae relative to non-transgenic siblings at 6 dpf. Scale bar 50 μm. **B**. Quantification for rostral *pars distalis* of the pituitary region in the ISH images in a. (N = 6). **C.** RT-qPCR analysis for *pomc* expression in whole larvae shows reduced expression in *Tg(2kbStARp:bPAC-2A-tdTomato)* transgenic larvae relative to non-transgenic siblings at 6 dpf. Data is shown as mean with standard error of the mean, analysed with unpaired t-test (p-value = 0.1). The individual values are depicted in circles, and the bar represents mean with standard error of the mean. N = 5 samples with 15 animals each. **D.** ISH images (single planes) showing differences in *fkbp5* brain expression between bPAC transgenic and non-transgenic larvae. The top panel shows the hypothalamic region marked with a yellow dotted line, the bottom panel shows the pituitary marked with an arrow. Scale bar 100 μm. **E.** Quantification for the hypothalamic (top, N = 14,16) and pituitary (bottom, N = 7) expression of *fkbp5* between bPAC transgenic and non-transgenic larvae. **F.** RT-qPCR analysis for *fkbp5* expression in whole larvae shows strong upregulation in *Tg(2kbStARp:bPAC-2A-tdTomato)* transgenic larvae relative to non-transgenic siblings at 6 dpf. Data is shown as mean with standard error of the mean, analysed with unpaired t-test (p-value<0.01). N = 5 samples with 15 animals each. Anterior is to the left, abbreviations: Hyp hypothalamus, pit pituitary.

activation could have pleiotropic effects on the adrenal/interrenal physiology. Briefly, the gain of function of the cAMP pathway in adrenal cells has been used to model Cushing's syndrome, which is the result of excessive production and release of cortisol (de Joussineau et al., 2012). This could occur at different levels of signal transduction: ectopic expression of GPCRs, activating mutations of G proteins and protein kinase A catalytic subunits and inactivating mutations of phosphodiesterases and protein kinase A regulatory subunits. The resultant adrenal overactivity in some of these models has been associated with adrenal tumorigenesis as well as developmental alterations (Calebiro et al., 2015; de Joussineau et al., 2012; Ronchi, 2019). For example, the adrenocortical-specific mouse knockout of protein kinase A regulatory subunit Prkar1a leads to the development of a pituitary-independent age- and sex-dependent model of Cushing's syndrome (Sahut-Barnola et al., 2010). The deregulation of adreno-cortical cell differentiation and hyperplasia in the model results in the improper

maintenance and expansion of fetal adrenal cells in adult adrenal glands, leading to the development of tumorous conditions. Also, the GC output of the HPA axis is closely linked to various physiological processes, including metabolism, immune function, osmoregulation and behaviour, as well as the stress response (Timmermans et al., 2019). Hence, our model could be leveraged to provide fresh insights in these diverse areas.

To understand and validate the underlying physiology for these chronic GC exposure-mediated phenotypes, we focused on two well-studied targets of GC, particularly in the context of the negative feedback loops of GC regulation – *pomca*-expressing pituitary corticotrophs and GR co-chaperone FKBP5. We observed strong and selective suppression of *pomca* expression in the rostral *pars distalis* of the pituitary gland in transgenic larval zebrafish at 6 dpf. This is similar to reports wherein larval zebrafish were exogenously treated with dexamethasone, a selective GR agonist (Liu et al., 2003; Peles et al., 2022; To et al., 2007), demonstrating the effect of greater GC negative feedback control and that this group of pituitary *pomca*-expressing cells regulated by cortisol. This suppression of *pomc*a expression thus could be a contributing factor for the blunted GC phenotype possibly due to lack of ACTH release upon stressor exposure. The RT-qPCR data for *pomc* expression from whole larval homogenate showed a similar trend. FKBP5 is a co-chaperone in the steroid receptor complex, whose expression is rapidly induced by GR activation. It exerts an inhibitory effect on intracellular GC signalling (Häusl et al., 2019; Matosin et al., 2018). There is also strong support in the literature for its role in shaping HPA axis function and overall stress reactivity (Brix et al., 2022; Häusl et al., 2021; Touma et al., 2011). Our observation of strong up-regulation of *fkbp5* gene expression at 6 dpf following chronic GC exposure mirrors previous findings of dramatic induction of FKBP5 expression in a number of brain regions after stimulation with dexamethasone or stress exposure (Scharf et al., 2011). *fkbp5* expression elevation was confirmed to be elevated in the pituitary and hypothalamus in transgenic larvae through in situ hybridization. This overall mimic of *fkbp5* disinhibition in our transgenic model may be of translational relevance since FKBP5 is a promising drug target for a variety of psychiatric and other disorders (Schmidt et al., 2012).

We can envisage that this zebrafish model will be useful to study mechanistically the developmental programming of the HPA axis as well as adult stress-related and social behaviour, particularly as a function of early-life GC exposure and chronic HPA axis activation (Gans & Coffman, 2021; Nagpal et al., 2019). Specifically, the genetic, developmental and high-throughput nature of the zebrafish system coupled to this model could be leveraged to reveal the molecular underpinnings of GC-mediated effects on brain and behaviour (Griffiths et al., 2012; Lopez et al., 2021; Swaminathan et al., 2023; van den Bos et al., 2020). Indeed, ELS in zebrafish, imposed via unpredictable mild electric stimuli (Chin et al., 2022) or chronic unpredictable early-life stress protocol (Fontana et al., 2021), has been demonstrated to affect anxiety-like behaviour in later life. Furthermore, chronic treatment of zebrafish embryos with cortisol has been linked to the development of a pro-inflammatory adult phenotype (Hartig et al., 2016) as well as aberrant regulation of GC-responsive genes such as *klf9* and *fkbp5* in adulthood (Hartig et al., 2020). The optogenetic model reported here provides a complementary approach that is targeted specifically at non-invasive HPA axis activation. In fact, we have used this model recently to uncover the effect of chronic early-life GC elevation on neurogenesis, brain transcriptome and adult behaviour (Choi et al., 2023; Eachus, Choi et al., 2023). This model can be leveraged in future studies to identify the critical period, intensity and duration of GC elevation leading to molecular, cellular and behavioural phenotypes resulting from early GC exposure. Lastly, relatively understudied brain–body communication factors such as the microbiome could be an important piece of the puzzle of GC – brain – behaviour axis, given that microbiome has been shown to strongly impact HPA axis function and stress behaviour (Nagpal & Cryan, 2021).

AUTHOR CONTRIBUTIONS

Jatin Nagpal: Conceptualization, Data curation, Investigation, Methodology, Supervision, Writing-original draft, Writing—review & editing, Funding acquisition. **Helen Eachus:** Investigation, Methodology, Writing—review & editing **Olga Lityagina:** Data curation, Investigation. **Soojin Ryu:** Conceptualization, Supervision, Writing—review & editing, Project administration, Funding acquisition.

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CONFLICT OF INTEREST STATEMENT

SR holds a patent, European patent number 2928288 and US patent number 10,080,355: "A novel inducible model of stress." The remaining authors declare that they have no known competing financial interests that could have appeared to influence the work reported in this paper.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/ejn.16301.

DATA AVAILABILITY STATEMENT

Data used in this report are freely available upon reasonable request to the corresponding author.

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