Coordinated Action of Corticotropin-Releasing Hormone and Cortisol Shapes the Acute Stress-Induced Behavioural Response in Zebrafish

Erin Faught Mathilakath M. Vijayan

Department of Biological Sciences, University of Calgary, Calgary, AB, Canada

Keywords

Stress · Corticotropin-releasing hormone · Corticotropin-releasing hormone receptor 1 · Glucocorticoid receptor · Mineralocorticoid receptor · Cortisol

Abstract

Introduction: The stress response mediated by the hypothalamus-pituitary-adrenal (HPA) axis activation is highly conserved in vertebrates. Hyperactivity is one such established acute stress response, and corticotropin-releasing hormone (CRH), the primary step in HPA activation, signalling has been implicated in this stressor-mediated behaviour. However, whether CRH mediates the acute behavioural effects either alone or in conjunction with glucocorticoids (GCs) are far from clear. We hypothesized that the CRH receptor 1 (CRHR1)-mediated rise in GCs post-stress is necessary for the initiation and maintenance of the acute stress-related behaviour. **Methods:** We first generated zebrafish (*Danio rerio*) with a mutation in the *CRHR1* gene (CRHR1-KO) to assess the function of CRH. The behavioural readout utilized for this study was the locomotor activity of larval zebrafish in response to an acute light exposure, a protocol that freezes the larvae in response to the light stimulus. To test whether cor-

tisol signalling is involved in the stress-mediated hyperactivity, we treated wildtype fish with metyrapone (MET), an inhibitor of 11 β -hydroxylase, to suppress cortisol production. The temporal role for cortisol signalling in the stress-related hyperactivity was tested using the glucocorticoid receptor knockout (GRKO) and mineralocorticoid receptor knockout (MRKO) zebrafish mutants. **Results:** CRHR1-KO larvae did not increase cortisol, the principal GC in teleosts, post-stress, confirming a functional knockout. An acute stress resulted in the hyperactivity of the larvae in light at 15, 60, and 240 min post-stress, and this was absent in CRHR1-KO larvae. Addition of MET effectively blocked the attendant rise in cortisol post-stress; however, the stress-mediated hyperactivity was inhibited only at 60 and 240 min but not at 15 min post-stress. Addition of human CRH peptide caused hyperactivity at 15 min, and this response was also abolished in the CRHR1-KO mutants. The stress-induced hyperactivity was absent in the MRKO fish, while GRKO mutants showed transient effects. **Conclusions:** The results suggest that the stress-induced hyperactivity is induced by the CRH/CRHR1 system, while the temporal activation of cortisol production and the associated GR/MR signalling is essential for prolonging the stressor-induced hyperactivity. This study underscores the importance of systems-level analysis to assess stress responsiveness.

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Introduction

Stress and the attendant rise in stress hormones are associated with a wide range of physiological and behavioural changes as part of the adaptive stress response [1]. Much of the work regarding stress and behaviour has focused on the effects of glucocorticoids (GCs), an endogenous stress hormone, which also has therapeutic applications [2–4]. While basal levels of GCs are generally tightly regulated, the activity of the hypothalamus-pituitary-adrenal (HPA) axis is upregulated diurnally and in response to stress [5]. The HPA axis activity is initiated by the release of corticotropin-releasing hormone (CRH) from the paraventricular nucleus of the hypothalamus, which promotes the release of adrenocorticotrophic hormone from the anterior pituitary via activation of CRH receptor 1 (CRHR1) [5]. Adrenocorticotrophic hormone in turn will act on the adrenals of mammals or inter-renal cells of teleosts to release GCs [5, 6]. GCs signal through 2 receptors, with different affinities and distinct but overlapping patterns of expression: the high-affinity mineralocorticoid receptor (MR; type I) and low-affinity glucocorticoid receptor (GR; type II) [7]. Because of the high affinity of MR for GCs, these receptors are believed to be mostly occupied at basal GC levels, while GR becomes occupied at higher GC levels such as during stress or at the circadian peak [7]. These receptor activations are essential in mediating the physiological changes that allow an animal to cope with stress, as disturbances to MR-GR-mediated effects impair physiological adaptations, including behaviour and endocrine function [8].

Several studies have established the importance of these corticosteroid receptors in mediating cortisol-driven behavioural responses in both mammals and lower vertebrates. In mice, overexpression of MR in the forebrain decreases anxiety-like behaviour and alters the stress response [9], whereas knockout of limbic MR impairs behavioural plasticity [10]. Mice with overexpression of GR in the forebrain exhibit no changes in basal or stress-induced corticosterone levels, but show increased anxiety-like behaviour and increase immobility in the forced swim test [11]. Overall, this led to the concept that MRs in the hippocampus control the initial stress reaction, which is important for appraisal of a novel situation, whereas the GR is essential for management of the later adaptive phase [7]. Interestingly, recent work has highlighted that both GR and MR display distinct temporal dynamics in different limbic regions of the brain after an *in vivo* stress, highlighting the importance for a systems-level analysis of stress responsivity [12].

Beyond GCs, the stress response involves the upregulation of a suite of hormones of behavioural relevance. These including amines and neuropeptides that are temporally distinct and have separate but overlapping roles with the intention of providing an adaptive advantage to the animal [13]. Dysregulation of the HPA axis has been associated with the pathology of several behavioural disorders, including major depressive disorders [4]. However, little is known about the temporal interactions between the different hormones of the HPA axis in modulating the stress-related behaviour.

CRH, the neuropeptide hormone at the apex of the HPA axis, is also known to play a role in the stress-related behaviours, including anxiety, arousal, motor function, and learning and memory [14–16]. CRH acts through either the CRHR1 or the CRH-receptor 2 (CRHR2), both G-protein coupled receptors which have different distributions and physiological functions [14, 15]. CRH binds with higher affinity to CRHR1, and loss of this receptor or overexpression of this receptor in mice either decreased or increased anxiety-related behaviour, respectively [15, 17]. Interestingly, mice lacking the *crh* gene exhibit normal stress-induced behaviour that is only blocked by a CRHR1 antagonist, suggesting that other CRH-like hormones can compensate if the receptor is present. Indeed, a knockout of CRHR1 in mice abolishes the stress-mediated increase of corticosterone levels [18]. The effect of CRH on behaviour appears to be conserved as exogenous CRH treatment stimulates locomotor activity in fish [19–21]. Given that CRH is the driver of GC production and signals rapidly upon the perception of a stressor, this neuropeptide may facilitate acute behavioural changes, as well as sustain these behavioural responses by the production of GCs [22, 23]. Here we explore the integration of CRH and GCs in mediating stress-related behaviour. Specifically, we tested the hypothesis that both these key hormones of the HPA activation act synergistically during an acute stress response to promote stress-related behaviours.

To determine the functional role of these hormones during an acute stress response, we used zebrafish (*Danio rerio*) lacking either CRHR1, MR, or GR to elucidate the temporal contribution and the interaction of these receptors, in the stress-related behaviour. Zebrafish have become a promising animal model to investigate neurobehavioural mechanisms of stress [24]. The larval fish are highly sensitive to light and show distinct behavioural responses to dark-light transitions. This includes reduced locomotion in the light that is increased by stressed levels of cortisol [25, 26]. Consequently, the larval activity in the

light was used as a read-out to test the stress-induced hyperactivity. Our systems-level approach indicates that CRHR1 and MR are involved in mediating the short-term behavioural response to an acute stress, while GR and MR play an important role in the temporal maintenance of the behavioural activity even after the recovery of the stressor-induced cortisol levels.

Materials and Methods

Animal Care

Zebrafish (Tupfel long fin strain) were held in a recirculating system (Tecniplast, Italy) on a 14 h:10 h light:dark cycle (light on 09:00; light off 23:00). Water was maintained at 28°C, 750 µS conductivity, and a pH of 7.5. Fish were fed twice daily with Gemma Micro (500) adult zebrafish food in the morning (Skretting, Canada) and live *Artemia* (San Francisco Bay Brand, Inc.) in the afternoon. For breeding, fish were set up the previous evening in breeding traps, and embryos were collected the next morning. Embryos were maintained in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄, and 0.1 ppm methylene blue as antifungal) in 10-cm Petri dishes (60–100 embryos per dish), also on a 14 h light:10 h dark cycle. Embryo media were refreshed daily (approx. 50%), and all dead embryos/larvae were removed. No significant treatment effect on mortality was observed. This maintenance continued until embryos or larvae were required for experiments, at 4 days post-fertilization (dpf).

Generation of CRHR1-KO Larvae

The generation of homozygous mutants lacking GR and MR using CRISPR/Cas9 mutagenesis was described previously [26]. The generation of the CRHR1-KO larvae was performed exactly as described for glucocorticoid receptor knockout (GRKO) and mineralocorticoid receptor knockout (MRKO) mutants. The sgRNA (bolded text) used was TAATACGACTCACTA TAG-G A A G G A T T C G G G A C A G G G C G T T T T A G A G C -TAGAAATAGC. Primers to amplify the target region were (FW: tgtaaacgacggccagtCCATTGATGGCATTGG and RV: gtgtcttTG-GTGGTGTG TATCGGAC). All embryos used in the study were spawned from homozygous mothers, making all mutants maternal zygotic, and eliminating any possible deposition of WT transcripts, of each respective gene, from a heterozygous mother.

Polymerase Chain Reaction

To determine the tissue distribution of CRHR1 in zebrafish, gene-specific primers were designed to amplify *CRHR1* mRNA (FW: CAGTCATCATCAACTACCTGGG and RV: AGCTGAACCACGAACCA). cDNA from brain, heart, gill, liver, muscle, testes, and ovary was made from extracted RNA as described previously [26]. β-Actin was amplified as a positive control. For both β-actin and *CRHR1*, a 20 µL PCR (10×Buffer [1×], 1.5 mM MgCl₂, 0.2 µM dNTPs, 0.2 µM primer pair, and 1 U/mL Taq DNA polymerase [10342020; Thermo Fisher Scientific]) was performed under the following conditions: 95°C, 5 min; 40× (94°C, 1 min; 60°C, 1 min, 72°C, and 30 s); 72°C, 5 min.

Treatments

To determine the stress effect on behavioural performance, we subjected zebrafish larvae (4 dpf) to an acute stressor, which has been standardized and used frequently to elicit a cortisol stress response in zebrafish larvae [26]. Briefly, the evening before (16:00–17:00), larvae were transferred from the rearing Petri dishes to 50 mL Falcon tubes. Each tube contained a final volume of 20 mL of the embryo medium. For determining the cortisol response to stress, the time 0 larvae (unstressed larvae) were immediately euthanized the following day (12:00; MS222, Millipore Sigma, final concentration 0.4 mg/mL), and the remaining larvae were transferred to a heated (28°C) floor shaker and subjected to a vortex stressor (250 rpm) for 1 min, allowed to recover, and euthanized at the appropriate time point (5, 10, or 30 min). All embryo media were removed, and larvae were frozen at –80°C until cortisol analysis. For the behavioural assessment, individual larvae were immediately transferred to a 96-well plate after the stressor, and locomotor behaviour was evaluated at 15, 60, or 240 min post-stressor. The stressor was always performed between 12:00 and 13:00. To block the attendant rise in cortisol post-stressor, larvae were treated with metyrapone (MET; 100 µM; Millipore Sigma) for 16 h prior to the stressor exposure. To determine whether cortisol was able to induce hyperactivity in the CRHR1-KO larvae, both CRHR1-KO and WT larvae were treated with cortisol (5 µg/mL) for 16 h prior to behavioural assessment, as described previously [26]. To determine potential players in the short-term hyperactivity, we (i) treated larvae with human CRH (hCRH; 5 µg/mL; GenScript, USA, RP10982) or (ii) stimulated the adrenergic pathway with epinephrine (30 µM; β-adrenoceptor agonist Millipore Sigma) or phenylephrine (30 µM; α-adrenoceptor agonist Millipore Sigma) and measured the locomotor activity at 15 min. To further test whether β-adrenoceptor signalling was involved in the stress-induced hyperactivity, larvae were treated with the β-adrenergic receptor antagonist propranolol (100 µM; Millipore Sigma) 1 h prior to either stressor or epinephrine (30 µM) addition.

Behavioural Assessment

All treatments and behavioural analyses were performed between 12:00 and 17:00, which was during the light phase of the 14 h light:10 h dark cycle when zebrafish larvae were 4 dpf. Unstressed larvae were transferred to clear 96-well plates with lids (1 individual per well; 100 µL total volume) the evening (16:00–17:00) prior to the assessment and were allowed to acclimate overnight. The larvae were stressed as described above and loaded into the 96-well plate immediately following the stressor. Analyses were performed in an isolated room maintained at 28.5°C. The movement of 4 dpf larvae was video captured using the Noldus Danio Vision Observation Chamber, and the subsequent video was analysed using Ethovision (Noldus, The Netherlands). For the light-dark response assay, larvae were subjected to short alternating periods of light and dark, and their movement in response to these stimuli was recorded. The protocol parameters consisted of alternating periods of light and dark every 7.5 min (450 s). This 15 min cycle of light and dark was repeated twice for a total of 30 min. Each treatment was repeated a minimum of 2 times (12–24 per treatment group per plate), and in all cases separate control and stressed groups were run to avoid the representation of duplicated data. To determine hyperactivity in the light phase, the total distance moved (in mm) was calculated per animal for the total duration they were subjected to the light stimulus (0–7.5 + 15–22.5 min).

Fig. 1. CRHR1-KO fails to elicit a cortisol response to stress. **a** Mutant for CRHR1 (CRHR1-KO) was created using CRISPR/Cas9 mutagenesis in the fourth exon of the *crhr1* gene. This results in a 1 bp deletion at position 78 of exon 4, which corresponds to the proline at position 82, in the extracellular domain of the CRHR1 protein. This caused a frameshift mutation resulting in a premature stop codon a further 153 bp downstream. **b** PCR amplicons showing that *crhr1* transcripts are ubiquitously expressed in the brain, heart, gill, liver, muscle, and gonads of zebrafish. β -Actin was used as the loading control. **c** Cortisol levels in response to stress. WT larvae show a peak in cortisol at 5–10 min post-stressor,

and this returns to resting by 30 min. CRHR1-KO failed to raise cortisol levels in response to stress. **d** Exogenous treatment with hCRH (5 μ g/mL; black bars) for 60 min increased cortisol levels above the sham (white bars) in WT larvae. CRHR1-KO larvae were unresponsive to hCRH stimulation. Bars represent mean $\pm$ SEM ($n = 4-8$ [each $n =$ pools of 10 larvae]). Data points with different letters/asterisks denote significant differences (2-way ANOVA, $p < 0.05$; not significant [ns]). hCRH, human corticotropin-releasing hormone; CRHR1-KO, corticotropin-releasing hormone receptor 1 knockout.

Cortisol Measurement

For cortisol quantification, pools of 10 embryos or larvae ($n = 4-6$) were collected, and all embryo media were removed. Embryos were stored at -80°C until analysis. Embryos or larvae were then partially thawed on ice and homogenized in 50 mM Tris buffer, pH 7.5 with protease inhibitors (10 μ L/embryo; Roche Diagnostics, Laval, QC, Canada). Samples were sonicated on ice to homogeneity, and cortisol was immediately quantified. Cortisol levels were measured using an in-house competitive enzyme-linked immunosorbent assay as described previously for zebrafish [27].

Statistics

Data are shown as mean $\pm$ SEM, and statistical (t test, 1-way ANOVA, 2-way ANOVA) analyses were carried out using Sigma Plot 13 (Systat Software, Inc.). A $p < 0.05$ was considered significant in all cases. All data were transformed to meet the assumptions of normality and equal variance. Untransformed data are shown in all figures.

Results

CRHR1-KO Fail to Elicit a Cortisol Response to Stress

We first established zebrafish lacking a functional CRHR1 by mutating the *CRHR1* gene (CRHR1-KO) using CRISPR/Cas9 mutagenesis (Fig. 1a). This receptor is ubiquitously expressed (Fig. 1b) and is the primary receptor for mediating the effects of CRH on the HPA axis in mammals [18]. Using the methods described previously [26], we edited the *CRHR1* gene at exon 4. This resulted in a 1 bp deletion at position 78 of exon 4, which corresponds to the proline at position 82, in the extracellular domain of the CRHR1 protein. In silico analysis revealed that this mutation causes a premature stop codon 153 bp downstream of this mutation site. In this case, a total of 133 amino acids would be translated out of a total 470

Fig. 2. CRHR1-KO inhibits acute stress-induced hyperactivity. Stress (black bars) causes hyperactivity (increased total distance moved) during intermittent light periods when WT-Sham larvae exhibit a freezing response (white bars). The total distance moved was calculated as distance each zebrafish larvae moved in the light during the 30-min behavioural assay (total time in light – 15 min). For movement in the dark, see online suppl. Fig. 3. CRHR1-KO larvae failed to elicit a behavioural response to stress (grey bars). Bars represent mean $\pm$ SEM ($n = 12$ –50 individual larvae). Data points with different letters/asterisks denote significant differences (2-way ANOVA for each time point, $p < 0.05$; not significant [ns]). CRHR1-KO, corticotropin-releasing hormone receptor 1 knockout.

amino acids, and therefore, it is unlikely that this truncated protein will persist in the animal.

To confirm this was a functional knockout, we stressed 4 dpf zebrafish larvae to determine if a cortisol response to stress (activation of HPI) could still be observed in larvae lacking CRHR1-KO (Fig. 1c). WT larvae had resting cortisol levels of 12.3 ± 1.2 pg/larva, which increased to 25 ± 3.9 pg/larva by 5 min post-stressor ($p = 0.002$). Larvae had similar cortisol levels at 10 min post-stressor (27 ± 1.8 pg/larva) compared to 5 min, but by 30 min, cortisol levels had returned to basal (14.7 ± 2.3 pg/larva). In contrast, there was no change in cortisol levels post-stress in the CRHR1-KO. Although resting cortisol levels in CRHR1-KO are higher (17.6 ± 1.5 pg/larva; $p = 0.053$; 2-way ANOVA, Holm-Sidak post hoc) compared to resting WT larvae, this was still less than what can be induced by stress in the WT (Fig. 1c). To further determine if CRHR1-KO larvae had impaired HPI axis function, we treated fish with hCRH ($5 \mu\text{g/mL}$), which raised cortisol levels 4-fold in WT larvae (31.8 ± 5.00 – 87.6 ± 30.6 ng/mL) at 60 min post-exposure ($p = 0.002$; Fig. 1d). hCRH failed to induce a rise in cortisol in CRHR1-KO larvae (Fig. 1d). hCRH also failed to induce an increase in cortisol levels in WT larvae at 15 min post-exposure (see online suppl. Fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000514778).

CRHR1-KO Inhibits Acute Stress-Induced Hyperactivity

To determine whether stress caused a behavioural change in larval zebrafish, we employed a light-dark paradigm to observe levels of zebrafish locomotion (online suppl. Fig. 2). This behaviour is unique to the larval stage of zebrafish and is an established protocol, in which a larval zebrafish will freeze in the light and move freely, within the bounds of each area, in the dark [28]. We found that the activity of stressed larvae in the light was nearly 6-fold greater compared to the control larvae ($p < 0.001$; Fig. 2). This hyperactive phenotype was evident at 15 min (acute behaviour) and persisted through 4 h (delayed behaviour; Fig. 2). In contrast, CRHR1-KO larvae did not exhibit a behavioural response to stress, with the stressed CRHR1-KO showing similar locomotor behaviour to unstressed CRHR1-KO and WT control larvae (Fig. 2). However, distinct from the later time points, the activity in the stressed CRHR1-KO larvae at 15 min was high enough to be similar to the stressed WT larvae. At both 1 and 4 h post-stress, CRHR1-KO abolished the stress-induced rise in locomotor activity. There was no effect of stress on the locomotor activity in the dark phase; however, in general, CRHR1-KO moved less compared to the WT larvae at both 15 and 60 min (online suppl. Fig. 3). At 4 h, stress in both WT and CRHR1-KO caused less movement compared to the unstressed larvae in the dark (online suppl. Fig. 3).

Fig. 3. Cortisol is involved in the acute stress-related behaviour. **a** To distinguish between CRHR1 signalling and GC-mediated signalling, we treated larvae with MET (11- β -hydroxylase inhibitor), which blocked the attendant rise in cortisol post-stress (inset denotes significant treatment effects; 2-way ANOVA; $n = 4-8$ [each $n =$ pools of 10 larvae]). **b** MET had no effect on stress-induced hyperactivity at 15 min (acute stress behaviour), but a lack of cortisol production abolished the hyperactivity observed at 60 or 240 min (delayed stress behaviour) (1-way ANOVA; $n = 12-50$ individual larvae). Bars represent mean $\pm$ SEM. Data points with different letters denote significant differences ($p < 0.05$). GC, glucocorticoid; MET, metyrapone.

Cortisol Is Involved in the Acute Stress-Related Behaviour

To determine whether it was a lack of CRH signalling or a lack of cortisol production which was driving the stress-mediated behavioural changes, we treated larvae with MET, a 11- β hydroxylase inhibitor, that blocks cortisol production from 11-deoxycortisol [29, 30]. Indeed, MET-treated larvae failed to elicit a cortisol response to

stress compared to its vehicle control (Fig. 3a). At both 60 min and 4 h post-stress, MET also reduced stress-induced hyperactivity (Fig. 3b). However, at 15 min, despite being treated with the drug for 16 h prior to the stressor, MET-treated fish were hyperactive in response to the acute stressor and comparable to the stressed group in the absence of MET ($p = 0.787$), which was significantly higher than the unstressed controls ($p = 0.027$).

Fig. 4. CRH stimulates acute stress-mediated hyperactivity. **a** Treatment with hCRH (hCRH, 5 $\mu\text{g}/\text{mL}$) increased larval activity 15 min post-treatment. This effect is abolished in the CRHR1-KO. **b** Treatment with propranolol (100 μM) 60 min prior to stress did not affect stress induced hyperactivity observed at 15 min. **c** Treatment with propranolol (PROP; 100 μM) 60 min prior to epinephrine (Epi; 30 μM) exposure abolished epinephrine-mediated hyperactivity at 15 min. Bars represent mean $\pm$ SEM ($n = 12\text{--}24$ individual larvae). Data points with different letters denote significant differences (1-way ANOVA, $p < 0.05$). CRHR1-KO, corticotropin-releasing hormone receptor 1 knockout; hCRH, human corticotropin-releasing hormone.

Fig. 5. Cortisol causes hyperactivity in fish lacking CRHR1. **a** To determine whether it was a lack of cortisol or another issue that prevented locomotion in the CRHR1-KO, we treated fish with cortisol (5 µg/mL) for 16 h prior to the behavioural assessment. Indeed, cortisol was able to increase hyperactivity in both the WT and CRHR1-KO larvae ($n = 12$ –24 individual larvae). **b** Cortisol levels in WT and CRHR1-KO larvae ($n = 4$ –8 [each n = pools of 10 larvae]). Bars represent mean $\pm$ SEM. Asterisks denote significant differences within each genotype, while the inset shows overall treatment effect between sham and cortisol independent of genotype (2-way ANOVA, $p < 0.05$). WT, wildtype; CRHR1-KO, corticotropin-releasing hormone receptor 1 knockout.

CRH Stimulates the Acute Stress-Mediated Hyperactivity

Given that MET did not block the stress-induced hyperactivity at 15 min, we sought to determine whether CRH directly stimulates the hyperactivity at this time point. To this end, we treated the larvae with hCRH and measured the hyperactivity at 15 min. Preliminary dose-response study showed that CRH-mediated hyperactivity was not evident below 1 µg/mL hCRH concentration (online suppl. Fig. 4). There was a 4-fold increase in hyperactivity in the larvae treated with 5 µg/mL ($p = 0.045$) CRH (Fig. 4a). This increase in activity was absent in CRHR1-KO larvae. Interestingly, CRHR1-KO larvae treated with 5 µg/mL hCRH exhibited a marked decrease in activity compared to CRHR1-KO controls ($p = 0.018$).

Acute Stress-Mediated Hyperactivity Is Not due to Adrenergic Stimulation

Acute stress effects, including the fight or flight response, are often mediated, in part, due to activation of the sympathetic nervous system. Therefore, we measured the acute effects of epinephrine (β -adrenergic agonist) and phenylephrine (α -adrenergic agonist) in eliciting the hyperactivity (online suppl. Fig. 5). While epinephrine caused a robust hyperactivity in light, phenylephrine failed to elicit any behavioural changes. Therefore, we used propranolol (an antagonist for β -adrenoreceptors) to determine whether epinephrine played a role in the stress-mediated hyperactivity. Propranolol (100 µM) failed to block the stress-induced hyperactivity at 15 min post stressor, with propranolol-treated fish exhibiting similar levels of hyperactivity post-stressor ($p = 0.801$; Fig. 4b). To confirm that propranolol was able to effectively block epinephrine-induced hyperactivity, we treated WT larvae with propranolol (100 µM) an hour prior to epinephrine (30 µM) exposure. Epinephrine caused a 7-fold increase in activity ($p < 0.001$), and this was abolished by propranolol (Fig. 4c). Propranolol treatment alone lowered the total distance moved by the larvae compared to controls ($p = 0.002$).

Cortisol Recovers Locomotor Activity in Fish Lacking CRHR1

To further confirm whether a lack of cortisol was responsible for the lack of hyperactivity in the CRHR1-KO larvae and not due to impaired cortisol signalling, we treated the fish with cortisol (5 µg/mL), which had been previously used to elicit hyperactivity in the light [25, 26]. As expected, cortisol treatment increased cortisol levels (~ 8 -fold) in both WT and CRHR1-KO larvae (Fig. 5b).

Cortisol treatment also increases the larval hyperactivity to light in both the WT and CRHR1-KO larvae ($p = 0.041$; Fig. 5a). The response to cortisol was stronger in the CRHR1-KO, and overall, the CRHR1-KO larvae moved a greater distance compared to the WT ($p = 0.017$; Fig. 5b).

MR and GR Signalling Are Essential for the Acute Stress-Induced Hyperactivity

We next wanted to determine the role of each corticosteroid receptor in the stress-induced behavioural changes. The stress response for both the GRKO and MRKO has been previously described [26]. Briefly, the MRKO larvae had a delayed cortisol peak post-stress, but had cortisol levels comparable to WT controls. The GRKO larvae are hypercortisolemic due to a loss of the negative feedback and have elevated cortisol levels even above that induced by a stressor [26]. Here, our results reveal that larvae lacking MR do not exhibit hyperactive behaviour at any time point post-stressor (Fig. 6). In the larvae lacking GR, there was no significant difference in the hyperactivity to light in the stressed larvae compared to the control larvae at 15 min and at 4 h post-stressor exposure (Fig. 6a, c). However, the hyperactivity of the stressed GRKO larvae were significant different from the control GRKO larvae at 1 h post-stressor (Fig. 6b), which was a similar hyperactive profile as seen in the WT larvae (Fig. 6a–c).

Discussion

Here, we demonstrate a key role for CRH/CRHR1 signalling in the onset of the acute stress-induced hyperactivity in zebrafish larvae. The results reveal that in addition to CRHR1 activation, both MR and GR signalling are an essential component of this acute stress-related behaviour. Thus, a coordinated effort by both CRH and GCs are essential to initiate and perpetuate the acute stress-induced hyperactivity even after the cortisol levels have returned to basal levels. This interdependency on multiple components of the HPI axis in shaping a stress-related behavioural response may provide valuable insights into

Fig. 6. MR and GR signalling are essential for acute stress-induced hyperactivity. **a** Locomotor activity at 15 min in sham (white bars) and post-stress (black bars) in WT, GRKO, and MRKO larvae. At this time point, only WT larvae exhibit hyperactivity. **b** Locomotion at 60 min post-stressor in WT, GRKO, and MRKO. At this time point, both WT and GRKO larvae exhibited hyperactivity post-stress. **c** Locomotion at 240 min post-stressor in WT, GRKO, and MRKO. At this time point, only WT exhibited hyperactivity. Bars represent mean $\pm$ SEM ($n = 12$ –24 individual larvae). Data points with asterisks denote significant differences (t test between sham and stressed larvae within each genotype, $p < 0.05$; ns). MR, mineralocorticoid receptor; GR, glucocorticoid receptor; GRKO, glucocorticoid receptor knockout; MRKO, mineralocorticoid receptor knockout; ns, not significant.

Fig. 7. Schematic representation of the temporal HPA axis contribution to the stress-induced hyperactivity. Acute stress behaviour (15 min), measured here as increased locomotor activity (hyperactivity) during a typical freezing response, in larval zebrafish involves the coordinated action of CRHR1, the MR, and the GR. Acute stress increases cortisol during this time (see Fig. 1c) but do not appear to be necessary for the hyperactivity as treatment with MET failed to block the behavioural response (see Fig. 3b). Treatment with CRH during this time caused an increase in locomotor activity, and this was blocked by CRHR1, suggesting acute CRH stimulation of stress-induced hyperactivity. The delayed behavioural response to stress is mediated by cortisol, primarily by MR

at 60 min post-stress and by both GR and MR at 240 min post-stress. However, without the presence of CRHR1, the attendant rise in cortisol does not happen and the hyperactivity is abolished post-stress. Therefore, CRHR1 directly mediates the acute behavioural response to stress and indirectly ensures the delayed response by stimulating cortisol production. Activation of each receptor within each time point is not to an exact temporal scale and is representative at best. MR, mineralocorticoid receptor; GR, glucocorticoid receptor; HPA, hypothalamus-pituitary-adrenal; CRHR1, corticotropin-releasing hormone receptor 1; MET, metyrapone.

the aetiology of stress-related behavioural disorders and potential therapeutic intervention.

Role of CRH/CRHR1

The role of CRH, from a neurobiological perspective, is thought to enhance attention to significant events, which may be novel or threatening [22]. The suite of hormones that is released at the onset of stress is spatially and temporally coordinated. How these hormones interact with each other to regulate processes along a certain timeline remains unclear [31]. As CRH is released at the onset of stress and will drive the production of cortisol via activation of the HPI axis, our hypothesis was that CRH-CRHR1 signalling would be an early mediator of the acute behavioural response to stress. Indeed, previous work on the fear response in mammals has established that the role of CRH is temporally distinct, with an initial signalling event meant to assess a novel situation, followed by a sustained CRH signalling that is driven by GCs to adapt to stress [22]. In the present study, we used a well-established freezing response in zebrafish larvae when exposed to light [28] as a model of stress-induced hyperactivity. Hyperactivity during this period can also be induced by

cortisol [25, 26], and we propose this stress-mediated behaviour as a fear response, in which the animal is motivated to override a basal biological response when faced with a stressor. To determine how stress-mediated behaviour could be temporally regulated, we first subjected larvae to a stressor and examined their responses to alternating periods of light and dark at 15, 60, and 240 min post-stressor. The WT larvae showed a robust increase in locomotor activity in light post-stressor exposure, moving a ~4-fold greater distance compared to the unstressed larvae (Fig. 2) and this persisted for the entire 240 min of testing. In contrast, CRHR1-KO larvae lacked this hyperactivity. It was not immediately clear whether this was due to CRHR1-specific signalling or activation of a downstream stress hormone, such as cortisol.

The loss of CRHR1 in mice has been shown to abolish the stress-induced increase in GC levels [18]. The same is true in zebrafish, where an acute stressor failed to elicit a cortisol stress response in the CRHR1-KO larvae (Fig. 1c) and treatment with exogenous CRH also failed to stimulate cortisol production (Fig. 1d). Interestingly, mammalian studies have shown that it is a loss of CRHR1 that causes a loss of HPA axis activation and not the loss of

CRH itself, implying that there may be compensatory mechanisms offsetting the loss of the hormone [18]. To inhibit the stress-mediated cortisol production, we treated WT larvae with MET, which effectively blocked the stress-induced cortisol response in larval zebrafish (Fig. 3a). However, MET abolished the stress-related behaviour only at 60 and 240 min but not at 15 min post-stressor, clearly indicating a role for cortisol in the maintenance of the behavioural response after stress.

The next step was to address whether this acute hyperactive behaviour was due to CRH and/or due to the activation of the sympathetic system. Catecholamines play an important role in the fight or flight response to stress, including the hyperactive behaviour [32]. We did see an increase in hyperactivity with epinephrine but not with phenylephrine suggesting a potential role for β -adrenergic stimulation in regulating the stress-induced hyperactivity (SI; online suppl. Fig. 5). Interestingly, we failed to abolish the stress-induced hyperactivity when the larvae were treated with propranolol (a β -adrenoceptor antagonist), suggesting the lack of epinephrine involvement in the observed behavioural response. Treatment of larvae with hCRH caused hyperactivity by 15 min, and this was completely abolished by the CRHR1-KO larvae (Fig. 2a). This clearly points to a key role for CRHR1 in the onset of the acute stress-induced hyperactivity, in addition to activating the HPA axis and initiating the cortisol-mediated stress coping mechanisms. Using optogenetic enhancement of pituitary corticotroph cell activity, previous work in zebrafish has also pointed to a role for CRH in dictating rapid locomotory changes [31]. However, clearly the role for CRHR1 extends beyond the acute behavioural phase as the loss of CRHR1 effectively blocks the persistence of the stress-induced hyperactivity for 240 min (Fig. 2). Our results suggest that this receptor is not only involved in the initial appraisal and reaction to stress but also complements the role of cortisol in the longer-term maintenance of the behavioural response to stress in zebrafish.

Role of GR and MR

Given that cortisol response to stress played a role in the longer-term maintenance of stress-induced hyperactivity, we sought to determine the role of GR and MR in mediating this behavioural response. MR is thought to play a role in evaluating novel objects, and for the onset of the stress reaction, while GR signalling follows to develop and dampen this stress response [7]. In the present study, in the absence of MR, the larvae were unable to elicit hyperactivity over the entire stress duration, suggesting an important role for this receptor in the develop-

ment and maintenance of the stress-mediated behaviour (Fig. 6a–c). Interestingly, the loss of GR also inhibited the stress-induced hyperactivity at 15 min and 4 h but not at 1 h post-stressor, suggesting a temporally distinct complementary role for both these receptors in maintaining the stress-induced hyperactivity. While this suggests that neither receptor can compensate for the other in this stress-related behaviour, it remains to be seen whether MR or GR requires the alternate receptor as a co-factor [33]. This support studies that showed exogenous cortisol is involved in hyperactive behaviour of larval zebrafish [25, 26], which was abolished in fish lacking either GR or MR [26]. Whether these receptors homodimerize and bind to distinct hormone response elements on the promoter region of target genes or whether they heterodimerise to mediate the behavioural effects remains to be determined. What is clear is that neither receptor can compensate for the other, and at both 15 and 240 min, a loss of either receptor resulted in a loss of stress-induced hyperactivity.

In the present study, hyperactivity observed in the light at the 1 h time point was independent of GR, suggesting a possible role for MR activation. Zebrafish lacking GRKO have an overactive HPI axis, which results in higher resting cortisol levels, higher than levels seen in the WT during stress [26]. Given that the behavioural hyperactivity post-stress seems to be mediated by cortisol, it was interesting to note that there was no change in resting zebrafish activity in light in the GRKO larvae despite the elevated cortisol levels, both in this study and in an earlier study [26]. It could be argued that cortisol alone is not sufficient to initiate hyperactivity, but the evidence against this is 2fold. Firstly, cortisol treatment alone increases hyperactivity to light in WT zebrafish larvae [25, 26], and secondly, in GRKO larvae, hyperactivity can be induced in response to an acute stressor at 1 h and not at 15 min, despite an intact CRHR1. The only corticosteroid receptor present in the GRKO larvae is MR, and given its high affinity for cortisol, it is likely that most MR are fully saturated in the GRKO larvae due to elevated resting cortisol levels [23]. This is important because MR activation is known to change with higher cortisol levels in that it will be more likely to bind to GC response elements in a gene-specific manner [33]. Despite having high cortisol levels, hyperactivity may still be induced at this time point in the GRKO larvae, suggesting that there may be other players involved. This may be due to compensatory mechanisms, which may not be relevant to the WT larvae. This is because, in WT larvae treated with MET, where GR would not be activated due to the absence of a rise in cortisol

levels post-stress, hyperactivity was abolished, pointing to at least some role for GR. Therefore, we propose that the loss of GR may be enhancing the sensitivity to other stress hormones. For instance, it is thought that hippocampal MRs, which are involved in the cognitive processes underlying the appraisal of novel situations [34], accomplish this task by amplifying the excitability induced by catecholamines, including noradrenaline [7]. It remains to be seen whether increased MR signalling in the GRKO fish may be sensitizing the larvae to other stress hormones, leading to the hyperactivity observed (Fig. 6b).

Both MR and GR are required for the longer-term (240 min) hyperactivity. This supports our previous study where hyperactivity was induced by 16 h of cortisol treatment, and this response was abolished in fish lacking either GR or MR [25, 26]. While the molecular targets modulated by these receptor activations are unclear, the 5HT1A receptor has been identified in mammals as a potential target for both GR and MR [34]. For instance, an acute rise in corticosterone facilitates 5-HT1A-mediated behavioural responses [34], but the effects were receptor dependent [35]. *In vitro* activation of the high-affinity MR suppresses 5HT1A receptor-mediated hyperpolarization, while subsequent activation of lower affinity GR enhances the effects of 5HT [35]. This underscores that the effects of corticosteroids can be highly divergent and depend on receptor type, brain region, cell type, and physiological context [36]. Despite this, our work here shows that there was a very clear behavioural agenda and that this required a coordinated effort at multiple levels of the HPA axis.

Finally, despite the acute behavioural response being dependent on CRHR1, a lack of MR and GR also abolished the stress-induced hyperactivity (Fig. 6a). Indeed, the interaction between the CRH system and GR/MR in the acute stress-induced behaviour is far from clear. The ability of GCs to act rapidly via membrane MRs implies that it may synergize with other mediators of the primary stress response, including nor(adrenaline), CRH, and vasopressin [7]. Rapid MR signalling is thought to be mediated by membrane MRs in the hippocampus, amplifying the excitatory glutamatergic responses via a pre-synaptic pathway [7]. These membrane MRs have a lower affinity and therefore respond to a stress-induced rise in GC levels, compared to the nuclear MR [7]. However, this may not be the case in our study as MET treatment, which abolished the cortisol response at 15 min, did not inhibit the acute stress-induced hyperactivity (Fig. 3). Neuronal MR activation is also required to maintain neuronal integrity and a stable excitatory tone [7] and may be neces-

sary for CRH signalling outside of the PVN [17, 37]. However, fish lacking MR have a relatively normal cortisol response to stress [26], implying functional CRHR1 signalling. Overall, while our results indicate a potential interaction between CRH and MR signalling in mediating the acute stress-induced hyperactivity in zebrafish larvae (Fig. 6), the mechanisms are unclear. CRHR1 activation may provide the cellular context necessary for membrane MRs to amplify the initial stress reaction [7, 37].

While GR signalling is primarily thought to mediate the longer-term effects of GCs on behaviour [7], our results indicate an acute stress-induced behavioural reaction mediated by this receptor (Fig. 6b). In mammals, the hypothalamic PVN, which mediates the activation of the HPI axis, expresses high levels of GR but virtually no MR, suggesting the primary role of GR is to mediate the negative feedback regulation of cortisol levels [8]. This receptor, similar to MR, can also translocate to the membrane of hypothalamic neurons [38]. The rapid, inhibitory effects of GCs on HPA axis activity have been previously established, and an infusion of dexamethasone, a synthetic GC, or a membrane-impermeable conjugate of DEX into the PVN cause this rapid inhibition [39]. This suggests that there may be a nongenomic role for GR, but whether this mediates the acute behavioural response observed in the present study needs further investigation.

Taken together, without CRHR1, GR, or MR, zebrafish larvae fail to exhibit the acute stress-induced hyperactivity, highlighting the interdependency of the HPA axis (Fig. 7). Given that the stress-mediated behavioural response is CRH mediated (Fig. 2), our results support the hypothesis that GR and MR signalling is also required for the acute stress-induced hyperactivity in zebrafish. Conversely, without CRHR1, cortisol will fail to increase, and long-term, stress-mediated behaviour dictated by GR and MR will also be absent. A limitation of this paper is the fact that we performed these studies in larvae, using a behavioural paradigm that is specific to larvae. Whether a similar integrated effort will be observed in adults in response to stress remains to be seen. Furthermore, the concept that the integration of multiple hormones may promote a particular biological agenda during the stress response almost certainly exists beyond behaviour. For example, in metabolism, both epinephrine and GCs promote increased glucose levels by increased hepatic glycogenolysis and gluconeogenesis [40]. The effects of CRH/CRHR1 signalling may also expand beyond its central role as CRHR1 is ubiquitously expressed in zebrafish tissues (Fig. 1b). Whether this receptor can act as a “first-

line” player in peripheral response to the stress warrants further study. Taken together, in vivo stress-mediated effects such as behaviour require complex coordination between different components of the HPA axis. We have identified key players and their temporal contribution to the acute stress-related behaviour, and this information may be useful when considering different treatment options for stress-related behavioural disorders such as anxiety and depression.

Statement of Ethics

All experimental protocols (Protocol #: AC17-0079) were approved by the Animal Care and Use Committee at the University of Calgary and were in accordance with the guidelines established by the Canadian Council on Animal Care.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Conception of study and experimental design: M.M.V. and E.F. Performed experiment and analysed results: E.F. Manuscript draft: M.M.V. and E.F. All authors approved the final version of the manuscript.

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