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rbfox1 loss of function in zebrafish leads to dysregulation in *bdnf/trkb2* and *pac1a* expression resulting in HPI axis hyperactivation, altered stress response and allostatic overload

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ABSTRACT

RBFOX1 regulates transcriptional networks linked to synaptic transmission and neurodevelopment. Mutations in the *RBFOX1* gene are associated with psychiatric disorders but how RBFOX1 influences psychiatric disorder vulnerability remains unclear. Recent studies showed that RBFOX1 mediates the alternative splicing of PAC1, a critical HPA axis activator. Further, RBFOX1 dysfunction is linked to dysregulation of BDNF/TrkB, a pathway promoting neuroplasticity, neuronal survival, and stress resilience. Hence, RBFOX1 dysfunction may increase psychiatric disorder vulnerability via HPA axis dysregulation, leading to disrupted development and allostatic overload. To test this hypothesis, we generated a zebrafish *rbfox1* loss of function (LoF) line and examined behavioural and molecular effects during development. In larvae and adults, *rbfox1* LoF resulted in hyperactivity, impulsivity and hyperarousal, and alterations in proliferation, fertility and survival, traits associated with allostatic overload. In larvae, *rbfox1* LoF disrupted expression of *pac1a*, *bdnf*, *trkb2,* and HPI axis genes. These latter were restored after chronic TrkB agonist/antagonist treatment. In adults, *bdnf*/*trkb2* and HPI axes dysregulation was only seen following acute stress. Our findings revealed a strict interplay between RBFOX1 and BDNF/TrkB in stress resilience and suggest that *RBFOX1* LoF predisposes to psychiatric diseases through HPA axis hyperactivation during development, impairing adaptation and heightening vulnerability to allostatic overload.

INTRODUCTION

Psychiatric disorders are influenced by multiple genes, with a substantial portion of their heritability linked to common genetic variations[1]. Identifying gene variants associated with multiple psychiatric disorders is crucial for understanding their shared genetic underpinnings and enhancing therapeutic strategies.

RNA binding fox-1 homologue 1 (RBFOX1) is a splicing factor highly conserved among vertebrates and expressed in the heart, brain and skeletal muscle, where it contributes to normal development and function[2,3]. Alterations in *RBFOX1* expression or function have been associated with susceptibility to psychiatric disorders, particularly those associated with changes in stress-related behaviour[4–6]. In a cross-disorder genome-wide association study (GWAS), *RBFOX1* emerged as the second most pleiotropic *locus*, showing association with seven out of the eight disorders studied: schizophrenia, bipolar disorder, depression, attention-deficit/hyperactivity disorder (ADHD), autism spectrum disorder (ASD), obsessive-compulsive disorder and Tourette syndrome[1]. In mice and zebrafish, *Rbfox1/rbfox1* knockout caused hyperactivity, increased anxiety-like behaviour and altered social behaviour[2,6]. However, the mechanisms by which *RBFOX1* genetic variants contribute to psychiatric disease are poorly understood.

In both mice and zebrafish, it has been shown that Rbfox1 regulates the alternative splicing of the Pituitary Adenylate Cyclase-Activating Polypeptide Type 1 Receptor (Pac1), an important mediator of corticotropin releasing hormone (CRH) synthesis in the hypothalamus[4]. CRH is the first hormone to be secreted in response to stress by the hypothalamic-pituitary-adrenal (HPA) axis (known in fish as the hypothalamic-pituitary-interrenal (HPI) axis)[7]. In mammals there are several PAC1 isoforms with the predominant isoforms in the brain being PAC1-hop (long isoform) and PAC1-short[8]. In mice and zebrafish, both *PAC1-hop* and *PAC1-short* increased during the early stress recovery phase, while at the late recovery phase, only *Pac1-hop* was still up-regulated[4].

Another regulator of the response to stress and a resilience factor against chronic stress-induced psychopathology is the brain derived neurotrophic factor (*BDNF*)/Tropomyosin receptor kinase B (TrkB) signalling pathway[9,10]. BDNF is a neurotrophin possessing a pivotal role in the modulation of neurotransmission and synaptic plasticity and dysregulation of the BDNF/TrkB pathway has been associated with several neuropsychiatric diseases, including anxiety/stress disorders[11,12]. In human neural stem cells, *RBFOX1* knockdown increased the expression levels of *BDNF*[13], while another study in mice identified TrkB as a target of Rbfox1 within the hippocampus[14]. Further, PAC1 activation can elevate *BDNF* levels and potentiate TrkB activity, enhancing the neuroprotective and plasticity-promoting effects of BDNF/TrkB signaling especially in the context of stress response and neuropsychiatric health[15].

Given that both PAC1 and BDNF/TrkB influence how the brain adapts to and manages stress, RBFOX1 variants may increase susceptibility to psychiatric disorders through dysregulation of the stress response, leading to adaptive plasticity and disrupted development in the short-term and allostatic overload in the long-term. Allostasis is the term used to describe the collective processes by which the body actively maintains stability (or homeostasis) in response to environmental stressors. When allostasis primary mediators (e.g., hormones of the HPA axis, catecholamines, and cytokines) are overactivated or fail to return to normal, it leads to an allostatic state[16,17]. The cumulative results of an allostatic state are referred to as allostatic load and, when maladaptive, allostatic overload[16]. Excess glucocorticoid exposure during early life and early life stress have been shown to cause prolonged activation of the allostatic systems, ultimately leading to allostatic overload[17]. Here, to explore the possibility that RBFOX1 loss of function (LoF) leads to increased vulnerability to psychiatric disease

through hyperactivation of the HPA axis and allostasis-induced adaptation during development, we generated a zebrafish line carrying a predicted *rbfox1* LoF mutation using CRISPR-Cas9 gene editing and assessed behavioural and molecular changes during development. We hypothesized that, in addition to PAC1 alternative splicing, RBFOX1 regulates HPA axis activity through an effect on BDNF/TrkB signalling, and that the combined effect leads to disrupted brain development.

RESULTS

Generation of a loss of function *rbfox1* line

CRISPR-Cas9 genome editing generated a 19 base pair deletion (NM_001005596.1, nts 120-138, TCCCATCGGCCCAGTTCGC) that introduced a premature termination codon (PTC) at position 58 in the *rbfox1* aminoacid sequence. Details regarding the generation of the line, nucleotide and aminoacid sequences can be found in our previous study[2]. Wild type animals are denoted throughout as $rbfox1^{+/+}$, heterozygous mutants as $rbfox1^{+/19del}$ and homozygous mutants as $rbfox1^{19del/19del}$.

To evaluate whether the PTC elicited mRNA non-sense mediated decay (NMD) and consequent reduction of *rbfox1* mRNA in mutant fish, we examined *rbfox1* expression by quantitative Real-Time polymerase chain reaction (qPCR) and by *in situ* hybridisation (ISH). qPCR showed that *rbfox1* transcript levels were significantly lower in mutant larvae compared to $rbfox1^{+/+}$ siblings ($p_{rbfox1^{+/+} \text{ vs } rbfox1^{+/19del}} < 0.05$, $p_{rbfox1^{+/+} \text{ vs } rbfox1^{19del/19del}} < 0.01$), and ISH showed that *rbfox1* was not detectable in $rbfox1^{19del/19del}$ fish, at either larval or adult stages (Supplementary Figure 1A-C).

These results confirm degradation of defective *rbfox1* mRNA in mutant fish.

rbfox1 mutant fish show hyperactivity, impulsivity and hyperarousal behaviour

As mutations in *RBFOX1* locus have been linked to several psychiatric diseases in humans, we examined *rbfox1* mRNA distribution in $rbfox1^{+/+}$ larvae and adults (Tübingen strain) and assessed zebrafish larvae and adult fish for phenotypic traits associated with such disorders.

In humans, *RBFOX1* copy number variants (CNVs) and LoF mutations are risk factors for ADHD[6]. Two major ADHD traits are hyperactivity and increased impulsivity. We therefore assessed hyperactive and impulsive behaviour of $rbfox1^{+/+}$, $rbfox1^{+/19del}$ and $rbfox1^{19del/19del}$ 5 days post fertilization (dpf) larvae and adults. When we measured larval locomotion, we observed a gene dosage effect on distance travelled whereby $rbfox1^{19del}$ larvae travelled greater distances ($p_{rbfox1^{+/+} \text{ vs } rbfox1^{+/19del}} < 0.05$, $p_{rbfox1^{+/+} \text{ vs } rbfox1^{19del/19del}} < 0.0001$, $p_{rbfox1^{+/19del} \text{ vs } rbfox1^{19del/19del}} < 0.05$) (Figure 1A), and a significant increase in the swimming speed of $rbfox1^{19del/19del}$ larvae ($p_{rbfox1^{+/+} \text{ vs } rbfox1^{19del/19del}} < 0.0001$, $p_{rbfox1^{+/19del} \text{ vs } rbfox1^{19del/19del}} < 0.05$) (Figure 1B). We also measured larval burst swimming, a parameter previously used as a measure to predict impulsive behaviour in zebrafish larvae to assess impulsivity[18]. We found a significant increase in the number of peaks (acceleration events when the fish travelled > 5 mm in < 12 s) of $rbfox1^{19del/19del}$ larvae compared to $rbfox1^{+/+}$ and $rbfox1^{+/19del}$ siblings ($p_{rbfox1^{+/+} \text{ vs } rbfox1^{19del/19del}} < 0.0001$, $p_{rbfox1^{+/19del} \text{ vs } rbfox1^{19del/19del}} < 0.0001$) (Figure 1C). This is in line with hyperactivity observed in adults $rbfox1^{19del/19del}$ in our previous study[2]. Impulsive behaviour was then assessed in adult (7 months old) fish using the 5-choice serial reaction time task (5-CSRTT)[19]. The 5-CSRTT consists of five stages (see Supplementary Table2) each run for at least a week until fish are promoted to the next stage. We found that 85% of the fish (100% $rbfox1^{+/+}$, 92% $rbfox1^{+/19del}$ and 74% $rbfox1^{19del/19del}$) learned the task within 9 weeks. During stages 2-3 there was a significant difference in the correct response (learning) ($p_{stage2} < 0.001$, $p_{stage3} < 0.05$) such that $rbfox1^{19del\text{-}19del}$ learned more slowly than $rbfox1^{+/+}$ ($p < 0.01$) and $rbfox1^{+/19del}$ ($p < 0.01$) in stage 2, and more slowly than $rbfox1^{+/+}$ ($p < 0.05$) in stage 3. We found no differences in the correct response in stages 4-5. In stage 5, we found a significant differences in the

number of premature responses (increased impulsivity) ($p < 0.01$) such that *rbfox1*$^{19del/19del}$ fish were more impulsive compared to *rbfox1*$^{+/+}$ ($p < 0.01$) and *rbfox1*$^{+/19del}$ ($p < 0.05$) siblings (Figure 1D-H).

RBFOX1 CNVs have also been identified in populations of individuals with schizophrenia[6,13]. Deficits in habituation to acoustic startle reflex are seen in both human schizophrenic patients and animal models of schizophrenia[20]. In zebrafish, the acoustic startle assay has been widely employed to measure habituation and can be used as an indicator of hyperarousal responses associated with increased anxiety (i.e., failure to habituate to the startles and increased distances travelled during the acoustic stimuli)[21]. We therefore tested 5dpf zebrafish larvae (*rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$ and *rbfox1*$^{19del/19del}$) in the habituation to acoustic startle response assay to assess differences in the rate of habituation. Consistent with our hyperactivity assay, during the baseline period (first 10 min of the assay), the genotype had a significant main effect on distance travelled [Effect of genotype: $\chi2(2) = 8.3397$, $p < 0.05$] whereby *rbfox1*$^{19del/19del}$ larvae travelled greater distances than *rbfox1*$^{+/+}$ siblings ($p < 0.05$). During the startle stimuli, we observed a main effect of genotype [Effect of genotype: $\chi2(2) = 33.536$, $p < 0.0001$] and stimulus number [Effect of stimulus number: $\chi2(9) = 487.968$, $p < 0.0001$] on distance travelled, and a significant two-way interaction between genotype and stimulus number [Effect of genotype by stimuli: $\chi2(18) = 31.511$, $p < 0.05$], such that *rbfox1*$^{19del/19del}$ larvae travelled greater distances than *rbfox1*$^{+/+}$ ($p < 0.001$) and *rbfox1*$^{+/19del}$ ($p < 0.001$) (Figure 1I). When assessed for the rate of habituation, larvae showed a habituation response to repeated acoustic startle consistent with previous reports[22]: 100% of *rbfox1*$^{+/+}$animals responded to the first acoustic stimulus, but only 22% responded to the last. When we examined the rate of habituation over time, we observed a significant effect of genotype [Effect of genotype: $\chi2(2) = 7.2676$, $p < 0.05$] and a significant two-way interaction between genotype and stimulus number [Effect of genotype by stimulus number: $\chi2(18) = 132.8476$, $p < 0.001$] whereby *rbfox1*$^{19del/19del}$ showed reduced rate of habituation and a greater proportion of responders compared to *rbfox1*$^{+/+}$ siblings ($p_{rbfox1^{+/+} \text{ vs } rbfox1^{19del/19del}} < 0.05$) (Figure 1J).

SNPs in *RBFOX1* locus have also been associated with anxiety[6,23]. Hence, we assessed anxiety-like behavior in 5dpf zebrafish larvae using the forced light-dark transition (FLDT) assay and in adult zebrafish using the novel tank diving (NTD) assay. In the FLDT assay, zebrafish are exposed to sudden transitions in illumination with effects on locomotion being used as a measure of anxiety-like behaviour: on transition from dark to light, a rapid startle response resulting in a brief, sharp increase in locomotion is observed, followed by a reduction in movement, while on transition from light to dark, larvae sharply increase their locomotion and then steadily decrease it. The increased locomotion upon the light-dark transition is attributed to increased stress/anxiety level[24–27]. Consistent with our acoustic startle assay, we found that during the baseline period (first 5 min of the assay) (Figure 1K) genotype had a significant main effect on distance travelled [Effect of genotype: $\chi2(2) = 8.8262$, $p < 0.05$] whereby *rbfox1*^{19del} larvae travelled greater distances than *rbfox1*$^{+/+}$ siblings ($p_{rbfox1^{+/+} \text{ vs } rbfox1^{+/19del}} < 0.05$, $p_{rbfox1^{+/+} \text{ vs } rbfox1^{19del/19del}} < 0.05$). We then examined the locomotion and amplitude of response during the 1min light flash (min 5-6 of the assay). We found a significant main effect of genotype on distance travelled [Effect of genotype: $\chi2(2) = 12.8139$, $p < 0.01$] with *rbfox1*$^{19del/19del}$ larvae travelling greater distances than WT ($p < 0.01$) siblings (Figure 1K). On transition from dark to light (examined at 1 sec resolution from sec 240 to sec 360) we found a significant main effect of time [Effect of time: $\chi2(58) = 705.091$, $p < 0.001$] and genotype [Effect of genotype: $\chi2(2) = 10.289$, $p < 0.01$], and a significant two-way interaction between time and genotype [Effect of time by genotype: $\chi2(2) = 188.008$, $p < 0.001$] on the amplitude of response, whereby *rbfox1*$^{19del/19del}$ larvae startled more than *rbfox1*$^{+/+}$ and *rbfox1*$^{+/19del}$ siblings ($p_{rbfox1^{+/+} \text{ vs } rbfox1^{19del/19del}} < 0.05$, $p_{rbfox1^{+/19del} \text{ vs } rbfox1^{19del/19del}} < 0.05$) (Figure 1L).

Anxiety-like behaviour in adult animals was assessed using the NTD assay. When introduced to a novel tank, zebrafish will first dive to the bottom of the tank, to seek protection, and then gradually increase

their swimming over time. During the first minute of the assay, we found a significant two-way interaction between genotype and the time spent at the bottom of the tank [Effect of genotype by proportion at the bottom tank: $\chi2(10) = 22.3333$, $p = 0.01349$], such that *rbfox1*$^{19del/19del}$ fish spent less time in the bottom than *rbfox1*$^{+/+}$ fish ($p < 0.001$) (Supplementary Figure 3A). However, during the remaining 5 minutes of the assay, we observed no significant differences in the time spent at the bottom of the tank ($p > 0.05$). In line with previous findings[2], we observed no significant differences in distance travelled ($p > 0.05$) (Supplementary Figure 3B), nor in the number of the transitions to the top area of the tank between *rbfox1* genotypes ($p > 0.05$) (Supplementary Figure 3C).

Thus, our data showed that loss of *rbfox1* resulted in behavioural changes that resemble psychiatric disorder traits in humans and suggest that adaptation occurs as the animals develop Consistent with our behavioural results and as seen previously[2,3], when we assessed *rbfox1* mRNA distribution in *rbfox1*$^{+/+}$ larvae and adults (Tübingen strain), we found that *rbfox1* was expressed in regions of the brain involved in the response to stress, in social and emotional behaviour, and in reward and learning (Supplementary Figure 2A-B) in agreement with data in rodents[28] and humans[29].

rbfox1 LoF disrupts zebrafish larvae HPI axis via *bdnf/trkb2* pathway

As larvae showed altered stress response and RBFOX1 has been linked to regulation of expression of HPA axis genes which may be mediated via BDNF/TrkB signalling, as well asBDNF/TrkB genes expression[4,13,14], we examined the expression of components of the HPI axis and of *bdnf/trkb2* following chronic developmental exposure to the TrkB agonist (7,8-DHF) and antagonist (ANA-12).

We performed qPCR experiments in 5dpf zebrafish larvae (*rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$, *rbfox1*$^{19del/19del}$) to assess changes in the expression levels of the HPI axis markers corticotropin releasing hormone (*crhb*), mineralocorticoid receptor (*mr*), and glucocorticoid receptor (*gr*), and of *bdnf* and *trkb2*. In teleosts, the duplication of the genome gave rise to two *CRH* genes, *crha* and *crhb,* and two TrkB genes, *trkb1* and *trkb2*[30,31]. However, only *crhb* and *trkb2* are regarded as the orthologues of the mammalian *CRH* and *TrkB* respectively[31,32]. Then, unlike other teleosts, zebrafish possess a single copy of the *MR* and *GR* genes, *mr* and *gr* respectively, and a single copy of the *BDNF* gene (*bdnf*)[31,33]. In mammals, several TrkB splicing isoforms are present, but the most abundant ones are the full-length TrkB.FL (TK+) and the truncated TrkB.T1 (TK-), this latter lacking the catalytic tyrosine kinase (TK) domain[34]. As in zebrafish the presence of both *trkb2* full-length and truncated forms has been demonstrated, here we used a pair of *trkb2 TK+/TK-* common primers, and another pair targeting only *TK+* to distinguish effects on the expression of the two isoforms[35].

In the absence of TrkB agonist/antagonist we found significant up-regulation of *crhb* in *rbfox1*$^{19del/19del}$ larvae ($p_{rbfox1^{+/+} \text{ vs } rbfox1^{19del/19del}} < 0.0001$, $p_{rbfox1^{+/19del} \text{ vs } rbfox1^{19del/19del}} < 0.0001$) and a significant up-regulation of *mr* in both *rbfox1*$^{+/19del}$ and *rbfox1*$^{19del/19del}$ larvae ($p < 0.05$), while we observed no significant changes in *gr* expression levels across genotypes ($p > 0.05$) (Figure 2A). Furthermore, we observed a significant up-regulation of *bdnf* in both *rbfox1*$^{+/19del}$ and *rbfox1*$^{19del/19del}$ larvae ($p < 0.05$) (Figure 2B) and significant down-regulation of *trkb2 TK-* (we could not detect any *trkb2 TK+* expression in 5dpf zebrafish larvae (Cq values ≥ 40)) in both *rbfox1*$^{+/19del}$ ($p_{rbfox1^{+/+} \text{ vs } rbfox1^{+/19del}} < 0.01$) and *rbfox1*$^{19del/19del}$ larvae ($p_{rbfox1^{+/+} \text{ vs } rbfox1^{19del/19del}} < 0.001$) (Figure 2B).

After chronic treatment from 5 hours post fertilisation (hpf) with either 7,8-DHF or ANA-12, *crhb* and *mr* expression levels of *rbfox1*$^{19del/19del}$ larvae were restored to levels comparable to controls (*rbfox1*$^{+/+}$) (Figure 2C-D). We observed no significant differences in the expression levels of *gr* ($p > 0.05$) (Figure 2C-D).

As the hypothalamus is the primary player in the stress response and *rbfox1* had only previously been shown to affect TrkB expression within the hippocampus, we used ISH to determine whether *trkb2* mRNA expression was reduced within the hypothalamic area. Since *trkb2* was similarly down-regulated by qPCR in both *rbfox1*^{19del} mutants, we employed only *rbfox1*$^{+/+}$ and *rbfox1*$^{19del/19del}$ larvae. In agreement with previous findings[36], in *rbfox1*$^{+/+}$ we found that *trkb2* is widely expressed in the brain of 5dpf larvae, whereas in *rbfox1*$^{19del/19del}$ larvae, consistently with our qPCR experiments, we found a significant overall reduction of *trkb2* mRNA in the whole brain (Figure 2E-H), including in the hypothalamus ($p < 0.01$) (Figure 2F, I, J).

Our findings showed that *rbfox1* LoF resulted in dysregulated *bdnf/trk2b* expression, leading to HPI axis hyperactivation.

***rbfox1* LoF alters *pac1a* expression levels of zebrafish larvae**

As RBFOX1 is a known regulator of *PAC1* alternative splicing[37] and PAC1 has been shown to regulate *BDNF* transcription and potentiate TrkB activity[15], we examined expression levels of *pac1a,* a zebrafish homologue of the mammalian *PAC-1*, in *rbfox1*$^{+/+}$ and *rbfox1*$^{19del/19del}$ 5dpf larvae by qPCR.

In mammals there are several PAC1 isoforms and their role in the regulation of stress is poorly understood[8]. In the brain, the predominant isoforms are *PAC-1-hop* (long isoform) and *PAC-1-short*[8]. Zebrafish possess two *pac1* genes, *pac1a* and *pac1b*, but only *pac1a* contains the *hop* cassette[8]. *PAC-1 short* is expressed during early stress response phase and enhances *CRH* transcription, while *PAC-1-hop* reduces *CRH* synthesis during stress recovery, ending the stress response[4]. We measured expression levels of both *pac1a-short* and *-hop* isoforms in *rbfox1*$^{+/+}$ and *rbfox1*$^{19del/19del}$ 5dpf larvae. We observed no differences in *pac1a-short* expression, but we found a significant up-regulation of *pac1a-hop* in *rbfox1*$^{19del/19del}$ mutant larvae ($p < 0.05$) (Figure 2K).

These results showed that *rbfox1* LoF altered *pac1a* expression in zebrafish larvae increasing selectively the *pac1-hop* isoform, which might be an adaptive response to terminate *crhb* transcription and decrease the HPI hyperactivation.

***rbfox1* mutants undergo adaptive mechanisms and allostatic overload during development**

The HPA axis possesses a vital role in the maintenance of allostasis, the process by which the body achieves stability in response to stress or environmental challenges. Dysregulation of the HPA axis often leads to disrupted allostasis during later life, also termed as allostatic load (i.e., the physiological consequence resulting from the cumulative "wear and tear" of the body in response to chronic stress)[38]. As we observed dysregulation of the HPI axis gene expression in *rbfox1*^{19del} mutant larvae and altered behavioural response to stress in *rbfox1*^{19del} mutant larvae but not in adults, we assessed expression of HPI axis components in adult zebrafish, in the presence and absence of acute stress, to explore the possibility of adaptation that may contribute to differences in allostatic load.

We first measured *crhb*, *mr*, *gr*, *bdnf* and *trkb2* mRNA expression levels in the brains of adult fish in physiological resting conditions and then, in the brains of adult fish exposed to a stressor (NTD). In physiological resting conditions, we observed no differences in HPI nor *bdnf/trkb2* axes gene expression levels ($p > 0.05$) (Figure 3A-B). However, after exposure to a stressor (NTD), with regards to the HPI axis, we observed the same dysregulation in *rbfox1*$^{19del/19del}$ adults as in *rbfox1* LoF larvae: we found significant up-regulation of *crhb* ($p_{rbfox1^{+/+} \text{ vs } rbfox1^{19del/19del}} < 0.001$), *mr* ($_{rbfox1^{+/+} \text{ vs } rbfox1^{19del/19del}} < 0.0001$), with no changes in *gr* expression levels ($p > 0.05$) (Figure 3A), and *bdnf* up-regulation ($p_{rbfox1^{+/+} \text{ vs } rbfox1^{19del/19del}} < 0.01$) (Figure 3B). Furthermore, in *rbfox1*$^{19del/19del}$ stressed adults, we found unbalanced *trkb2 TK+/TK-* levels ($p_{rbfox1^{+/+} \text{ vs } rbfox1^{19del/19del}} < 0.001$) not seen in *rbfox1*$^{+/+}$ fish, with a stark increase in *trkb2 TK+* ($p_{rbfox1^{+/+} \text{ vs } rbfox1^{19del/19del}} < 0.05$), while trkb2 TK- was still down-regulated ($p_{rbfox1^{+/+} \text{ vs } rbfox1^{19del/19del}} < 0.05$) (Figure 3C).

When the cumulative burden of chronic stress (i.e., the allostatic load) becomes excessive, the body's ability to maintain allostasis is compromised, resulting in significant physiological and psychological harm, also known as allostatic overload[39]. Excess glucocorticoid (GC) exposure during early developmental stages or early life stress (ELS) can lead to allostatic overload in the long-term[17].

One of the effects caused by GC- or ELS-induced allostatic overload is reduced cell proliferation during adulthood[17]. This effect has been suggested to be developmentally dynamic, since it is often preceded by increased cell proliferation during early stages[17]. Further, Bdnf signalling has been shown to regulate neural stem cell proliferation through TK-, suggesting that *rbfox1* LoF fish that have altered levels of *TK*- expression, may show altered proliferation across the life course. We therefore assessed the rate of proliferation at both larval and adult stages using qPCR for proliferating cell nuclear antigen (*pcna*). We observed a significant increase in *pcna* expression levels in 5dpf *rbfox1*$^{19del/19del}$ zebrafish larvae ($p_{rbfox1^{+/+} \text{ vs } rbfox1^{19del/19del}} < 0.05$) and a significant decrease in *pcna* expression levels in the brains of adult *rbfox1*$^{19del/19del}$ fish when compared to *rbfox1*$^{+/+}$ siblings ($p_{rbfox1^{+/+} \text{ vs } rbfox1^{19del/19del}} < 0.01$) (Figure 3D).

In zebrafish, other effects caused by GC-induced allostatic overload include reduced fertility and survival rates[17]. Therefore, we assessed fertility and survival of *rbfox1*$^{19del/19del}$ zebrafish versus *rbfox1*$^{+/+}$ siblings. We found that both fertility ($p_{rbfox1^{+/+} \text{ vs } rbfox1^{19del/19del}} < 0.05$) and survival ($p_{rbfox1^{+/+} \text{ vs } rbfox1^{19del/19del}} < 0.001$) of *rbfox1*$^{19del/19del}$ fish were significantly reduced compared to *rbfox1*$^{+/+}$ siblings (Figure 3E-F).

Consistent with our data showing hyperactivation of the stress response system, these findings suggest that, during development, *rbfox1* mutants undergo adaptive changes like those caused by GC-exposure, leading to altered development and lasting differences in response to environmental stressors.

DISCUSSION

In this study we generated a CRISPR-Cas9 LoF *rbfox1* zebrafish line (*rbfox1*^{19del}) to investigate the mechanisms by which *RBFOX1* LoF increases susceptibility to psychiatric disorders.

Allostatic load has been linked with several cognitive disorders including depression, schizophrenia, anxiety and PTSD[40]. Overactivity of the HPA axis is a key factor in the onset of the allostatic load[38]. RBFOX1 is known to regulate the stress response by influencing expression of genes related to stress signaling pathways such as PAC1 and BDNF/TrkB. Therefore, alterations in RBFOX1 function may increase vulnerability to psychiatric disorders through alterations in stress response systems, such as the HPA axis, predisposing to vulnerability to allostatic overload in later life.

Similarly to *Rbfox1* deficient mice[6], we found that *rbfox1*$^{19del/19del}$ mutants were hyperactive and impulsive. These findings are consistent with studies showing *RBFOX1* genetic variants (both common and rare) as risk factors for psychiatric disorders like ASD, ADHD, schizophrenia and anxiety/stress disorders[5,6,41]. In the response and habituation to acoustic startle assay, *rbfox1*$^{19del/19del}$ larvae startled more and had a higher response rate over time compared to *rbfox1*$^{+/+}$ siblings. In a forced light-dark assay, although baseline hyperactivity of *rbfox1*$^{19del/19del}$ larvae confounds results, *rbfox1*$^{19del/19del}$ larvae showed an increased startle response upon dark to light transition, consistent with heightened anxiety. These results suggest that *rbfox1* LoF leads to heightened arousal, aligning with studies linking *RBFOX1* to mood and anxiety disorders, including PTSD[42]. In the 5-CSRTT we also found that *rbfox1*$^{19del/19del}$ mutants learn the task more slowly and were more impulsive than *rbfox1*$^{+/+}$ siblings, consistent with RBFOX1 variants being associated with impulsivity and related disorders such as ADHD and ASD[6].

In *rbfox1* LoF larvae we observed dysregulation of the fish HPI axis, analogous to the HPA axis in mammals. The HPA axis is crucial for managing stress, with CRH triggering cortisol secretion, affecting metabolism, immunity, and behaviour[43,44]. In zebrafish larvae under resting physiological conditions, we found increased *crhb* and *mr*, with no changes in *gr* levels. In adults, although resting levels were unchanged, *rbfox1* LoF showed an exaggerated response to stress. Elevated CRH levels suggest a chronic anxiety state and increased CRH and MR levels are linked to mood disorders like depression and PSTD[45–48]. These findings support RBFOX1 role in CRH regulation and suggest that RBFOX1 LoF may contribute to mood disorders via HPA axis hyperactivation and disrupted stress resilience.

BDNF and TrkB are key regulators of the stress response[49]. BDNF levels often increase during stress to promote neuronal survival and plasticity, buffering negative effects of stress on the brain via TrkB[9–11,49–51]. Dysfunction of BDNF/TrkB signalling is linked to several stress-related disorders[11]. The increased *bdnf* expression we observed in *rbfox1*$^{19del/19del}$ larvae under resting physiological conditions suggests a compensatory response to cope with *crhb* increase and maintain homeostasis. In line with previous findings in zebrafish, we could not detect any *trkb2 TK+* transcripts in 5 days old *rbfox1*$^{+/+}$ larvae[35]. Therefore, we hypothesised that the behavioural and molecular effects observed here were mediated by the *trkb2 TK-* form and suggest that TrkB.T1 (TK-) can contribute to adaptive/repair mechanisms and disease. Indeed, TrkB.T1 (TK-) is up-regulated with ageing and in injured brains[52] and TrkB.T1 (TK-) expression is altered in brain samples from suicide victims[34]. In contrast to studies in rodents where Rbfox1 up-regulation led to increased TrkB.T1 (TK-) expression in the hippocampus and no changes were observed following Rbfox1 deletion[14], we observed widespread down-regulation of *trkb2 TK-* expression in *rbfox1*$^{19del/19del}$ larvae. The difference between these studies may be due to i) differences in the tissue examined (zebrafish larvae whole head versus rodent hippocampus only), ii) the developmental stage of the animal (larval versus adult stage), iii) the genotype of the animals studied (*rbfox1*$^{19del/19del}$ in our study versus *Rbfox1*$^{+/-}$ in the study in rodents), or iv) presence of compensatory mechanisms (in the hippocampal mouse model *Rbfox1* knockdown resulted in up-regulation of *Rbfox2*). As for this latter hypothesis, like in previous studies in zebrafish[2], we did not see any changes in *rbfox2* expression upon *rbfox1* LoF (Supplementary Figure4) suggesting that this compensatory mechanism does not occur in larval fish.

Consistent with our hypothesis that changes in HPI axis genes were mediated by Bdnf/Trkb2 signalling, chronic exposure to TrkB agonist (7,8-DHF) and antagonist (ANA-12) restored HPI axis genes expression to that seen in *rbfox1*$^{+/+}$. ANA-12 is a selective non-competitive antagonist of TrkB exerting central TrkB blockade and producing rapid and long-lasting anxiolytic and antidepressant effects[53]. Mice treated with ANA-12 showed reduced anxiety-like behaviour and in stressed rats, ANA-12 blocked CRH increase in the hypothalamus and amygdala[54]. 7,8-DHF is a potent selective agonist of TrkB used in the treatment of several disorders, including depression and schizophrenia, and has been shown to enhance memory consolidation and emotional learning in healthy rodents[55]. In a similar fashion, after chronic treatment with ANA-12, we observed that the expression of HPI axis genes in *rbfox1*$^{19del/19del}$ larvae was restored to *rbfox1*$^{+/+}$ levels. Contrary to expectation, a similar result was seen in the presence of either chronic agonist or antagonist exposure. However, chronic exposure to agonists may lead to desensitization and inhibition of TrkB signalling. Despite our inability to detect TK+ in *rbfox1*$^{19del/19del}$ 5dpf larvae, agonist/antagonist effects on HPI axis seen here suggest an action via TK+, as previous studies have indicated phosphorylation of TrkB.FL/TK+ upon BDNF binding regulates *CRH* expression[10].It is of note that neither TrkB agonist nor antagonist had any effect on gene expression in *rbfox1*$^{+/+}$ larvae. One possible explanation is that, as the larvae were not exposed to any stressors, any effect on trkb2 signalling had limited effect on HPI axis activity.

In *rbfox1*$^{19del/19del}$ larvae, we also observed up-regulation of *pac1a-hop* with no changes in *pac1a-short* levels. Previous studies in zebrafish have shown that Pac1-short gain of function caused persistent *crh* increase, while overexpression of Pac1-hop prevented stress-induced *crh* transcription activation[4].

The up-regulation of *pac1a-hop* observed in our study suggests an adaptive response to *crh* increase through an unknown mechanism or, alternatively, a compensatory mechanism to Pac1-hop protein deficiency caused by *rbfox1* LoF. This latter hypothesis needs further investigation.

In contrast to larvae, differences in HPI axis and *bdnf/trkb2* gene expression were not seen in adult animals under resting conditions. However, HPI dysregulation was evident in *rbfox1*$^{19del/19del}$ adults on challenge with a stressor, aligning with previous findings that Rbfox1 is required for the termination of the acute stress response[4] and that chronic stress during development leads to adult HPI axis adaptation, often at a cost in later life (e.g., reduced fertility and survival)[17,32]. This was evidenced by reduced fertility and survival rates in *rbfox1*$^{19/19del}$ adults. Another long-term effect of excess GC-exposure/early life stress is reduced neural stem cell proliferation in adult animals, often accompanied by increased proliferation at early stages[17]. Our findings that *rbfox1*$^{19/19del}$ showed increased *pcna* levels at larval staged but reduced *pcna* levels during adulthood, corroborate this latter hypothesis of a dynamic developmental effect of early life stress. Interestingly, after acute stress, *rbfox1*$^{19del/19del}$ adults showed unbalanced *trkb2* expression, with up-regulation of *trkb2.TK+* and down-regulation of *trkb2.TK-* (similar to *rbfox1*$^{19del/19del}$ larvae). Although both TrkB.FL/TK+ and TrkB.T1/TK- are thought to contribute to the stress response, and altered expression of TrkB.FL/TK+ and TrkB.T1/TK- is seen in the brain of suicide victims and deceased schizophrenic patients [56], the association between altered TrkB.FL/TK+ and TrkB.T1/TK- expression, stress response and psychiatric disease warrants further investigation.

In conclusion, given the conservation between fish HPI axis and the mammalian HPA axis, our data unveils a pivotal new finding in *CRH* regulation, revealing an interplay between RBFOX1 and BDNF/TrkB in the context of chronic stress and stress resilience and suggest that RBFOX1 plays a crucial role in adaptive stress mechanism. In response to stressful challenges, RBFOX1 functions as a "switch" maintaining the balance between short/long isoforms of both PAC1 and TrkB receptors: upon stress, RBFOX1 promotes PAC1-short alternative splicing and *TrkB.FL/TK+* mRNA stability, inducing *CRH* transcription; during the recovery phase, *RBFOX1* switches the balances in support of of *PAC1-hop* and *TrkB.T1/TK-* isoforms, decreasing *CRH* levels and turning off the HPA axis (Figure 4). Furthermore, as loss of *rbfox1* in zebrafish led to behavioral phenotypes resembling those associated with human psychiatric diseases (hyperactivity, impulsivity, reduced habituation, heightened arousal and impaired learning), our findings also suggest that *RBFOX1* contributes to the liability of such disorders through HPA axis hyperactivation and BDNF/TrkB dysregulation, leading to disrupted development and allostatic overload.

METHODS

Animal maintenance

Wild type (WT) and mutant zebrafish (Tübingen strain/background) were maintained in a recirculating system (Tecniplast, UK) with a 14h:10h light/dark cycle and a constant temperature of 28°C. Fish were fed with ZM-400 fry food (Zebrafish Management Ltd.) in the morning and brine shrimps in the afternoon. Breeding was set up in the evening, in sloping breeding tanks (Tecniplast) provided with dividers for timed mating. The following morning, dividers were removed to allow spawning. Eggs were collected in Petri dishes (max 50 eggs/dish). Infertile eggs were removed, and fertile ones were incubated at 28°C. Petri dishes were checked daily to ensure consistent developmental stage across groups. If reared, larvae were moved to the recirculating system at 5 days post fertilization (dpf) and fed as stated above. All procedures were carried out under license in accordance with the Animals (Scientific Procedures) Act, 1986 and under guidance from the Local Animal Welfare and Ethical Review Board at Queen Mary University of London.

Generation of *rbfox1* loss of function zebrafish line

The zebrafish *rbfox1* loss of function (LoF) mutant line was generated as described previously[57]. CRISPR RNA (crRNA) (Merck) was designed to target *rbfox1* exon 2 (**CCC**AGTTCGCTCCCCCTCAGAAC, PAM sequence in bold, MwoI recognition site underlined). A 3 µL injection mix containing 1 µL (F_C 83 ng/µL) crRNA, 1 µL (F_C 83 ng/µL) tracrRNA (Merck, Cat.TRACRRNA05N), 1 µL (F_C 1.67 µM) Cas9 protein (New England Biolabs, Cat.M0646) was freshly prepared on the morning of the injection procedure. Then, 1 nL of the injection mix was injected into one-cell stage zebrafish embryos (~ 100-150 embryos). Injection efficacy was assessed at 24 hours post fertilization (hpf) by polymerase chain reaction (PCR) from genomic DNA (*rbfox1*_Forward, 5'-TAATCAAGACGCCCCAGCAC–3'; *rbfox1*_Reverse, 5'-GTACTCAGCAGGAATGCCGT- 3') followed by MwoI restriction enzyme digestion. Successful injections will introduce indel mutations disrupting the recognition site of the restriction enzyme, preventing this latter from cutting the PCR amplicon. Once reached sexual maturity (at ~ 3 months of age) injected (F_0) fish were outcrossed with WT to generate F_1 embryos. This progeny will carry different mutations due to the mosaic nature of the F_0 parents. F_1 fish were therefore screened for mutations leading to premature termination codon (PTC) via cloning into pGEM-T Easy vector (Promega, Cat.A1360), followed by transformation, colony PCR, DNA purification (Monarch® Plasmid Miniprep Kit, NEB, Cat. T1010) and sequencing (Source BioScience PLC). Quantitative real time PCR (qPCR) was used to confirm reduction of *rbfox1* mRNA expression and non-sense RNA mediated decay (NMD).

Fish breeding and genotyping

For adult experiments, mixed sexes were used. When possible, animals were associated with an identification number and genotype was assigned after data analysis. For larval experiments, fish were generated by heterozygous in-cross and genotyped prior to data analysis. Genotyping primers were the same as the ones used to identify founder carriers.

Drug Treatment

Zebrafish embryos were treated from 5 hpf to 5 dpf with 20 µM TRKB antagonist ANA-12 (abcam, Cat.ab146196), or 20 µM TRKB agonist 7,8-dihydroxyflavone (7,8-DHF) (abcam, Cat. ab120996), or dimethylsulfoxide (DMSO, vehicle 0.01%) (Merck, Cat. 34869). Treatment was performed in Petri dishes (max 30 embryos x dish) and drugs or vehicle were dissolved in fish water. Solutions were replaced daily.

Total Nucleic Acid Extraction

Total Nucleic Acid Extraction was performed at 5dpf. DNA was employed for genotyping and RNA for qPCR analysis. DNA was extracted using the HotSHOT method. Samples were incubated at 95 °C in 50 mM NaOH for 30 min, followed by 1 min at 10 °C. Reaction was stopped using 1M Tris HCl, pH 8. Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, United States) following manufacturer's instructions. Briefly, after homogenization, RNA was isolated by precipitation, rinsed, and resuspended in RNase free water. Total RNA was then quantified using BioDrop (Biochrom Ltd.), and up to 1 µg was reverse transcribed to cDNA using the ProtoScript II First Strand cDNA Synthesis Kit (NEB, Cat.E6560) following manufacturer's instructions. Resulting cDNA yield and quality were evaluated using BioDrop (Biochrom Ltd.). Quantification of trkb2 splice variants was performed as described previously[58].

Quantitative Real-Time PCR

Quantitative Real-time PCR (qPCR) of target RNA was performed on 5dpf WT (*rbfox1*$^{+/+}$), *rbfox1* heterozygous (*rbfox1*$^{+19del}$) and *rbfox1* homozygous (*rbfox1*$^{19del/19del}$) larvae, using Luna® Universal One-Step RT-qPCR Kit (NEB, Cat.M3005) and a Bio-Rad 96-well qPCR machine (CFX96 Touch Real-Time PCR Detection System). All reactions included 5 biological replicates and 3 technical replicates. For experiments in larvae, each biological replicate consisted of 15 larval heads (eyes and jaw removed). For experiments in adults, each biological replicate consisted of a single brain. Actin – β 2 (*actb2*) and ribosomal protein L13a (*rpl13a*) were employed as reference genes. For the NMD, *rbfox1* primers were designed upstream of the CRISPR insertion. Accession numbers, primer sequences and amplification efficiencies for all the reference and target genes can be found in Supplementary Table 1.

***In situ* hybridization**

In situ hybridization (ISH) was carried out on whole mount zebrafish larvae and on sagittal sections of adult zebrafish brain as described previously[2]. The original clone to generate the riboprobe was provided by Dr William Norton (University of Leicester). A riboprobe to identify *rbfox1* mRNA (NM_001005596) was synthetized by *in vitro* transcription (IVT) using using MAXIscript™ SP6/T7 kit (Invitrogen by Thermo Fisher Scientific–Catalogue number AM1322, Carlsbad, CA, USA), following manufacturer's instructions and using a DIG RNA Labeling Mix, 10× conc (Roche, Basel, Switzerland) containing digoxigenin labeled uracil.

ISH on zebrafish larvae

In situ hybridization was carried out on 28 hpf, 2- 3- 4- and 5 dpf *rbfox1*$^{+/+}$ and *rbfox1*$^{19del/19del}$ larvae. To prevent skin pigmentation, embryos were incubated in 0.2 mM 1-phenyl 2-thiourea (PTU) (Sigma) from 24 hpf. When they reached the desired ages, larvae were fixed in 4% paraformaldehyde (PFA) (Merck, Cat. 158127) overnight (ON) at 4°C. The following day, larvae were rinsed in 1x phosphate buffered saline (PBS) supplemented with Tween (0.05% v/v), dehydrated in ascending methanol series (25%, 50%, 70%, 80%, 90%, 100% methanol, 5 min each) and stored in 100% methanol at -20 °C. To perform ISH experiments, larvae were rehydrated in descending methanol series (100%, 90%, 80%, 70%, 50%, 25% methanol), 5 min each, and washed in 1xPBS, 5 min. Larvae were permeabilized using proteinase K (PK) (stock 20 µg/mL in 1xPBS) as follows: 28 hpf larvae were permeabilized in PK 1:2000 in 1xPBS for 20 min at room temperature (RT), older stages were permeabilized in PK 1:1000 at 37°C for at least 30 min. Then, larvae were post fixed in 4% PFA for 20 min and washed in 1xPBS at RT, 5 x 5 min. Prehybridization was carried out in hybridization solution (HB) containing 50% formamide, 5% saline sodium citrate buffer (SSC), 50 µg/mL heparin, 0.05 mg/mL yeast RNA, 0.1% Tween 20, and 0.92% citric acid at 68 °C for 2h. Thereafter, larvae were incubated in HB containing *rbfox1* riboprobe (500 pg/µL), ON at 68°C. Post hybridization washes were performed at 68°C with a gradient of 2xSSC and formamide (50%, 25% and 0% formamide), 10 min each, and then twice with 0.02xSSC, 30 min each. Subsequently, larvae were blocked in blocking solution (BS) containing 10% normal sheep serum and 2 µg/µL bovine serum albumin, for 1h at RT. After blocking step, larvae were incubated in anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (Roche, Cat. 11093274910), 1:2000 in BS, 1h at RT and then ON at 4°C. The following day, larvae were washed in 1xPBS, 6 x 15 min each, and then in alkaline phosphatase (NTMT) buffer (100 mM Trish HCl, 50 mM MgCl2, 100 mM NaCl, 0.1% Tween), 3 x 5 min each. The chromogenic reaction was carried out by incubating the larvae in BCIP/NBT solution (Merck, Cat. 203790) in NTMT buffer, at RT in the dark, and were observed every 20 min until the signal detection. After reaching the desired staining, larvae were washed in 1xPBS at

RT, post fixed in 4% PFA for 2h, cleared and stored in 80% glycerol at 4°C. For sagittal cryosections, larvae were embedded in 1.5% low melting point agarose (Merck) supplemented with 5% Sucrose in 1xPBS. Sections (5 µm) were collected on adhesive microscope slides Superfrost® Plus Gold (Epredia).

ISH on adult zebrafish brain

ISH was conducted on paraffin embedded *rbfox1*$^{+/+}$ and *rbfox1*$^{19del/19del}$ adult brains. Fish were culled by overdose of tricaine prior to head removal. Brains were dissected and fixed in 4% PFA in 1xPBS, ON at 4°C. Brains were then rinsed in 1xPBS and dehydrated in ascending ethanol series (15 min in each of 30%, 50%, 70%, 80%, 90%, 100% ethanol) and embedded in paraffin. Transverse sections of 12 µm thickness were cut using a microtome (Leica). To perform ISH, slides were de-waxed in xylene (twice, 10 min each), rehydrated in descending ethanol series (2 x 5 min in absolute ethanol, then 90%, 80%, 70%, 50% and 25% ethanol, 5 min each), and rinsed in 1xPBS for 5 min. Then, sections were permeabilized using PK (0.05 µg/µL) for 8 min at RT, washed with 2 mg/mL glycine twice (5 min each), post fixed in 4% PFA for 20 min and washed in 1xPBS at RT. Prehybridization was carried out in HB, for 1h at 68°C. Thereafter, sections were incubated in HB containing *rbfox1* riboprobe (500 pg/µL), ON at 68°C. Post hybridization washes were performed at 68°C twice for 20 min in 1xSSC, twice for 20 min in 0.2xSSC, and several washes were performed in 1xPBS, 5 min each at RT. Then, sections were blocked in BS for 30 min at RT and incubated in a 1:2000 dilution of anti-digoxigenin Fab fragments conjugated with alkaline phosphatase in BS, ON at 4°C. The following day, sections were washed in 1xPBS, 5 x 10 min each. The chromogenic reaction was carried out by incubating the slides in BCIP/ NBT solution in NTMT buffer, at RT in the dark, and were observed every 20 min until the signal detection. When the desired staining was obtained, sections were washed in 1xPBS at RT, dehydrated in ascending ethanol series (25%, 50%, 70%, 80%, 90%, 100% ethanol, 5 min each), cleared in xylene (twice, 5 min each) and mounted with DPX mounting medium (Merck).

Image acquisition and processing

Pictures of whole mount ISH on zebrafish larvae were acquired by Leica MZ75 microscope. For ISH on sections, pictures were acquired using a Leica DMRA2 upright epifluorescent microscope with color QIClick camera (Leica) and processed with Velocity 6.3.1 software (Quorum Technologies Inc). Quantification of the ISH staining signal intensity was performed as described previously[59], using Fiji software[60]. Adult anatomical structures were identified according to the Neuroanatomy of the Zebrafish Brain by Wullimann [61].

Behavioral Assays

For larvae, all behavioural experiments were conducted on *rbfox1*$^{+/19del}$ in-cross progenies. Larvae were genotyped prior to data analysis. For adults, as the genotype of the animals was known, fish were pseudorandomised across testing systems with all trials having an approx. equal number of each genotype. The adult fish were weight- and age-matched, with approximately equal numbers of both sexes.

Larval behavioural experiments

Patterns of locomotor activity of 5 dpf *rbfox1*$^{+/+}$ and *rbfox1*^{19del} mutant zebrafish larvae were investigated as described previously[18,22,62]. Tests were conducted between 9 a.m. and 4 p.m. At 5 dpf, larvae were placed in individual wells of a 24-well plate. To reduce stress due to manipulation, larvae

were acclimatised for at least 1 h before testing. Then, plates were placed into the DanioVision observation chamber (Noldus). Locomotion parameters such as distance travelled and swimming velocity were recorded using EthoVision XT software (Noldus). Data were exported in 1 min and 1 sec time bins and analysed with R programming language[63]. For the hyperactivity assay, larval basal locomotion was tracked for 15 min in dark conditions. Larval swimming burst was assessed as described previously[18] and peaks were considered as the acceleration events when larvae travelled > 5 mm in < 12 s. The forced light dark transition assay was performed as described previously[19,64,65] with modifications: after an initial 5 min period of dark (baseline), larvae were exposed to one light/dark cycle of 1 min light (Noldus white light device) followed by 5 min dark. The response and habituation to acoustic startle stimuli was performed as described previously[22]: after 10 min of baseline (no stimuli, dark conditions), larvae were subjected to 10 sound/vibration stimuli (Noldus tapping device) over 20 s (2 s intervals between each stimulus).

5-CSRTT

As deficit in impulse control and learning are associated with many psychiatric diseases including ADHD and bipolar disorder, we measured impulsive action using a zebrafish version of the 5-Choice Serial Reaction Time Task (5-CSRTT)[19]. Adult zebrafish (7 months old, mixed-sex, $N_{rbfox1^{+/+}}$= 28, $N_{rbfox1^{+/19del}}$= 28 (26 learned the task and were used for the final analysis), $N_{rbfox1^{19del/19del}}$= 50 (37 learned the task and were used for the final analysis), were singly housed for a week prior to experiment and remained singly housed for the whole duration of the assay (up to 11 weeks). Fish were tested using the Zantiks AD unit (Zantiks, Supplementary Figure5). Each unit was provided with a small tank with five apertures and a food hopper insert. The five apertures created five different entry points for the fish, acting like the five nose poke holes of the rodent version of the assay. The food hopper was placed at the opposite side of the five apertures and formed an area for the fish to enter and collect food reward. Below the testing tank there was an integrated screen, used to display white light (stimulus) into the five apertures. Responses were detected when a fish enters these apertures and recorded with an integrated camera placed at the top of the tank. The experiment consisted of five training stages: i) habituation, ii) initiator training, iii) stimulus light training, iv) 5-CSRTT/no delay, v) 5-CSRTT/delay. Details of each stage are provided in Supplementary Table 2.

Novel tank diving

Novel tank diving is a behavioural test to assess anxiety level in adult fish. Response to novel tank was assessed in 9 months old zebrafish, mixed-sexes, *rbfox1*$^{+/+}$ and *rbfox1*^{19del} mutant fish (20 fish x genotype), as described previously[62]. Fish were singly housed for a week prior to performing the experiment and acclimatized for at least 1 h in the behavioral room on the testing day. Behavioural assays were conducted between 9 a.m. and 2 p.m. During the test, fish were individually placed into a 1.5 L tank and their behaviour was tracked and recorded using EthoVision system (Noldus). Data were exported in 1 min time bin and analysed as previously described[62]. Experimental groups were randomised during testing. We analyzed three behaviours in response to the novel tank: i) time spent in the bottom of the tank, ii) total distance traveled, and iii) number of transitions to the top–bottom area of the tank.

Statistical analysis

For qPCR, relative mRNA expressions were calculated using Pfaffl method[66]. Differences in gene expression were assessed using a one-way ANOVA followed by Tukey's post-hoc test. The p-values were generated and adjusted for multiple comparison using the Dunn-Sidak method[67]. For

behavioural analysis, all data were analysed with R programming language[63]. Scripts are available on GitHub (https://github.com/AdeleLeg). For models where distance moved, distance to zone, velocity, or top halves visits were the response variable, we fitted data to mixed linear models using the "*lme4*" package, and where proportion of responders or proportion of time spent in the bottom third were our response variable, we fitted data to beta regression models using the "*betareg*" package. In all instances, we used genotype (stimulus number for the response and habituation to acoustic startle assay) as fixed effects, and fish ID and day of experiment as random effects. As in García-González et al.[62], we reported significant fixed effects as Type II Wald χ^2 from models using the package "*car*," post hoc Tukey's tests were also conducted as necessary with the package "*emmeans*". For the 5-CSRTT, overall correct responses (learning) and anticipatory responses (impulsivity) were assessed using the formulas in Supplementary Table 2. Power analysis was conducted using G*Power 3.1[68]. Sample size needed to achieve adequate statistical power for detecting a significant effect was determined based on data from previous research or pilot studies. Accepted α level and power were respectively ≤ 0.05 and ≥ 0.80.

Fertilisation and survival analysis

Fertilisation and survival rates were measured as described previously[17]. For fertilization rate we measured the percentage of fertilized eggs when pairing *rbfox1*$^{19del/19del}$ and compared it with fertilization rate of *rbfox1*$^{+/+}$ siblings. Data were collected upon 3 different mating trials, each trial comprising 3-5 trios (1 male and 2 females). Trials were performed on different weeks, using different trios for each test. Eggs were collected in different Petri dishes, properly labelled to distinguish between trios. Fertilisation rate was determined by averaging across Petri dishes from the same trio and calculated at 6-8 hpf. For survival analysis, we measured the percentage of survival animals (3 stocks x 50 larvae x genotype) from when larvae were added to the nursery (at 5dpf) until 2 months of age, when fish were transferred into adult aquarium.

CONTRIBUTIONS

AL conceived the project, designed and conducted the experiments, analysed the data, and wrote the manuscript. JGG designed and performed the CRISPR/Cas9 experiment generating the line. SH, PA, SA, XW and WH contributed to the experiments. NFC and BC edited the manuscript. CHB directed the study, designed the experiments, edited the manuscript, and secured funding. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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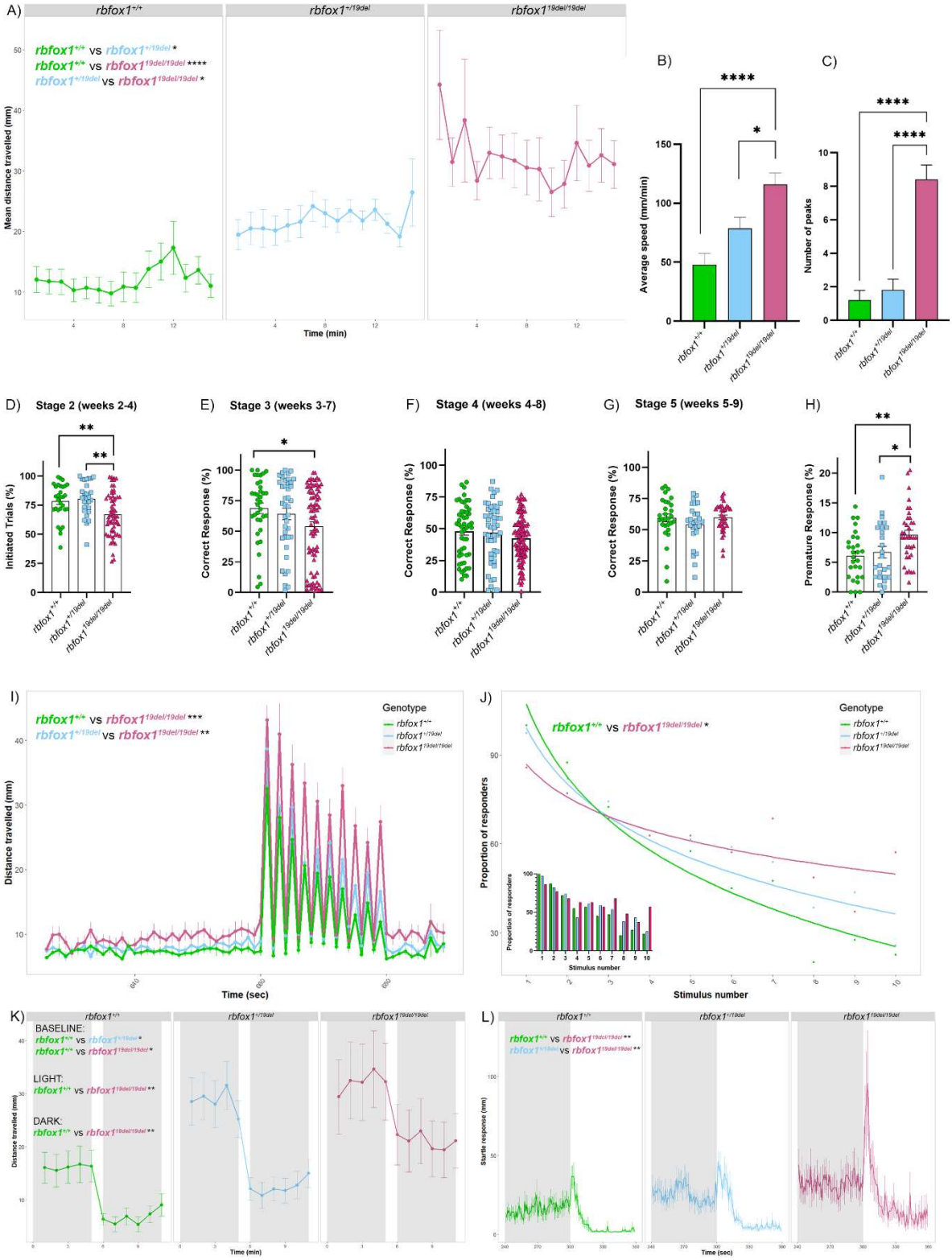


Figure 1. *rbfox1* mutant fish show hyperactivity, impulsivity and hyperarousal behaviour. A-C) Locomotion assay in 5 days post fertilization (dpf) zebrafish larvae (*rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$, *rbfox1*$^{19del/19del}$). **A)** distance travelled during 15 min assay; **B)** average speed; **C)** number of peaks (acceleration events when the fish travelled > 5 mm in < 12 s). N = *rbfox1*$^{+/+}$ 28 (all learned the task); *rbfox1*$^{+/19del}$ 28 (26 learned the task); *rbfox1*$^{19del/19del}$ 50 (37 learned the task). **D, H)** 5-Choice Serial Reaction Time Task in adult zebrafish (*rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$, *rbfox1*$^{19del/19del}$): **D)** initiated trial during

stage 2 (weeks 2-4); **E-G)** correct response/learning for stages 3-5 (weeks 3-9); **H)** premature response/impulsivity during stage 5 (weeks 5-9). Dots/square/triangles in D) and E) represent single adult fish. N = *rbfox1*$^{+/+}$ 19; *rbfox1*$^{+/19del}$ 17; *rbfox1*$^{19del/19del}$ 32. **I, J)** Response and habituation to acoustic startle assay in 5 dpf zebrafish larvae (*rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$, *rbfox1*$^{19del/19del}$): **I)** mean distance travelled during the assay; **J)** rate of habituation over time/stimuli. N = *rbfox1*$^{+/+}$ 40; *rbfox1*$^{+/19del}$ 39; *rbfox1*$^{19del/19del}$ 35. **K-L)** Forced light transition assay in 5dpf zebrafish larvae (*rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$, *rbfox1*$^{19del/19del}$): **H)** distance travelled during the 10 min assay; **I)** 1-second time bin resolution plots of the dark-light transition. N = *rbfox1*$^{+/+}$ 43; *rbfox1*$^{+/19del}$ 34; *rbfox1*$^{19del/19del}$ 37. All larvae employed were progeny of *rbfox1*$^{+/19del}$ in-cross and were genotyped prior to data analysis. Bars represent standard error of the mean (SEM). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

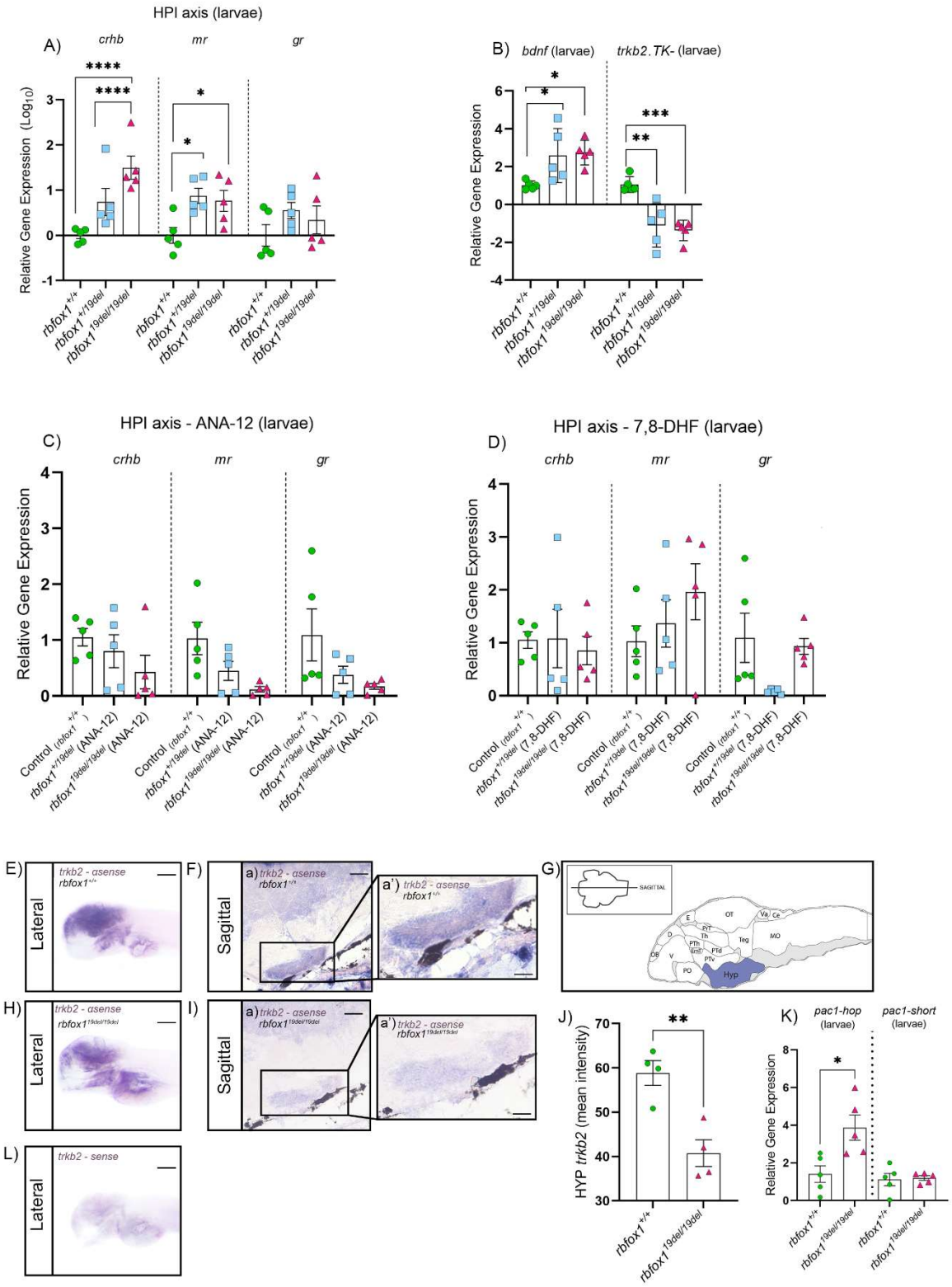


Figure 2. *rbfox1* LoF disrupts zebrafish larvae HPI axis via *bdnf/trkb2* pathway. **A)** Expression levels of HPI axis genes corticotropin releasing hormone b (*crhb*), mineral corticoid receptor (*mr*) and glucocorticoid receptor (*gr*) in 5 days post fertilisation (dpf) zebrafish larvae (*rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$, *rbfox1*$^{19del/19del}$). **B)** Expression levels of *bdnf* and *trkb2* in 5 dpf zebrafish larvae (*rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$, *rbfox1*$^{19del/19del}$). **C-D)** Expression levels of HPI axis genes *crhb*, *mr* and *gr* in 5 dpf zebrafish larvae (*rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$, *rbfox1*$^{19del/19del}$) in presence of the **C)** TrkB selective antagonist ANA-12 (N-(2-((2-oxoazepan-3-yl)carbamoyl)phenyl)benzo[b]thiophene-2-carboxamide) and **D)** TrkB selective

agonist 7,8-DHF (7,8-Dihydroxyflavone). Actin – β 2 (*actb2*) and ribosomal protein L13a (*rpl13a*) were used as reference genes. Bars represent standard error of the mean (SEM). All larvae employed were progeny of *rbfox1*$^{+/19del}$ in-cross and were genotyped prior to data analysis. Dots/square/triangles represent pool of 15 larval heads (eyes and jaw removed). Where indicated, we used Log_{10} transformation to normalize the data facilitating a clearer visualization of trends within the dataset. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. **E, H)** Whole mount *in situ* hybridisation (ISH) for *trkb2* (anti-sense (αsense) riboprobe in **E)** *rbfox1*$^{+/+}$ and **H)** *rbfox1*$^{19del/19del}$ 5 dpf larvae. **F, I)** Sagittal cryosection of *trkb2* ISH in **F)** *rbfox1*$^{+/+}$ and **I)** *rbfox1*$^{19del/19del}$ 5 dpf larvae. Black boxes in **F-a)** and **I-a)** represent the region of the brain showed in higher magnification panels in **F-a')** and **I-a')**. Scale bars: 200 µm in E) and H); 100 µm in F)a) and I)a); 50 µm in F)a) and I)a). **G)** Schematic depiction (sagittal) of zebrafish larval brain indicating position of levels illustrated by ISH on sagittal cryosections. **J)** *trkb2* ISH intensity mean in the hypothalamus of 5dpf zebrafish larvae, *rbfox1*$^{+/+}$ versus *rbfox1*$^{19del/19del}$. ** $p < 0.01$. Bars represent SEM. N = 5 larvae x genotype. **K)** Expression levels of *pac1-hop* and *pac1-short* in *rbfox1*$^{+/+}$ and *rbfox1*$^{19del/19del}$ 5 dpf larvae. Dots/triangles represent pool of 15 larval heads (eyes and jaw removed). Bars represent SEM. * $p < 0.05$. **L)** Whole mount ISH using *trkb2* sense riboprobe used as negative control. Scale bar: 200 µm.

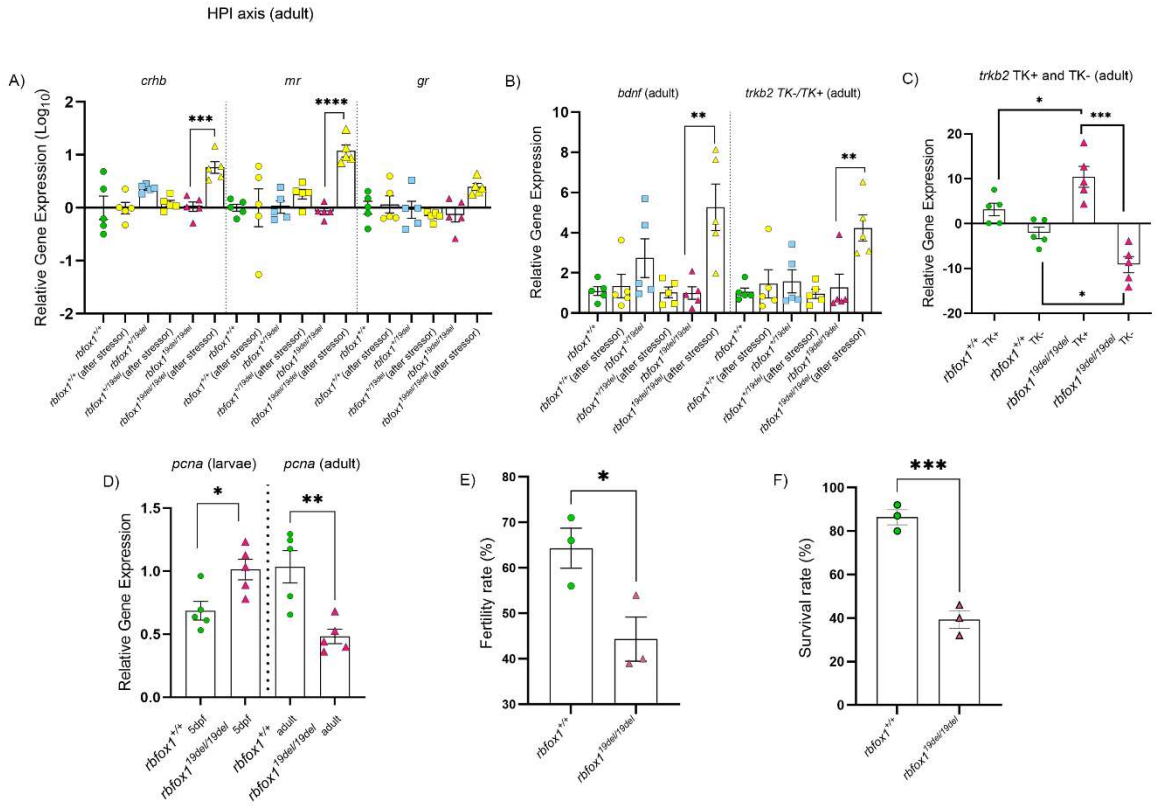


Figure 3. *rbfox1* mutants undergo adaptive mechanisms and allostatic overload during development. A) Expression levels of HPI axis genes corticotropin releasing hormone b (*crhb*), mineral corticoid receptor (*mr*) and glucocorticoid receptor (*gr*) in adult zebrafish brain (*rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$, *rbfox1*$^{19del/19del}$) in normal resting conditions and after exposure to a stressor (novel tank diving). **B)** Expression levels of *bdnf* and *trkb2 TK-/TK+* (common primers) in adult zebrafish brain (*rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$, *rbfox1*$^{19del/19del}$) showing increased *bdnf* levels and increased *trkb2 TK-/TK+* levels in *rbfox1*$^{19del/19del}$. **C)** Expression levels of the two *trkb2* isoforms (*TK+*, including the kinase domain, and

TK- without the kinase domain) in adult zebrafish brain (*rbfox1*$^{+/+}$ and *rbfox1*$^{19del/19del}$). **D)** Expression levels of proliferating cell nuclear antigen (*pcna*) in adult zebrafish brain (*rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$, *rbfox1*$^{19del/19del}$) in normal resting conditions. **E)** Fertility rate and **F)** survival rate of *rbfox1*$^{19del/19del}$ versus *rbfox1*$^{+/+}$. For A-D) Actin – β 2 (*actb2*) and ribosomal protein L13a (*rpl13a*) were used as reference genes and dots/square/triangles represent pool of 15 heads (eye and jaw removed) for the larvae and single brains for the adults. For E) each dot/triangle represents average fertility of 3-5 trios (1 male and 2 females) assessed over 2-3 petri dish (50 embryos per dish) per trio. For F) each dot/triangle represent represent percentage of survival of a single fish stock comprising 50 larvae. Bars represent standard error of the mean (SEM). Where indicated, we used Log_{10} transformation to normalize the data facilitating a clearer visualization of trends within the dataset. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

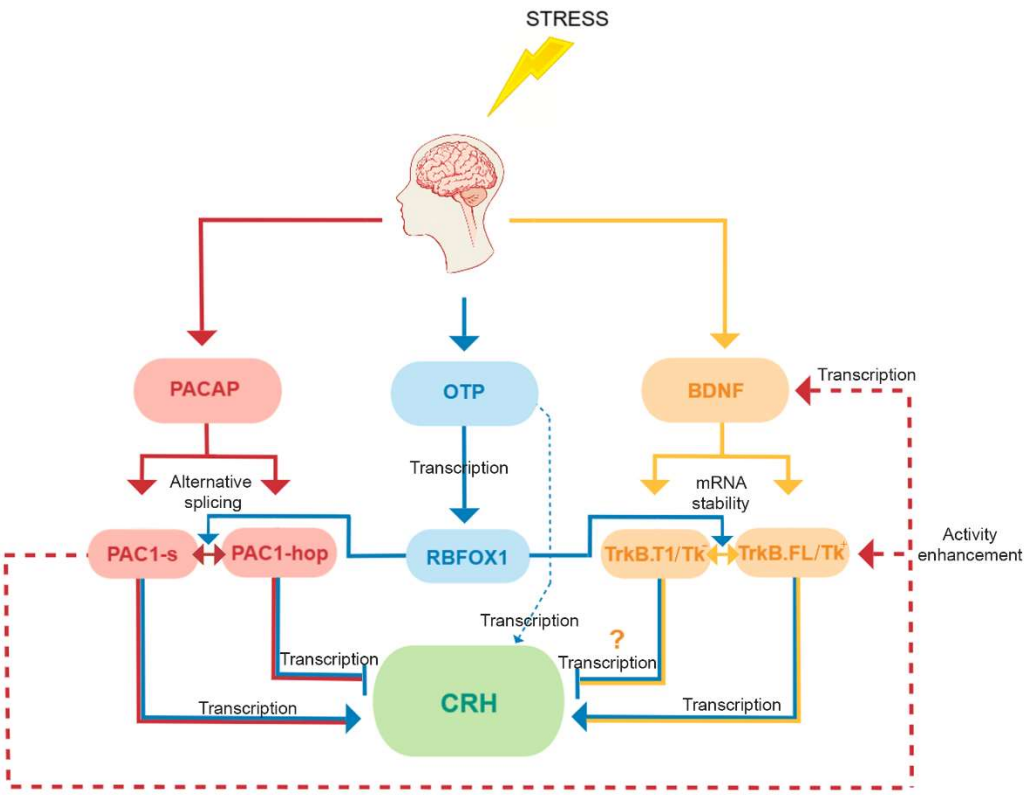
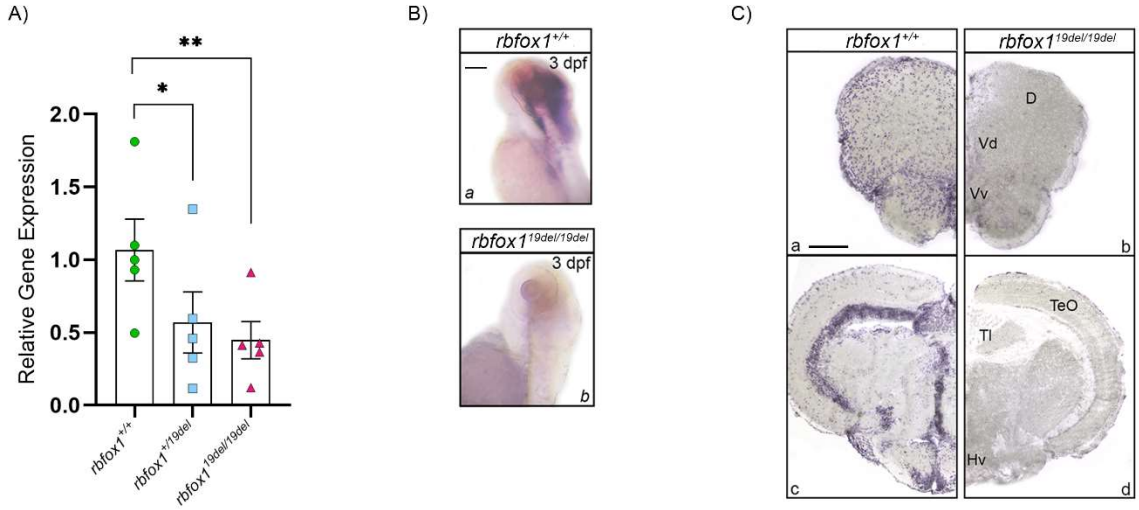
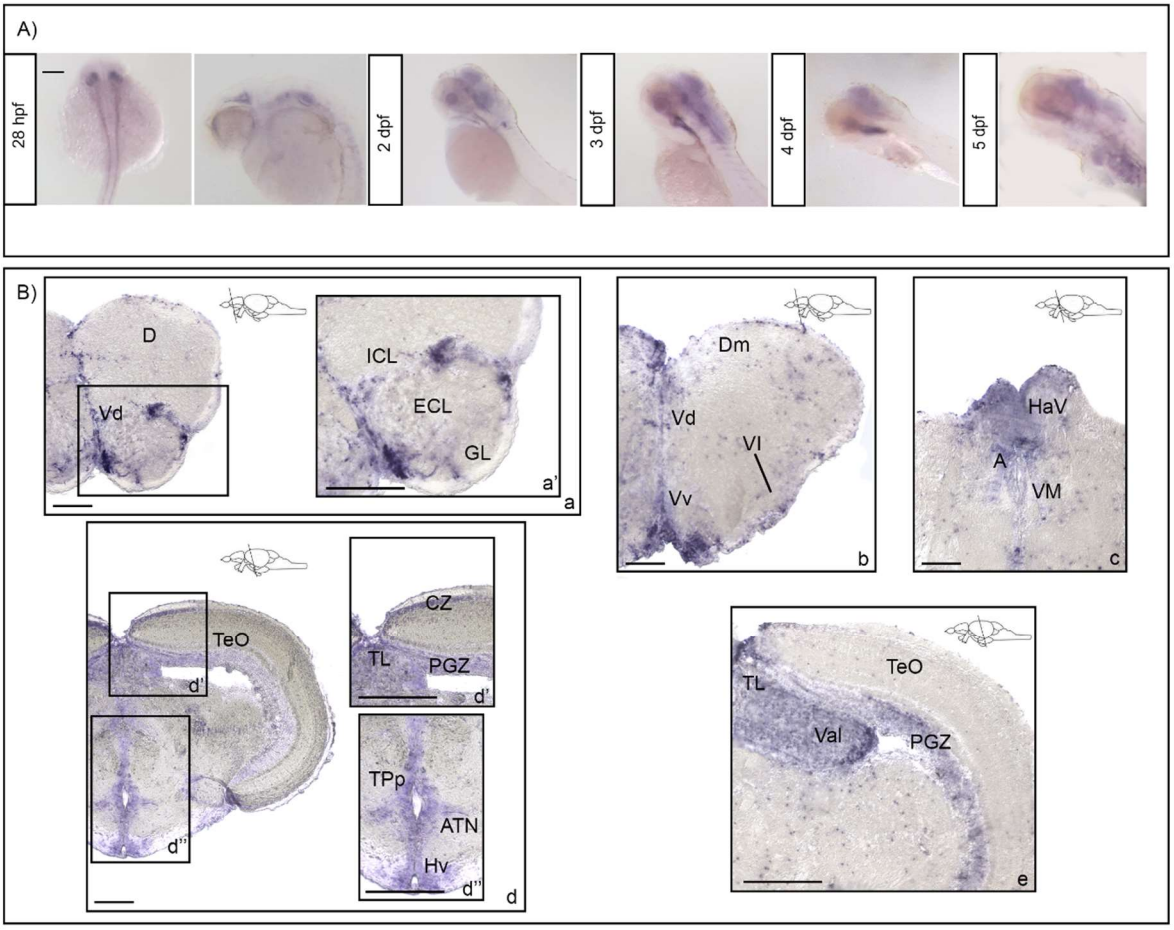


Figure 4. RBFOX1 role in stress resilience and chronic stress recovery: proposed mechanism of action. In response to stressful challenges, BDNF expression levels increase, stimulating *CRH* transcription via TrkB.FL (yellow pathway). At the same time, stress triggers also *PACAP* transcription (red pathway) and Otp-mediated transcription of *RBFOX1* and *CRH* (blue pathway). PACAP stimulates *CRH* transcription via PAC1-short and inhibits *CRH* transcription via PAC1-hop. PACAP binding to PAC1-short also increases *BDNF* transcription and enhances TrkB.FL activity. RBFOX1 functions as "switch" maintaining the balance between short/long isoforms of both PAC1 (regulating PAC1 alternative splicing) and TrkB receptors (promoting TrkB mRNA stability). Following stress, RBFOX1 promotes PAC1-short alternative splicing and *TrkB.FL* mRNA stability, inducing *CRH* transcription. Then, during the recovery phase, *RBFOX1* switches the balances in support of *PAC1-hop* and *TrkB.T1* isoforms, decreasing *CRH* levels and turning off the HPA axis. Our data suggest that upon chronic stress, other regulatory mechanisms step in to compensate for the absence of RBFOX1 in the alternative splicing of

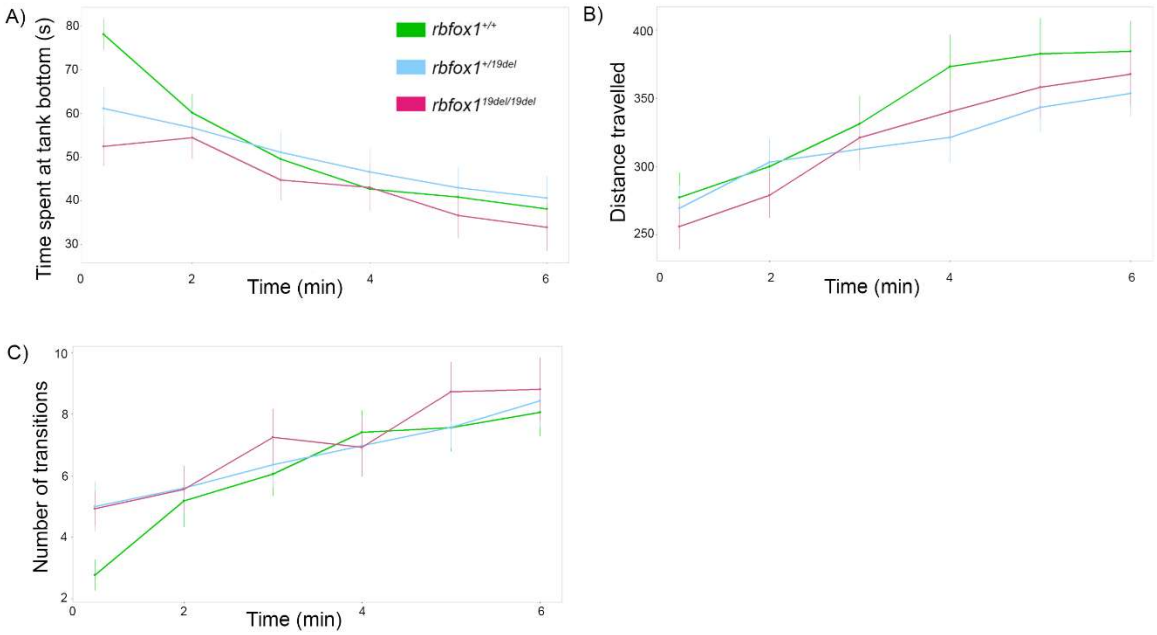
PAC-1, and that RBFOX1-mediated regulation of *TrkB.T1* mRNA stability is necessary to promote neuroplasticity and resilience.



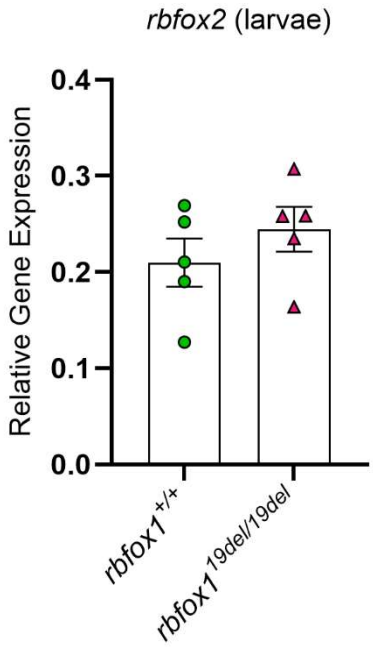
Supplementary Figure 1. Generation of a *rbfox1* loss of function line. A) mRNA non-sense mediated decay in *rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$ and *rbfox1*$^{19del/19del}$ 5 days post fertilisation (dpf) larvae. Actin – β 2 (*actb2*) and ribosomal protein L13a (*rpl13a*) were used as reference genes. Dots/square/triangles represent pool of 15 larval heads. Bars represent standard error of the mean (SEM). * $p < 0.05$, ** $p < 0.01$. *In situ* hybridisation on **B)** whole mount zebrafish larvae at 3 dpf, a) *rbfox1*$^{+/+}$ and b) *rbfox1*$^{19del/19del}$ and **C)** adult transverse sections of *rbfox1*$^{+/+}$ (a) fore- and (c) mid- brain and *rbfox1*$^{19del/19del}$ (b) fore- and (d) mid-brain. Scale bars: 200 µm in B); 100 µm in C). Abbreviations: D, dorsal area of dorsal telencephalon; Hv, ventral hypothalamus; TeO, optic tectum; Tl, longitudinal tori; Vd, ventral area of dorsal telencephalon; Vv, ventral area of ventral telencephalon.



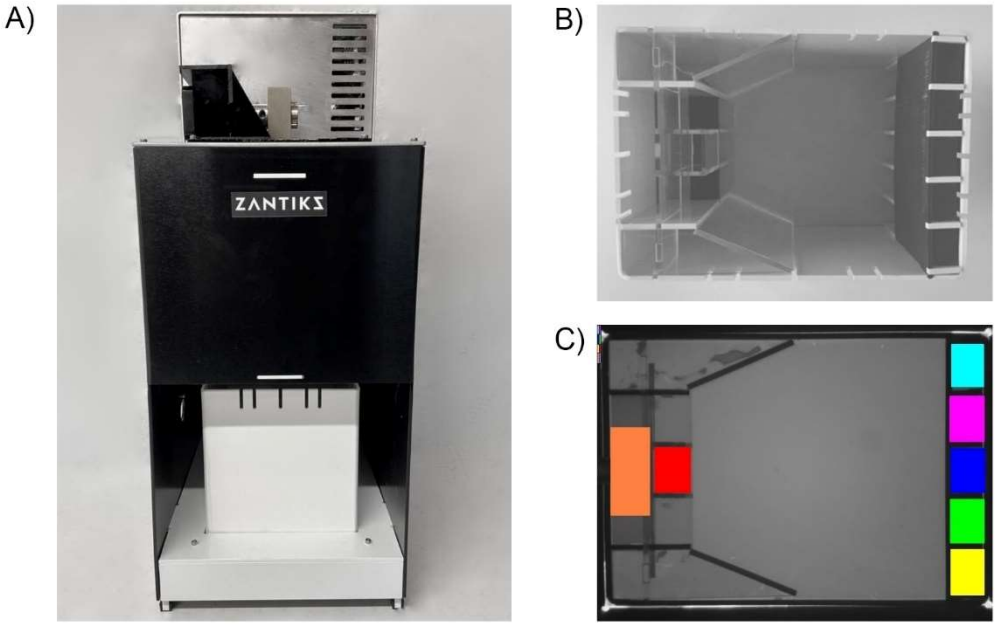
Supplementary Figure 2. *rbfox1* is expressed in regions of the brain associated with the stress response, social and emotional behaviour, reward and learning. A) *rbfox1* whole mount *in situ* hybridization (ISH) on zebrafish larvae at 28 hours post fertilization and 3-4-5 days post fertilization. **B)** *rbfox1* ISH on adult zebrafish (a - c) forebrain and (d-e) mid-/hind-brain transverse sections. Black boxes represent the region of the brain showed in higher magnification panels. Schematic depictions of the lateral view of the zebrafish brain indicate position of levels illustrated by ISH. Scale bars: 200 µm in A), 100 µm in B) a-d); 50 µm in B) a', c, d', d'', e.



Supplementary Figure 3. Novel tank diving assay in adult *rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$ and *rbfox1*$^{19del/19del}$ fish. A) Time spent at the bottom of the tank. **B)** Distance travelled during the assay and **C)** number of transitions between the top and bottom of the tank. N = *rbfox1*$^{+/+}$, 50; *rbfox1*$^{+/19del}$ 54; *rbfox1*$^{19del/19del}$ 54.



Supplementary Figure 4. *rbfox2* qPCR in 5 days old *rbfox1*$^{+/+}$ and *rbfox1*$^{19del/19del}$. Actin – β 2 (*actb2*) and ribosomal protein L13a (*rpl13a*) were used as reference genes. Dots/triangles represent pool of 10 larval heads. Bars represent standard error of the mean (SEM).



Supplementary Figure 5. Zantiks 5-Choice Serial Reaction Time Task A) unit and B-C) tank. **A)** Zantiks AD unit. **B)** Top view of the testing tank; 5 apertures are on the right and food-reward delivery zone on the left. **C)** Typical target zones: stimulus initiator zone (red), feeding area (orange), five apertures (turquoise, purple, blue, green, yellow). NOTE: colours do not represent the stimulus light color used in the task, the stimulus is represented by white light. Source: https://zantiks.com

Supplementary Table 1. List of primers used for mRNA non-sense mediated decay and other qPCR experiments.

Gene (purpose)	Gene acronym	Accession number	Sequence (5' to 3')	Product size (bp)
actin, β 2 (housekeeping)	*actb2*	NM_181601.5	Forward: CGAGCTGTCTTCCCATCCA	86
			Reverse: TCACCAACGTAGCTGTCTTTCTG	
ribosomal protein L13a (housekeeping)	*rpl13a*	NM_212784.1	Forward: TCTGGAGGACTGTAAGAGGTATGC	148
			Reverse: AGACGCACAATCTTGAGAGCAG	
RNA binding fox-1 homolog 1	*rbfox1*	NM_001005596.1	Forward: TGCTGCAGGGATTGGGACTA	91
			Reverse: TGCTGGGGCGTCTTGATTAC	
corticotropin releasing hormone	*crhb*	NM_001007379.1	Forward: CGAGACATCCCAGTATCCAAAAAG	60

			Reverse: TCCAACAGACGCTGCGTTAA	
mineralcorticoid receptor	*mr*	NM_001100403	Forward: CTTCCAGGTTTCCGCAGTCTAC	75
			Reverse: GGAGGAGAGACACATCCAGGAAT	
glucocorticoid receptor	*gr*	NM_001020711.3	Forward: ACTCCATGCACGACTTGGTG	90
			Reverse: GCATTTCGGGAAACTCCACG	
brain derived neurotrophic factor	*bdnf*	XM_009303234.3	Forward: AGCAGACTGTCTTTTGTTGTGTT	123
			Reverse: CTTAAGCTCACACAGGTGCC	
neurotrophic tyrosine kinase, receptor, type 2b (common primers)	*trkb2*	NM_001197161.2	Forward: CGCATACCTGACGTGGATCA	130
			Reverse: AAGCAACCCCAGCTATTCCC	
neurotrophic tyrosine kinase, receptor, type 2b (tyrosine kinase domain primers)	*trkb2.KD-*	NM_001197161.2	Forward: TGAAAGTGGGCGAGCAGATT	173
			Reverse: CATCTGGACCATGAGCCCTG	
adenylate cyclase activating polypeptide 1a (pituitary) receptor type I (adcyap1r1a) transcript variants 1 and 2	*pac1a-hop*	NM_001142926.1 NM_001013444.2	Forward: CAGAAATGCTTCAGTGAGCCC	82
			Reverse: CCAGACGTAGCGTGATGGTA	
adenylate cyclase activating polypeptide 1a (pituitary) receptor type I (adcyap1r1a), transcript variant 3	*pac1a-short*	NM_001142925.1	Forward: GATTCTGGCTGCTGGGACAT	183
			Reverse: GCGCGCCAGACGTAGATAAA	
RNA binding fox-1 homolog 2	*rbfox2*	NM_214746.2	Forward: CCGTATGCTAACGGGGACAG	106
			Reverse: ACCCTGGAACTGCGTAGAAC	

Supplementary Table 2. 5-Choice Serial Reaction Time Task stages (5-CSRTT) and corresponding descriptions. Source: https://zantiks.com

Stage	Name of the stage	Description
Stage 1	Habituation	All lights are illuminated (initiator light, five choice lights and food light) for 20 sec and the food. Entry into any illuminated area is reinforced with a small food reward. Stage1 is run for 30 trials per day with an inter-trial interval (ITI) of 10 sec with no light on. All fish are moved to the next stage of training.
Stage 2	Initiator training	Initiation light is on for 20 sec. If the fish swims to the illuminated initiation area, an initiated trial is recorded and the feeder releases food with the food light on for 20 sec. This is followed by 10 sec ITI. Fish move to the next stage when the average of initiated trials to total trials over five days is > 50%.
Stage 3	Stimulus light training	The initiation light is on for 20 sec. If the fish swims to the illuminated initiation area, an initiated trial is recorded and all five choice lights are illuminated for 20 sec. If the fish swims to any illuminated choice area, a correct response is recorded and the feeder releases food with the food light on for 20 sec. This is followed by 10 sec ITI. Fish move to the next stage when the average of initiated trials to total trials and correct responses to initiated trials are > 50%.
Stage 4	5-CSRTT/no-delay	The initiation light is on for 20 sec. If the fish swims to the initiation area, an initiated trial is recorded and one of five choice lights is illuminated for 20 sec. If the fish swims to the illuminated choice area, a correct response is recorded and the feeder releases food with the food light on for 20 sec. This is followed by 10 sec ITI. Fish move to the next stage when the average of initiated trials to total trials and correct responses to initiated trials are > 50%.
Stage 5	5-CSRTT/delay	The initiation light is on for 20 sec. If the fish swims to the initiation area, an initiated trial is recorded and after a pre-stimulus interval of 0-8 sec, one of five choice lights is illuminated for 20 sec. If during the pre-stimulus interval, the fish swims to any unilluminated choice area, a premature response is recorded. This is followed by 10 sec ITI. The average of premature responses to initiated trials over four days is reported as a measure of impulsive action.