rbfox1 loss in zebrafish drives HPI axis hyperactivation via *bdnf/trkb2* pathway and allostatic overload during later life

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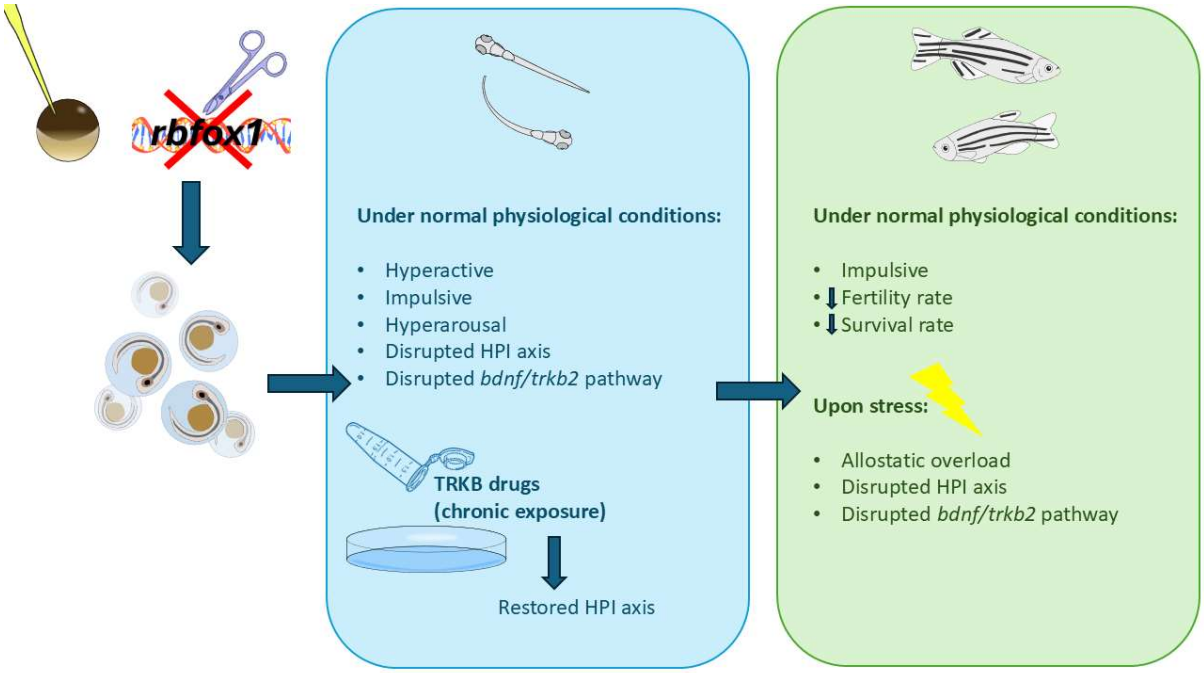

ABSTRACT

Mutations in the *RBFOX1* gene are associated with psychiatric disorders but how RBFOX1 influences psychiatric disorder vulnerability remains unclear. Recent studies showed that RBFOX proteins mediate 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. We found that *rbfox1* LoF mutants exhibited hyperactivity, impulsivity and heightened arousal, alongside alterations in proliferation – traits associated with neurodevelopmental and stress-related disorders. In adults, loss of *rbfox1* function led to decreased fertility and survival, consistent with allostatic overload. At the molecular level, at larval stages *rbfox1* LoF disrupted the expression of *bdnf*, *trkb2, pac1a-hop* and HPI axis genes. Pharmacological intervention targeting TRKB restored HPI axis and *bdnf/trkb2* gene expression. In adults, dysregulation of *bdnf*/*trkb2* and HPI axis genes was only seen following acute stress exposure. Our findings revealed a fundamental role for RBFOX1 in integrating stress responses through its regulation of BDNF/TRKB and neuroendocrine signalling. By uncovering how RBFOX1 modulates stress adaptation from development to adulthood, our study provides novel insights into the molecular basis of stress resilience and psychiatric disease vulnerability, suggesting *RBFOX1* loss predisposes to psychiatric diseases through HPA axis hyperactivation during development, impairing adaptation and heightening vulnerability to allostatic overload in later life.

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), also known as FOX1 or ataxin 2-binding protein 1 (A2BP1), 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 suggested that Rbfox1 regulates the Pituitary Adenylate Cyclase-Activating Polypeptide Type 1 Receptor (Pac1) alternative splicing, an important mediator of corticotropin releasing hormone (CRH) synthesis in the hypothalamus[4,7]. CRH is the first hormone to be secreted in response to stress by the hypothalamic-pituitary-adrenal (HPA) axis (hypothalamic-pituitary-interrenal (HPI) axis in fish)[8]. In mammals there are several PAC1 isoforms with the predominant isoforms in the brain being PAC1-hop (long isoform) and PAC1-short[9]. In mice and

zebrafish, following acute stress exposure, both *Pac1-hop/pac1-hop* and *Pac1-short/pac1-short* expression increased, while at the late recovery phase, only *Pac1-hop* was still up-regulated[4].

Another regulator of the stress response and a resilience factor against chronic stress-induced psychopathology is the brain derived neurotrophic factor (BDNF)/Tropomyosin receptor kinase B (TRKB) pathway[10–12]. 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[13,14]. In human neural stem cells, *RBFOX1* knockdown increased *BDNF* expression levels[15], while another study in mice identified *TrkB* as a target of RBFOX1 within the hippocampus[16]. Further, PAC1 activation can elevate *BDNF* levels and potentiate TRKB activity, enhancing BDNF/TRKB neuroprotective and plasticity-promoting effects, especially in the context of stress response and neuropsychiatric health[17].

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 refers to the collective processes by which the body actively maintains stability (or homeostasis) in response to environmental stressors[12]. When allostasis primary mediators (e.g., HPA axis hormones, catecholamines, cytokines) are overactivated or fail to return to normal, it leads to an allostatic state[18,19]. The cumulative results of an allostatic state are referred to as allostatic load and, when maladaptive, allostatic overload[18]. 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[19–22], while neurotrophic factors such as BDNF play a key role in regulating adaptive plasticity and mechanisms counteracting damage caused by allostatic overload[12]. Here, to explore the possibility that *RBFOX1* loss of function (LoF) leads to increased vulnerability to psychiatric disease through HPA axis hyperactivation 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 at different developmental stages. We hypothesized that RBFOX1 regulates HPA axis activity through an effect on BDNF/TRKB signalling leading 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, nucleotides 120-138, TCCCATCGGCCCAGTTCGC) that introduced a premature termination codon (PTC) at position 58 in the *rbfox1* amino acid sequence. Details regarding the generation of the line, nucleotide and amino acid 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). As *RBFOX1* itself is alternatively spliced to generate nuclear and cytoplasmic isoforms[7], we designed primers targeting all *rbfox1* zebrafish isoforms available on NCBI (see Supplementary Table1). 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).

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

112

113

114 ***rbfox1* mutant fish show hyperactivity, impulsivity and hyperarousal behaviour**

115 As mutations in *RBFOX1* locus have been linked to several psychiatric diseases in humans, we assessed
116 zebrafish larvae and adult fish for phenotypic traits associated with such disorders and examined
117 *rbfox1* mRNA distribution in *rbfox1*$^{+/+}$ larvae and adults (Tübingen strain).
118
119 In humans, *RBFOX1* copy number variants (CNVs) and LoF mutations are risk factors for ADHD[6]. Two
120 major ADHD traits are hyperactivity and increased impulsivity. We therefore assessed hyperactive and
121 impulsive behaviour of *rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$ and *rbfox1*$^{19del/19del}$ 5 days post fertilization (dpf) larvae
122 and adults. When we measured larval locomotion, we observed a gene dosage effect on distance
123 travelled, whereby *rbfox1* mutant larvae travelled greater distances than wild type siblings ($p_{rbfox1^{+/+} \text{ vs } rbfox1^{+/19del}} < 0.05$, $p_{rbfox1^{+/+} \text{ vs } rbfox1^{19del/19del}} < 0.0001$, $p_{rbfox1^{+/19del} \text{ vs } rbfox1^{19del/19del}} < 0.05$) (Figure 1A), and a
125 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). This is in line with hyperactivity observed in adult
127 *rbfox1*$^{19del/19del}$ in our previous study[2]. We also measured larval burst swimming, a parameter
128 previously used as a measure to predict impulsive behaviour in zebrafish larvae[23]. We found a
129 significant increase in the number of peaks (acceleration events when the fish travelled > 5 mm in <
130 12 sec) in *rbfox1*$^{19del/19del}$ larvae ($p_{rbfox1^{+/+} \text{ vs } rbfox1^{19del/19del}} < 0.0001$, $p_{rbfox1^{+/19del} \text{ vs } rbfox1^{19del/19del}} < 0.0001$)
131 (Figure 1C). Impulsive behaviour was then assessed in adult (7 months old) fish using the 5-choice
132 serial reaction time task (5-CSRTT)[24]. The 5-CSRTT consists of five stages (Supplementary Table2) each
133 run for at least a week until fish are promoted to the next stage. We found that 79% *rbfox1*$^{+/+}$, 72%
134 *rbfox1*$^{+/19del}$ and 62% *rbfox1*$^{19del/19del}$ learned the task within 9 weeks. We found no significant
135 differences in the correct responses (stages 2-5, Figure 1D-G). In stage 5, we found a significant
136 difference in the number of premature responses (increased impulsivity) such that *rbfox1*$^{19del/19del}$ fish
137 were more impulsive compared to *rbfox1*$^{+/+}$ siblings ($p < 0.05$) (Figure 1H).

138 *RBFOX1* CNVs have also been identified in individuals with schizophrenia[6,15]. Deficits in habituation to
139 acoustic startle reflex are seen in both schizophrenic patients and animal models of schizophrenia[25].
140 The acoustic startle assay has been widely employed to measure habituation in animals, including
141 zebrafish[26]. The magnitude of the startle response and the extent of habituation can also serve as
142 indicators of hyperarousal linked to heightened stress responses[27]. We therefore tested 5dpf zebrafish
143 larvae (*rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$ and *rbfox1*$^{19del/19del}$) in the habituation to acoustic startle response assay.
144 Consistent with our hyperactivity assay, during the baseline (first 10 min), the genotype had a
145 significant main effect on distance travelled [Effect of genotype: $\chi2(2) = 8.3397$, $p < 0.05$] whereby
146 *rbfox1*$^{19del/19del}$ larvae travelled greater distances than *rbfox1*$^{+/+}$ siblings ($p < 0.05$). During the startle
147 stimuli, we observed a main effect of genotype [Effect of genotype: $\chi2(2) =33.536$, $p < 0.0001$] and
148 stimulus number [Effect of stimulus number: $\chi2(9) = 487.968$, $p < 0.0001$] on distance travelled, and
149 a significant two-way interaction between genotype and stimulus number [Effect of genotype by
150 stimuli: $\chi2(18) = 31.511$, $p < 0.05$], whereby *rbfox1*$^{19del/19del}$ larvae travelled greater distances than
151 *rbfox1*$^{+/+}$ ($p < 0.001$) and *rbfox1*$^{+/19del}$ ($p < 0.01$) (Figure 1I). When assessed for the rate of habituation,
152 larvae showed a habituation response to repeated acoustic startle consistent with previous reports[28]:
153 100% of *rbfox1*$^{+/+}$animals responded to the first acoustic stimulus, but only 22% responded to the last.
154 When we examined the rate of habituation over time, we observed a significant genotype effect
155 [Effect of genotype: $\chi2(2) =7.2676$, $p < 0.05$] and a significant two-way interaction between genotype
156 and stimulus number [Effect of genotype by stimulus number: $\chi2(18) =132.8476$, $p < 0.001$] whereby

157 *rbfox1*$^{19del/19del}$ showed reduced rate of habituation and a greater proportion of responders compared
158 to *rbfox1*$^{+/+}$ siblings ($p_{rbfox1^{+/+} \text{ vs } rbfox1^{19del/19del}} < 0.05$) (Figure 1J).

159 SNPs in *RBFOX1* locus have also been associated with anxiety[6,29]. Hence, we assessed anxiety-like
160 behavior in 5dpf zebrafish larvae using the forced light-dark transition (FLDT) assay and in adult
161 zebrafish using the novel tank diving (NTD) assay. In the FLDT assay, zebrafish are exposed to sudden
162 transitions in illumination with effects on locomotion and amplitude of response on transition from
163 dark to light being used as a measure of anxiety-like behaviour: the increased locomotion/startle
164 response upon the light-dark transition is attributed to increased stress/anxiety level[30,31]. Consistent
165 with our acoustic startle assay, we found that during the baseline (first 5 min) (Figure 1K) genotype
166 had a significant main effect on distance travelled [Effect of genotype: $\chi2(2) = 8.8262$, $p < 0.05$]
167 whereby *rbfox1*^{19del} larvae travelled greater distances than *rbfox1*$^{+/+}$ siblings ($p_{rbfox1^{+/+} \text{ vs } rbfox1^{+/19del}} <$
168 0.05, $p_{rbfox1^{+/+} \text{ vs } rbfox1^{19del/19del}} < 0.05$). During the 1min light flash (min 5-6 of the assay) we found a
169 significant main effect of genotype on distance travelled [Effect of genotype: $\chi2(2) = 12.8139$, $p <$
170 0.01] with *rbfox1*$^{19del/19del}$ larvae travelling greater distances than *rbfox1*$^{+/+}$ siblings ($p < 0.01$) (Figure
171 1K). On transition from dark to light (from sec 240 to sec 360) we found a significant main effect of
172 time [Effect of time: $\chi2(118) = 705.091$, $p < 0.001$] and genotype [Effect of genotype: $\chi2(2) = 10.289$,
173 $p < 0.01$], and a significant two-way interaction between time and genotype [Effect of time by
174 genotype: $\chi2(236) = 188.008$, $p < 0.001$] on the amplitude of response, whereby *rbfox1*$^{19del/19del}$ larvae
175 startled more than *rbfox1*$^{+/+}$ ($p < 0.01$) and *rbfox1*$^{+/19del}$ ($p < 0.01$) siblings (Figure 1L). As there was a
176 significant difference in basal locomotion, we also examined the amplitude of response on transition
177 from dark to light normalizing the data against baseline, as reported previously[32]. We observed similar
178 results as in absence of normalization: significant main effect of time [Effect of time: $\chi2(118) =$
179 1705.943, $p < 0.001$] and genotype [Effect of genotype: $\chi2(2) = 12.756$, $p < 0.01$], and a significant
180 two-way interaction between time and genotype [Effect of time by genotype: $\chi2(236) = 336.523$, $p <$
181 0.001] whereby *rbfox1*$^{19del/19del}$ larvae startled more than *rbfox1*$^{+/+}$ siblings ($p_{rbfox1^{+/+} \text{ vs } rbfox1^{19del/19del}} <$
182 0.01).
183
184 Anxiety-like behaviour in adult animals was assessed using the NTD assay. When introduced to a novel
185 tank, zebrafish will first dive to the bottom of the tank, to seek protection, and then gradually increase
186 their swimming over time[33]. We found no significant differences between *rbfox1* genotypes over the
187 entire duration of the NTD assay ($p > 0.05$). However, during the first minute of the assay, we found a
188 significant two-way interaction between genotype and the time spent at the bottom of the tank [Effect
189 of genotype by proportion at the bottom tank: $\chi2(10) = 22.3333$, $p < 0.05$], such that *rbfox1*$^{19del/19del}$
190 fish spent less time in the bottom than *rbfox1*$^{+/+}$ fish ($p < 0.001$) (Supplementary Figure 3A). In line
191 with previous findings[2], we observed no significant differences in distance travelled ($p > 0.05$)
192 (Supplementary Figure 3B), nor in the number of the transitions to the top area of the tank between
193 *rbfox1* genotypes ($p > 0.05$) (Supplementary Figure 3C).
194
195 Thus, our data showed that loss of *rbfox1* resulted in behavioural changes that resemble human
196 psychiatric disorder traits and suggest that adaptation occurs as the animal develop. Consistent with
197 our behavioural results and as seen previously[2,3], when we assessed *rbfox1* mRNA distribution in
198 *rbfox1*$^{+/+}$ larvae and adults (Tübingen strain), we found that *rbfox1* was expressed in regions of the
199 brain involved in the response to stress, in social and emotional behaviour, and in reward and learning
200 (Supplementary Figure 2A-B) in agreement with data in rodents[34] and humans[35].

201
202 ***rbfox1* LoF disrupts zebrafish larvae HPI axis and *bdnf/trkb2* pathway**

203 As larvae showed altered stress response and RBFOX1 has been linked to regulation of HPA axis and
204 *BDNF/TRKB* gene expression[4,15,16], we examined the expression of components of the HPI axis and of
205 *bdnf/trkb2.* Since we did not observe significant differences in behaviour between wild type and
206 heterozygous animals, we employed wild type and homozygous animals only.

207 We performed qPCR experiments in 5dpf zebrafish larvae to assess changes in the expression levels
208 of the HPI axis markers corticotropin releasing hormone (*crhb*), mineralocorticoid receptor (*mr*), and
209 glucocorticoid receptor (*gr*), and of *bdnf* and *trkb2*. In teleosts, the duplication of the genome gave
210 rise to two *CRH* genes, *crha* and *crhb,* and two *TRKB* genes, *trkb1* and *trkb2*[36,37]*.* However, only *crhb*
211 and *trkb2* are regarded as the orthologues of the mammalian *CRH* and *TRKB* respectively[37,38]. Then,
212 unlike other teleosts, zebrafish possess a single copy of the *MR* (*mr*) and *GR* (*gr*) genes, and a single
213 copy of the *BDNF* gene (*bdnf*)[37,39]. In mammals, several *TRKB* splicing isoforms are present, but the
214 most abundant ones are the full-length (TRKB.FL/TK+) and the truncated (TRKB.T1/TK-), this latter
215 lacking the catalytic tyrosine kinase (TK) domain[40]. As in zebrafish the presence of both *trkb2* full-
216 length and truncated forms has been demonstrated[41], here we used a pair of *trkb2 TK+/TK-* common
217 primers, and another pair targeting only *TK+* to distinguish effects on the expression of the two
218 isoforms.

219 In *rbfox1*$^{19del/19del}$ larvae, in we observed a significant up-regulation of *bdnf* ($p < 0.05$) and *TK+* ($p <$
220 0.05), and a significant down-regulation of *TK-* ($p < 0.05$) expression levels (Figure 2A). Regarding HPI
221 axis, in *rbfox1*$^{19del/19del}$ larvae we found a significant up-regulation of *crhb* ($p < 0.0001$) and a significant
222 up-regulation of *mr* ($p < 0.01$) (Figure 2C, D). We observed no significant changes in *gr* expression
223 levels ($p > 0.05$) (Figure 2C, D).

224 To assess *rbfox1* LoF effects on zebrafish larvae during early developmental stages, we performed
225 *crhb*, *bdnf* and *trkb2* qPCR experiments also in 3dpf larvae. Similarly to 5dpf larvae, in 3dpf
226 *rbfox1*$^{19del/19del}$ larvae we observed a significant up-regulation of *crhb* ($p < 0.01$), *bdnf* ($p < 0.05$) and
227 *TK+* ($p < 0.05$), and a significant down-regulation of *TK-* ($p < 0.05$) (Figure 2B).

228 These findings showed that *rbfox1* LoF led to alterations in stress-related gene expression and
229 hyperactivation of the HPI axis, in line with our behavioural data and implicating *RBFOX1* as a critical
230 regulator of stress response mechanisms.

231 **Treatment with TRKB agonist/antagonist restores HPI axis gene expression in *rbfox1* LoF larvae**

232 To assess whether changes in HPI axis gene expression seen in *rbfox1* LoF larvae were mediated by
233 bdnf/trkb2 signalling, we examined the expression of HPI axis components (*crhb*, *mr*, *gr*) in 5dpf
234 zebrafish larvae following chronic exposure (from 5 h to 5dpf) to the TRKB agonist 7,8-DHF or
235 antagonist ANA-12.

236 In *rbfox1*$^{+/+}$ larvae, neither TRKB agonist 7,8-DHF or antagonist ANA-12 influenced HPI axis gene
237 expression ($p > 0.05$) (Figure 2C, D). In *rbfox1*$^{19del/19del}$ larvae, chronic exposure to either TRKB agonist
238 or antagonist significantly reduced *crhb* ($p_{rbfox1^{19del/19del} \text{ vs } rbfox1^{19del/19del + ANA\text{-}12}} < 0.0001$, $p_{rbfox1^{19del/19del} \text{ vs}}$
239 $_{rbfox1^{19del/19del + 7,8\text{-}DHF}} < 0.0001$) and *mr* ($p_{rbfox1^{19del/19del} \text{ vs } rbfox1^{19del/19del + ANA\text{-}12}} < 0.0001$, $p_{rbfox1^{19del/19del} \text{ vs}}$
240 $_{rbfox1^{19del/19del + 7,8\text{-}DHF}} < 0.05$) expression levels relative to untreated mutant larvae, restoring HPI axis
241 gene expression to levels comparable with wild type controls ($p > 0.05$) (Figure 2C, D). Following
242 identification and removal of outliers (1 outlier in the *rbfox1*$^{+/+}$ + ANA-12 group, 1 outlier in the
243 *rbfox1*$^{+/+}$ + 7,8-DHF group and 1 outlier in the *rbfox1*$^{19del/19del}$+ 7,8-DHF group), we observed a significant
244 down-regulation of *mr* expression levels in *rbfox1*$^{19del/19del}$ + ANA-12 versus *rbfox1*$^{+/+}$ un-exposed
245 controls ($p < 0.01$) (Figure 2C) and a significant up-regulation of *mr* expression levels in *rbfox1*$^{19del/19del}$+
246 7,8-DHF versus both exposed ($p < 0.01$) and un-exposed ($p < 0.05$) controls (Figure 2D).

247 As the hypothalamus is the primary player in the stress response and Rbfox1 had only previously been
248 shown to affect TRKB expression within the hippocampus[16], we used ISH to determine whether *trkb2*
249 mRNA expression was reduced within the hypothalamic area. In agreement with previous findings[42],
250 in *rbfox1*$^{+/+}$ we found that *trkb2* is widely expressed in the brain of 5dpf larvae, whereas in
251 *rbfox1*$^{19del/19del}$ larvae, consistently with our qPCR experiments, we found a significant overall reduction
252 of *trkb2* mRNA in the whole brain (Figure 2E-H), including in the hypothalamus ($p < 0.01$) (Figure 2F, I,
253 K, Supplementary Figure 5).

254 Our findings showed that the dysregulation in HPI axis gene expression caused by *rbfox1* LoF was
255 prevented by TRKB agonists and antagonists suggesting the increased CRH expression is mediated by
256 an action of *RBFOX1* LoF on BDNF signalling.

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

258 As RBFOX proteins have been shown to be able to regulate *Pac1* alternative splicing to include the *hop*
259 cassette[7] and PAC1 has been shown to regulate *Bdnf* transcription and potentiate TRKB activity[17], we
260 examined expression levels of *pac1a,* the zebrafish homologue of the mammalian *PAC1*, in *rbfox1*$^{+/+}$
261 and *rbfox1*$^{19del/19del}$ 5dpf larvae by qPCR.
262
263 In mammals there are several PAC1 isoforms and their role in the regulation of stress is poorly
264 understood[9]. In the brain, the predominant isoforms are *PAC1-hop* (long isoform) and *PAC1-short*[9].
265 Zebrafish possess two *pac1* genes, *pac1a* and *pac1b*, but only *pac1a* contains the *hop* cassette[9]. *PAC1-*
266 *short* enhances *CRH* transcription, while *PAC1-hop* reduces *CRH* synthesis during late stress recovery
267 phase[4]. We measured expression levels of both *pac1a-short* and *-hop* isoforms and we observed no
268 differences in *pac1a-short* expression, but we found a significant up-regulation of *pac1a-hop* in
269 *rbfox1*$^{19del/19del}$ mutant larvae ($p < 0.05$) (Figure 2L).

270 This finding strengthens previous data[7] suggesting that RBFOX1 is not the main regulator, or at least
271 not the sole regulator of, *PAC1* alternative splicing and suggests that changes in *crhb* expression seen
272 here are the result of the altered *bdnf/trkb2* expression.

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

274 The HPA axis possesses a vital role in the maintenance of allostasis, the process by which the body
275 achieves stability in response to stress or environmental challenges. Dysregulation of the HPA axis
276 often leads to disrupted allostasis during later life, also termed as allostatic load (i.e., the physiological
277 consequence resulting from the cumulative "wear and tear" of the body in response to chronic
278 stress)[22]. As we observed dysregulation of the HPI axis gene expression in *rbfox1* mutant larvae and
279 altered behavioural responses in *rbfox1* mutant larvae but not in adults, we assessed expression of
280 HPI axis components in adult zebrafish, in the presence and absence of acute stress (NTD), to explore
281 the possibility of adaptation that may contribute to differences in allostatic load.

282 We first measured *crhb*, *mr*, *gr*, *bdnf* and *trkb2* mRNA expression levels. In physiological resting
283 conditions, we observed no differences in HPI nor *bdnf/trkb2* expression levels ($p > 0.05$) (Figure 3A,
284 B). However, after stress exposure, regarding the HPI axis, in *rbfox1*$^{19del/19del}$ adults we observed the
285 same dysregulation seen in *rbfox1* LoF larvae: we found significant up-regulation of *crhb* ($p < 0.001$)
286 and *mr* ($p < 0.0001$) and no changes in *gr* expression levels ($p > 0.05$) (Figure 3A), and significant up-
287 regulation of *bdnf* ($p < 0.01$) and *TK+* ($p < 0.05$), and significant down-regulation of *TK-* ($p < 0.05$) (Figure
288 3B, C).

289 Excess glucocorticoid (GC) exposure during early developmental stages or early life stress (ELS) can
290 lead to vulnerability to allostatic overload in the long-term[19,22]. One of the effects caused by GC- or
291 ELS-induced allostatic overload is reduced cell proliferation during adulthood[19]. This effect has been

suggested to be developmentally dynamic, since it is often preceded by increased cell proliferation during early stages[19]. Further, BDNF signalling has been shown to regulate neural stem cell proliferation through TK-, suggesting that *rbfox1* LoF fish showing altered levels of *TK-* expression, may show altered proliferation across the life course[43]. 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 up-regulation of *pcna* expression levels in 5dpf *rbfox1*$^{19del/19del}$ zebrafish larvae ($p < 0.05$) and a significant down-regulation of *pcna* expression levels in the brains of adult *rbfox1*$^{19del/19del}$ fish ($p < 0.01$) when compared to *rbfox1*$^{+/+}$ siblings (Figure 3D).

In adult zebrafish, other effects caused by GC-induced allostatic overload include reduced fertility and survival rates[19]. Therefore, we assessed fertility and survival of *rbfox1*$^{19del/19del}$ zebrafish and *rbfox1*$^{+/+}$ siblings. We found that both fertility ($p < 0.05$) and survival ($p < 0.001$) rates 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[44]. HPA axis overactivity is a key factor in the onset of the allostatic load[22]. RBFOX1 has been shown to influence expression of stress related genes such as PAC1 and BDNF/TRKB. Therefore, alterations in RBFOX1 function may increase psychiatric disorders vulnerability through alterations in stress response systems, such as the HPA axis, pre-disposing to allostatic overload vulnerability in later life (Figure 4).

Similarly to *Rbfox1* deficient mice[6], we found that *rbfox1*$^{19del/19del}$ mutants were hyperactive and impulsive. These findings are consistent with studies implicating common and rare *RBFOX1* genetic variants as risk factors for psychiatric disorders like ASD, ADHD, schizophrenia and anxiety/stress disorders[5,6,45]. At larval stages *rbfox1*$^{19del/19del}$ were hyperactive and had increased burst swimming, indicative of increased impulsivity. 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 makes interpretation difficult, *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[46]. Interestingly, *rbfox1* $^{19del/19del}$ adults showed no significant difference in the novel tank diving assay, an adult measure of stress-reactive behaviour, suggesting behavioural adaptation. In the 5-CSRTT we found that *rbfox1*$^{19del/19del}$ 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 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[47,48]. 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 molecular response to stress. Elevated *crhb* levels as seen in *rbfox1*$^{19del/19del}$ larvae suggest a chronic anxiety state. Increased CRH and MR levels are linked

340 to mood disorders like depression and PSTD[49–52]. These findings support a role for *RBFOX1* in *CRH*
341 regulation and suggest that *RBFOX1* LoF may contribute to mood disorders via HPA axis
342 hyperactivation and disrupted stress resilience.
343
344 BDNF and TRKB are also key regulators of the stress response[53]. BDNF levels often increase during
345 stress to promote neuronal survival and plasticity, buffering negative effects of stress on the brain via
346 TRKB[10,11,13,53–55]. Dysfunction of BDNF/TRKB signalling is linked to several stress-related disorders[13].
347 The increased *bdnf* expression we observed in *rbfox1*$^{19del/19del}$ larvae suggests a compensatory
348 response to cope with *crhb* increase and maintain homeostasis. At 3 and 5dpf, in *rbfox1*$^{+/+}$ larvae we
349 detected both *trkb2* truncated (*TK-*) and full-length (*TK+*) forms, and in *rbfox1*$^{19del/19del}$ larvae we
350 observed a significant down-regulation of *TK-* and a significant up-regulation of *TK+*, which is predicted
351 to lead to an increased activation of TRKB signalling, consistent with the increased *crhb* expression as
352 seen here[56]. Studies in rodents showed that *Rbfox1* up-regulation led to increased TrkB.T1 (TK-)
353 expression in the hippocampus and no changes were observed following *Rbfox1* deletion[16]. In
354 contrast, we observed down-regulation of *trkb2 TK-* expression in *rbfox1*$^{19del/19del}$ larvae in resting
355 physiological conditions. The difference between these studies may be due to i) differences in the
356 tissue examined (zebrafish larvae whole head versus rodent hippocampus), ii) the developmental
357 stage of the animals (larval versus adult stage), iii) the genotype of the animals studied (*rbfox1*$^{19del/19del}$
358 in our study versus *Rbfox1*$^{+/-}$ in the study in rodents), or iv) presence of compensatory mechanisms (in
359 the hippocampal mouse model *Rbfox1* knockdown resulted in up-regulation of *Rbfox2*). As for this
360 latter hypothesis, as in previous studies in zebrafish[2], we did not see any changes in *rbfox2* expression
361 upon *rbfox1* LoF (Supplementary Figure4) suggesting that this compensatory mechanism does not
362 occur in larval fish.
363
364 Consistent with our hypothesis that *RBFOX1* LoF leads to hyperactivation of the HPA axis through the
365 BDNF/TRKB pathway, chronic exposure to TRKB agonist (7,8-DHF) or antagonist (ANA-12) restored HPI
366 axis gene expression to that seen in *rbfox1*$^{+/+}$ larvae. ANA-12 is a selective non-competitive antagonist
367 of TRKB exerting central TRKB blockade and producing rapid and long-lasting anxiolytic and
368 antidepressant effects[57]. Mice treated with ANA-12 showed reduced anxiety-like behaviour[57] and in
369 stressed rats, ANA-12 blocked *Crh* increase in the hypothalamus and amygdala[58]. 7,8-DHF is a potent
370 selective agonist of TRKB used in the treatment of several disorders, including depression and
371 schizophrenia, and has been shown to enhance memory consolidation and emotional learning in
372 healthy rodents[59]. In a similar fashion to the effects of ANA-12 seen in rats (blocking *Crh* increase)[57],
373 after chronic treatment with ANA-12, we observed that the expression of HPI axis genes in
374 *rbfox1*$^{19del/19del}$ larvae was restored to *rbfox1*$^{+/+}$ levels. Contrary to expectation, a similar result was
375 seen in the presence of chronic agonist exposure. However, chronic exposure to agonists may lead to
376 desensitization and inhibition of TRKB signalling[60]. Interestingly, *rbfox1*$^{19del/19del}$ larvae were
377 differentially sensitive to the effect of TRKB drugs on *mr* expression, most likely due to the unbalanced
378 *TK+/TK-* levels. These findings warrant further investigations. It is of note that neither TRKB agonist
379 nor antagonist had a significant effect on gene expression in *rbfox1*$^{+/+}$ larvae. One possible explanation
380 is that, as the larvae were not exposed to any stressors, any effect on Trkb2 signalling had limited
381 effect on HPI axis activity.
382
383 In *rbfox1*$^{19del/19del}$ larvae, we also observed up-regulation of *pac1a-hop* with no changes in *pac1a-short*
384 levels. Previous studies in zebrafish have shown that Pac1-short gain of function caused persistent *crh*
385 increase, while overexpression of Pac1-hop prevented stress-induced *crh* transcription activation[4].
386 These previous studies also suggest that RBFOX1 regulates the alternative splicing of *PAC1* promoting
387 the formation of *PAC1-hop*[4]. However, the increase in *pac1-hop* seen in our study argues against this
388 suggestion and suggests an adaptive response to the increased expression of *crh* either through
389 *rbfox2*[7] (whose expression was not altered by *rbfox1* LoF (Supplementary Figure4)) or through an
390 unknown mechanism.

In contrast to larvae, differences in HPI axis and *bdnf/trkb2* gene expression were not seen in adult animals under resting conditions. However, despite no difference in stress-reactive behaviour as determined by the novel tank diving assay, *rbfox1*$^{19del/19del}$ adults showed exaggerated HPI activation on challenge with a stressor, aligning with the previous suggestion that Rbfox1 is required for the termination of the acute endocrine 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)[19,38]. This latter was evidenced by reduced fertility and survival rates in *rbfox1*$^{19del/19del}$ 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[19]. Our findings that *rbfox1*$^{19del/19del}$ 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. Consistent with the *crhb* increase observed in stressed *rbfox1*$^{19del/19del}$ adults, and as seen in larvae, after acute stress, *rbfox1*$^{19del/19del}$ adults also showed unbalanced *trkb2* expression, with up-regulation of *TK+* and down-regulation of *TK-* .

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 suggests that RBFOX1 plays a crucial role in adaptive stress mechanisms. In response to stressful challenges, RBFOX1 functions as a "switch" regulating the balance between short/long isoforms of TRKB receptors: during the stress recovery phase, RBFOX1 switches the balance towards *TRKB.T1/TK-* isoform, 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 and heightened arousal), our findings also suggest that *RBFOX1* contributes to the liability of psychiatric disorders through HPA axis hyperactivation and BDNF/TRKB dysregulation, which leads to disrupted development and vulnerability to allostatic overload in later life. The suite of behavioural effects seen in *rbfox1* LoF animals coupled with exaggerated HPI axis response on exposure to a stressor are reminiscent of reported traits in passive versus active copers[62] suggesting that RBFOX1 activity may also play a key role in individual differences in coping styles. Although we show a key role for RBFOX1 in regulation of the HPA axis consistent with this being a primary mechanism by which *RBFOX1* variants pre-disposes to psychiatric disorders, RBFOX1 regulates the splicing of a wide range of additional genes[15] which may also play a part of disease vulnerability.

METHODS

Animal maintenance

All fish 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.

439

440 **Generation of *rbfox1* loss of function zebrafish line**

441 The zebrafish *rbfox1* loss of function (LoF) mutant line was generated as described previously using
442 Tübingen strain as background [63]. CRISPR RNA (crRNA) (Merck) was designed to target *rbfox1* exon 2
443 (**CCC**AGTTCGCTCCCCCTCAGAAC, PAM sequence in bold, MwoI recognition site underlined). A 3 µL
444 injection mix containing 1 µL (F_C 83 ng/µL) crRNA, 1 µL (F_C 83 ng/µL) tracrRNA (Merck,
445 #TRACRRNA05N), 1 µL (F_C 1.67 µM) and 1 µL Cas9 protein (New England Biolabs, #M0646) was freshly
446 prepared on the morning of the injection procedure. Then, 1 nL of the injection mix was injected into
447 one-cell stage zebrafish embryos (~ 100-150 embryos). Injection efficacy was assessed at 24 hours
448 post fertilization (hpf) by polymerase chain reaction (PCR) from genomic DNA (*rbfox1*_Forward, 5'-
449 TAATCAAGACGCCCCAGCAC–3'; *rbfox1*_Reverse, 5'- GTACTCAGCAGGAATGCCGT- 3') followed by MwoI
450 (New England Biolabs, #R0573S) restriction enzyme digestion. Successful injections will introduce
451 indel mutations disrupting the recognition site of the restriction enzyme, preventing this latter from
452 cutting the PCR amplicon. Once reached sexual maturity (at ~ 3 months of age) injected fish (F_0) were
453 outcrossed with wild type to generate F_1 embryos. The progeny may carry different mutations due to
454 the mosaic nature of the F_0 parents. F_1 fish were therefore screened for mutations leading to
455 premature termination codon (PTC) via cloning into pGEM-T Easy vector (Promega, #A1360), followed
456 by transformation, colony PCR, DNA purification (Monarch® Plasmid Miniprep Kit, New England
457 Biolabs, #T1010) and sequencing (Source BioScience PLC). Quantitative real time PCR (qPCR) was used
458 to confirm reduction of *rbfox1* mRNA expression.
459

460 **Fish breeding and genotyping**

461

462 For adult experiments, mixed sexes were used. When possible, animals were associated with an
463 identification number and genotype was assigned after data analysis. For larval experiments, fish were
464 generated by heterozygous in-cross and genotyped prior to data analysis. Genomic DNA was
465 extracted from fins and using the HotSHOT method. Briefly, samples were incubated at 95 °C in 50
466 mM NaOH for 30 min, followed by 1 min at 10 °C. Reaction was stopped using 1M Tris HCl (1/10 of
467 the initial NaOH volume), pH 8.00. Genotyping primers were the same as the ones used to identify
468 founder carriers.
469

470 **Drug Treatment**

471

472 Zebrafish embryos of each genotype (*rbfox1*$^{+/+}$ and *rbfox1*$^{19del/19del}$) were treated from 5hpf to 5dpf
473 with 20 µM TRKB antagonist ANA-12 (abcam, Cat.ab146196), or 20 µM TRKB agonist 7,8-
474 dihydroxyflavone (7,8-DHF) (abcam #ab120996), or dimethylsulfoxide (DMSO, vehicle 0.01%) (Merck
475 #34869). Treatment was performed in Petri dishes (max 30 embryos x dish) and drugs or vehicle were
476 dissolved in fish water. Solutions were replaced daily.
477

478 **Quantitative Real-Time PCR**

479 Quantitative Real-time PCR (qPCR) of target RNA was performed on 5dpf *rbfox1* wild type (*rbfox1*$^{+/+}$)
480 and *rbfox1* homozygous (*rbfox1*$^{19del/19del}$) larvae, using Luna® Universal One-Step RT-qPCR Kit (New
481 England Biolabs #M3005) and a Bio-Rad 96-well qPCR machine (CFX96 Touch Real-Time PCR Detection
482 System). Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific) following
483 manufacturer's instructions. Briefly, after homogenization, RNA was isolated by precipitation, rinsed
484 in ethanol and resuspended in RNase free water. Total RNA was then quantified using BioDrop

485 (Biochrom Ltd.), and up to 1 µg was reverse transcribed to cDNA using the ProtoScript II First Strand
486 cDNA Synthesis Kit (New England Biolabs, #E6560) following manufacturer's instructions. The resulting
487 cDNA yield and quality were evaluated using BioDrop (Biochrom Ltd.). As for zebrafish *trkb2 TK+* and
488 *TK-* isoforms quantification, Ct values resulting from the amplification of the *TK+* specific product were
489 subtracted from the Ct values resulting from the amplification of the *TK+/TK-* common product. All
490 reactions included 5 biological replicates and 3 technical replicates. For experiments in larvae, each
491 biological replicate consisted of 15 larval heads (eyes and jaw removed). For experiments in adults,
492 each biological replicate consisted of a single brain. Actin – β 2 (*actb2*) and ribosomal protein L13a
493 (*rpl13a*) were employed as reference genes. For assessing *rbfox1* mRNA expression, *rbfox1* primers
494 were designed upstream of the CRISPR deletion. Accession numbers, primer sequences and
495 amplification efficiencies for all the reference and target genes can be found in Supplementary Table
496 1.
497
498 ***In situ* hybridization**

499 *In situ* hybridization (ISH) was carried out on whole mount zebrafish larvae and on larval (sagittal) and
500 adult brain (transverse) sections as described previously[2]. For *rbfox1* ISH, the original plasmid used to
501 generate the riboprobe was provided by Dr William Norton (University of Leicester). The plasmid used
502 to produce the *trkb2* riboprobe was generated in our laboratory (Forward primer: 5'-
503 GTTCGTGGAATGGCTTGCTG-3', Reverse primer: 5'-TCTGGCCCACGATGTTTTCA-3') using the pGEM®-T
504 Easy Vector System (Promega, #A1360) and *in-house* generated *E. coli* DH5α competent cells.
505 Riboprobes to identify *rbfox1* (NM_001005596) and *trkb2* (NM 01197161.2) mRNA were synthetized
506 by *in vitro* transcription (IVT) using MAXIscript™ SP6/T7 kit (Invitrogen by Thermo Fisher Scientific,
507 #AM1322), following manufacturer's instructions and using a DIG RNA Labeling Mix, 10× conc (Roche,
508 #11277073910) containing digoxigenin labeled uracil.
509
510 *In situ* hybridization on whole mount zebrafish larvae
511
512 *In situ* hybridization was carried out on 28hpf, 2- 3- 4- and 5dpf *rbfox1*$^{+/+}$ and *rbfox1*$^{19del/19del}$ larvae. To
513 prevent skin pigmentation, embryos were incubated in 0.2 mM 1-phenyl 2-thiourea (PTU) (Sigma,
514 #S527335) from 24hpf. When they reached the desired age, larvae were fixed in 4% paraformaldehyde
515 (PFA) (Merck, #158127) overnight (ON) at 4°C. The following day, larvae were rinsed in 1x phosphate
516 buffered saline (PBS) (Thermo Fisher Scientific, #18912014) supplemented with Tween 20 (Sigma,
517 #P1379) (0.05% v/v), dehydrated in ascending methanol series (25%, 50%, 70%, 80%, 90%, 100%
518 methanol, 5 min each) and stored in 100% methanol at -20 °C. To perform ISH experiments, larvae
519 were rehydrated in descending methanol series (100%, 90%, 80%, 70%, 50%, 25% methanol), 5 min
520 each, and washed in 1xPBS, 5 min. Larvae were permeabilized using proteinase K (PK) (ITW Reagents,
521 #A3830) (stock 20 µg/mL in 1xPBS) as follows: 28hpf larvae were permeabilized in PK 1:2000 in 1xPBS
522 for 20 min at room temperature (RT), older stages were permeabilized in PK 1:1000 at 37°C for at least
523 30 min. Then, larvae were post fixed in 4% PFA for 20 min and washed in 1xPBS at RT, 5 x 5 min.
524 Prehybridization was carried out in hybridization solution (HB) containing 50% formamide, 5% saline
525 sodium citrate buffer (SSC), 50 µg/mL heparin, 0.05 mg/mL yeast RNA, 0.1% Tween 20, and 0.92%
526 citric acid at 68 °C for 2h. Thereafter, larvae were incubated in HB containing *rbfox1* riboprobe (500
527 pg/µL), ON at 68°C. Post hybridization washes were performed at 68°C with a gradient of 2xSSC and
528 formamide (50%, 25% and 0% formamide), 10 min each, and then twice with 0.02xSSC, 30 min each.
529 Subsequently, larvae were blocked in blocking solution (BS) containing 10% normal sheep serum
530 (Gibco, #16070096) and 2 µg/µL bovine serum albumin, for 1h at RT. After blocking step, larvae were
531 incubated in anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (Roche,

532 #11093274910), 1:2000 in BS, 1h at RT and then ON at 4°C. The following day, larvae were washed in
533 1xPBS, 6 x 15 min each, and then in alkaline phosphatase (100 mM NaCl, 100 mM Tris HCl, 50 mM
534 MgCl2, 0.1% Tween 20) (NTMT) buffer, 3 x 5 min each. The chromogenic reaction was carried out by
535 incubating the larvae in BCIP/NBT solution (Merck, #203790) in NTMT buffer, at RT in the dark, and
536 were observed every 20 min until the signal detection. After reaching the desired staining, larvae were
537 washed in 1xPBS at RT, post fixed in 4% PFA for 2h, cleared and stored in 80% glycerol at 4°C. For
538 sagittal cryosections, larvae were embedded in 1.5% low gelling temperature agarose (Scientific
539 Laboratories Supplies, #A9414) supplemented with 5% Sucrose in 1xPBS. Sections (5 µm) were
540 collected on adhesive microscope slides Superfrost® Plus Gold (Epredia).
541
542 *In situ* hybridization on sections
543
544 ISH was conducted on paraffin embedded *rbfox1*$^{+/+}$ and *rbfox1*$^{19del/19del}$ adult brains (transverse
545 sections) and 5dpf *rbfox1*$^{+/+}$ and *rbfox1*$^{19del/19del}$ zebrafish larvae (sagittal sections). Fish were culled by
546 an overdose of tricaine prior to head removal. Dissected adult brains and larvae were fixed in 4% PFA
547 in 1xPBS, ON at 4°C. Tissues were then rinsed in 1xPBS and dehydrated in ascending ethanol series (15
548 min in each of 30%, 50%, 70%, 80%, 90%, 100% ethanol) and embedded in paraffin. Transverse (adult
549 brains) or sagittal (5 days old larvae) sections of 12 µm (adult brains) or 7 µm (5 days old larvae)
550 thickness were cut using a microtome (Leica). To perform ISH, slides were de-waxed in xylene (twice,
551 10 min each), rehydrated in descending ethanol series (2 x 5 min in absolute ethanol, then 90%, 80%,
552 70%, 50% and 25% ethanol, 5 min each), and rinsed in 1xPBS for 5 min. Then, sections were
553 permeabilized using PK (0.05 µg/µL) for 8 min at RT, washed with 2 mg/mL glycine twice (5 min each),
554 post fixed in 4% PFA for 20 min and washed in 1xPBS at RT. Prehybridization was carried out in HB, for
555 1h at 68°C. Thereafter, sections were incubated in HB containing *rbfox1* riboprobe (500 pg/µL), ON at
556 68°C. Post hybridization washes were performed at 68°C twice for 20 min in 1xSSC, twice for 20 min
557 in 0.2xSSC, and several washes were performed in 1xPBS, 5 min each at RT. Then, sections were
558 blocked in BS for 30 min at RT and incubated in a 1:2000 dilution of anti-digoxigenin Fab fragments
559 conjugated with alkaline phosphatase in BS, ON at 4°C. The following day, sections were washed in
560 1xPBS, 5 x 10 min each. The chromogenic reaction was carried out by incubating the slides in BCIP/
561 NBT solution in NTMT buffer, at RT in the dark, and were observed every 20 min until the signal
562 detection. When the desired staining was obtained, sections were washed in 1xPBS at RT, dehydrated
563 in ascending ethanol series (25%, 50%, 70%, 80%, 90%, 100% ethanol, 5 min each), cleared in xylene
564 (twice, 5 min each) and mounted with dibutyl phthalate polystyrene xylene mounting medium (Sigma,
565 #06522).

566 **Image acquisition and processing**

567 Pictures of whole mount ISH on zebrafish larvae were acquired by Leica MZ75 microscope. For ISH on
568 sections, pictures were acquired using a Leica DMRA2 upright epifluorescent microscope with colour
569 QIClick camera (Leica) and processed with Velocity 6.3.1 software (Quorum Technologies Inc).
570 Quantification of the ISH staining signal intensity was performed as described previously[64], using Fiji
571 software[65]. Adult anatomical structures were identified according to the Neuroanatomy of the
572 Zebrafish Brain by Wullimann [66].

573 **Behavioral Assays**

574 For larvae, all behavioural experiments were conducted on the progeny of a *rbfox1*$^{+/19del}$ in-cross.
575 Larvae were genotyped prior to data analysis. For adults, were the genotype of the animals was
576 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 5dpf *rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$ and *rbfox1*$^{19del/19del}$ mutant zebrafish larvae were investigated as described previously[23,28,67]. 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[68]. For the hyperactivity assay, larval basal locomotion was tracked for 15 min in dark conditions. Larval swimming burst was assessed as described previously[23] and peaks were considered as the acceleration events when larvae travelled > 5 mm in < 12 sec. The forced light-dark transition assay was performed as described previously[69,70] 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[28]: after 10 min of baseline (no stimuli, dark conditions), larvae were subjected to 10 sound/vibration stimuli (Noldus tapping device) over 20 sec (2 sec intervals between each stimulus).

5-Choice Serial Reaction Time Task

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)[24]. Adult *rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$ and *rbfox1*$^{19del/19del}$ zebrafish, 7 months old, mixed sexes, were singly housed for a week prior to experiment and remained singly housed for the whole duration of the assay (9 weeks). Fish were tested using the Zantiks AD units. 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 entered 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/variable-delay. Details of each stage are provided in Supplementary Table 2.

Novel tank diving

Novel tank diving is a behavioural test to assess anxiety-like behaviour in adult fish. Response to novel tank was assessed in *rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$ and *rbfox1*$^{19del/19del}$ 9 months old zebrafish, mixed-sexes, as described previously[67]. 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[67]. Experimental groups were randomised during testing. We analyzed three behaviours in response to the novel tank: i) time spent in the bottom

620 of the tank, ii) total distance traveled, and iii) number of transitions to the top–bottom area of the
621 tank.

622

623 **Statistical analysis**

624 For qPCR, relative mRNA expressions were calculated using the Pfaffl method[71]. Outliers were
625 identified and removed using Dixon's test ($\alpha = 0.05$)[72]. Differences in gene expression were assessed
626 using a one-way ANOVA followed by Tukey's post-hoc test using GraphPad Software (Prism). For
627 behavioural analysis, all data were analysed with R programming language[68]. Scripts used for analysis
628 are available on GitHub (https://github.com/AdeleLeg). For models where distance moved, distance
629 to zone, velocity, or top halves visits were the response variable, we fitted data to mixed linear models
630 using the "*lme4*" package, and where proportion of responders or proportion of time spent in the
631 bottom third were our response variable, we fitted data to beta regression models using the "*betareg*"
632 package. In all instances, for all experiments, we used genotype as fixed effect, and fish ID and day of
633 experiment as random effects. In the response and habituation to acoustic startle, we used also the
634 stimulus number as fixed effect. As in García-González et al.[67], we reported significant fixed effects as
635 Type II Wald χ^2 from models using the package "*car*," post hoc Tukey's tests were also conducted as
636 necessary with the package "*emmeans*". For the 5-CSRTT, overall correct responses (learning) and
637 anticipatory responses (impulsivity) were assessed using the formulas in Supplementary Table 2.
638 Statistical analysis of mean intensity in *in situ* hybridization images of zebrafish larval sections was
639 performed using ImageJ. Mean intensity values were quantified across specified regions of interest,
640 indicated in Supplementary Figure 5, ensuring that comparable areas of similar dimensions were used
641 for each sample to calculate differences in expression. The resulting values were compared across
642 groups using a paired t-test to determine significant differences using GraphPad Software (Prism). For
643 all experiments, sample size needed to achieve adequate statistical power for detecting a significant
644 effect was determined based on data from previous research or pilot studies. Accepted α level and
645 power were respectively ≤ 0.05 and ≥ 0.80.

646 **Fertilisation and survival analysis**

647 Fertilisation and survival rates were measured as described previously[19]. For fertilization rate we
648 measured the percentage of fertilized eggs when pairing *rbfox1*$^{19del/19del}$ and compared it with
649 fertilization rate of *rbfox1*$^{+/+}$ siblings. Data were collected upon 3 different mating trials, each trial
650 comprising 3-5 trios (1 male and 2 females). Trials were performed on different weeks, using different
651 trios for each test. Eggs were collected in different Petri dishes, properly labelled to distinguish
652 between trios. Fertilisation rate was determined by averaging across Petri dishes from the same trio
653 and calculated at 6-8hpf. For survival analysis, we measured the percentage of surviving animals
654 (across 3 tanks for each genotype for each batch, to avoid tank effect) from when larvae were added
655 to the nursery (at 5dpf) until 2 months of age, when fish were transferred into adult aquarium.
656

657 **CONTRIBUTIONS**

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

ACKNOWLEDGMENTS

CHB, AL, SH, PA and WH were supported by the NIH grant U01 DA044400-03. JGG was supported by a 2022 NARSAD Young Investigator Grant (Number #30749) by the Brain & Behavior Research Foundation. NFC and BC were supported by the grants PID2021-127776OB-I100, 202218 31 and 2021-SGR-01093 from the Ministry of Science, Innovation and Universities (Spain), Fundació La Marató de TV3, and AGAUR-Generalitat de Catalunya, respectively, and by ICREA Academia 2021. Authors would like to acknowledge SciDraw (https://scidraw.io/) for images used to create the graphical abstract.

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.

REFERENCES

1. Cross-Disorder Group of the Psychiatric Genomics Consortium. Electronic address: plee0@mgh.harvard.edu & Cross-Disorder Group of the Psychiatric Genomics Consortium. Genomic Relationships, Novel Loci, and Pleiotropic Mechanisms across Eight Psychiatric Disorders. *Cell* **179**, 1469-1482.e11 (2019).
2. Antón-Galindo, E. *et al.* Pleiotropic contribution of rbfox1 to psychiatric and neurodevelopmental phenotypes in two zebrafish models. *Transl. Psychiatry* **14**, 1–11 (2024).
3. Thisse, B. & Thisse, C. ZFIN Publication: Thisse et al., 2004. *ZFIN* http://zfin.org/ZDB-PUB-040907-1.
4. Amir-Zilberstein, L. *et al.* Homeodomain Protein Otp and Activity-Dependent Splicing Modulate Neuronal Adaptation to Stress. *Neuron* **73**, 279–291 (2012).
5. Abi-Dargham, A. *et al.* Candidate biomarkers in psychiatric disorders: state of the field. *World Psychiatry* **22**, 236–262 (2023).
6. O'Leary, A. *et al.* Behavioural and functional evidence revealing the role of RBFOX1 variation in multiple psychiatric disorders and traits. *Mol. Psychiatry* **27**, 4464–4473 (2022).
7. Lee, J.-A., Tang, Z.-Z. & Black, D. L. An inducible change in Fox-1/A2BP1 splicing modulates the alternative splicing of downstream neuronal target exons. *Genes Dev.* **23**, 2284–2293 (2009).

691 8. Shaughnessy, C. A., Myhre, V. D., Hall, D. J., McCormick, S. D. & Dores, R. M. Hypothalamus-
692 pituitary-interrenal (HPI) axis signaling in Atlantic sturgeon (Acipenser oxyrinchus) and sterlet
693 (Acipenser ruthenus). *Gen. Comp. Endocrinol.* **339**, 114290 (2023).
694 9. Biran, J. *et al.* Splice-specific deficiency of the PTSD-associated gene PAC1 leads to a paradoxical
695 age-dependent stress behavior. *Sci. Rep.* **10**, 9559 (2020).
696 10. Linz, R. *et al.* Acute psychosocial stress increases serum BDNF levels: an antagonistic relation to
697 cortisol but no group differences after mental training. *Neuropsychopharmacology* **44**, 1797–
698 1804 (2019).
699 11. Jeanneteau, F. D. *et al.* BDNF and glucocorticoids regulate corticotrophin-releasing hormone
700 (CRH) homeostasis in the hypothalamus. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 1305–1310 (2012).
701 12. Karatsoreos, I. N. & McEwen, B. S. Psychobiological allostasis: resistance, resilience and
702 vulnerability. *Trends Cogn. Sci.* **15**, 576–584 (2011).
703 13. Miao, Z., Wang, Y. & Sun, Z. The Relationships Between Stress, Mental Disorders, and Epigenetic
704 Regulation of BDNF. *Int. J. Mol. Sci.* **21**, 1375 (2020).
705 14. Suliman, S., Hemmings, S. & Seedat, S. Brain-Derived Neurotrophic Factor (BDNF) protein levels
706 in anxiety disorders: systematic review and meta-regression analysis. *Front. Integr. Neurosci.* **7**,
707 55 (2013).
708 15. Fogel, B. L. *et al.* RBFOX1 regulates both splicing and transcriptional networks in human
709 neuronal development. *Hum. Mol. Genet.* **21**, 4171–4186 (2012).
710 16. Tomassoni-Ardori, F. *et al.* Rbfox1 up-regulation impairs BDNF-dependent hippocampal LTP by
711 dysregulating TrkB isoform expression levels. *eLife* **8**, e49673 (2019).
712 17. Solés-Tarrés, I., Cabezas-Llobet, N., Vaudry, D. & Xifró, X. Protective Effects of Pituitary
713 Adenylate Cyclase-Activating Polypeptide and Vasoactive Intestinal Peptide Against Cognitive
714 Decline in Neurodegenerative Diseases. *Front. Cell. Neurosci.* **14**, (2020).

715 18. Mcewen, B. S. Protection and Damage from Acute and Chronic Stress: Allostasis and Allostatic
716 Overload and Relevance to the Pathophysiology of Psychiatric Disorders. *Ann. N. Y. Acad. Sci.*
717 **1032**, 1–7 (2004).
718 19. Eachus, H. *et al.* Elevated glucocorticoid alters the developmental dynamics of hypothalamic
719 neurogenesis in zebrafish. *Commun. Biol.* **7**, 1–14 (2024).
720 20. Danese, A. & McEwen, B. S. Adverse childhood experiences, allostasis, allostatic load, and age-
721 related disease. *Physiol. Behav.* **106**, 29–39 (2012).
722 21. Bornstein, S. R. *et al.* Stress-inducible-stem cells: a new view on endocrine, metabolic and
723 mental disease? *Mol. Psychiatry* **24**, 2–9 (2019).
724 22. Kinlein, S. A., Wilson, C. D. & Karatsoreos, I. N. Dysregulated Hypothalamic–Pituitary–Adrenal
725 Axis Function Contributes to Altered Endocrine and Neurobehavioral Responses to Acute Stress.
726 *Front. Psychiatry* **6**, (2015).
727 23. Lange, M. *et al.* The ADHD-susceptibility gene lphn3.1 modulates dopaminergic neuron
728 formation and locomotor activity during zebrafish development. *Mol. Psychiatry* **17**, 946–954
729 (2012).
730 24. Parker, M. O. *et al.* Development and automation of a test of impulse control in zebrafish. *Front.*
731 *Syst. Neurosci.* **7**, (2013).
732 25. Halberstadt, A. L. & Geyer, M. A. Habituation and sensitization of acoustic startle: opposite
733 influences of dopamine D1 and D2-family receptors. *Neurobiol. Learn. Mem.* **92**, 243–248
734 (2009).
735 26. Burgess, H. A. & Granato, M. Sensorimotor gating in larval zebrafish. *J. Neurosci. Off. J. Soc.*
736 *Neurosci.* **27**, 4984–4994 (2007).
737 27. Cohen, H. *et al.* Blunted HPA axis response to stress influences susceptibility to posttraumatic
738 stress response in rats. *Biol. Psychiatry* **59**, 1208–1218 (2006).
739 28. Leggieri, A. *et al.* Ankk1 Loss of Function Disrupts Dopaminergic Pathways in Zebrafish. *Front.*
740 *Neurosci.* **16**, (2022).

741 29. Davies, M. N. *et al.* Generalised Anxiety Disorder – A Twin Study of Genetic Architecture,
742 Genome-Wide Association and Differential Gene Expression. *PLOS ONE* **10**, e0134865 (2015).
743 30. Basnet, R. M., Zizioli, D., Taweedet, S., Finazzi, D. & Memo, M. Zebrafish Larvae as a Behavioral
744 Model in Neuropharmacology. *Biomedicines* **7**, E23 (2019).
745 31. Lee, H. B. *et al.* Novel zebrafish behavioral assay to identify modifiers of the rapid, nongenomic
746 stress response. *Genes Brain Behav.* **18**, e12549 (2019).
747 32. Hillman, C., Kearn, J. & Parker, M. O. A unified approach to investigating 4 dpf zebrafish larval
748 behaviour through a standardised light/dark assay. *Prog. Neuropsychopharmacol. Biol.*
749 *Psychiatry* **134**, 111084 (2024).
750 33. Baiamonte, M., Parker, M. O., Vinson, G. P. & Brennan, C. H. Sustained Effects of Developmental
751 Exposure to Ethanol on Zebrafish Anxiety-Like Behaviour. *PLoS ONE* **11**, e0148425 (2016).
752 34. Gene Detail :: Allen Brain Atlas: Mouse Brain. https://mouse.brain-map.org/gene/show/92602.
753 35. Brain tissue expression of RBFOX1 - Summary - The Human Protein Atlas.
754 https://www.proteinatlas.org/ENSG00000078328-RBFOX1/brain.
755 36. Grone, B. P. & Maruska, K. P. Divergent evolution of two corticotropin-releasing hormone (CRH)
756 genes in teleost fishes. *Front. Neurosci.* **9**, 365 (2015).
757 37. Sahu, M. P. *et al.* Neurotrophin receptor Ntrk2b function in the maintenance of dopamine and
758 serotonin neurons in zebrafish. *Sci. Rep.* **9**, 2036 (2019).
759 38. Eachus, H., Ryu, S., Placzek, M. & Wood, J. Zebrafish as a model to investigate the CRH axis and
760 interactions with DISC1. *Curr. Opin. Endocr. Metab. Res.* **26**, 100383 (2022).
761 39. Faught, E. & Vijayan, M. M. The mineralocorticoid receptor is essential for stress axis regulation
762 in zebrafish larvae. *Sci. Rep.* **8**, 18081 (2018).
763 40. Tessarollo, L. & Yanpallewar, S. TrkB Truncated Isoform Receptors as Transducers and
764 Determinants of BDNF Functions. *Front. Neurosci.* **16**, (2022).
765 41. Martin, S. C., Marazzi, G., Sandell, J. H. & Heinrich, G. Five Trk Receptors in the Zebrafish. *Dev.*
766 *Biol.* **169**, 745–758 (1995).

767 42. Thisse, C. & Thisse, B. High-resolution in situ hybridization to whole-mount zebrafish embryos.
768 *Nat. Protoc.* **3**, 59–69 (2008).
769 43. Islam, O., Loo, T. X. & Heese, K. Brain-Derived Neurotrophic Factor (BDNF) has Proliferative
770 Effects on Neural Stem Cells through the Truncated TRK-B Receptor, MAP Kinase, AKT, and
771 STAT-3 Signaling Pathways. *Curr. Neurovasc. Res.* **6**, 42–53.
772 44. Finlay, S., Rudd, D., McDermott, B. & Sarnyai, Z. Allostatic load and systemic comorbidities in
773 psychiatric disorders. *Psychoneuroendocrinology* **140**, 105726 (2022).
774 45. Elia, J. *et al.* Rare structural variants found in attention-deficit hyperactivity disorder are
775 preferentially associated with neurodevelopmental genes. *Mol. Psychiatry* **15**, 637–646 (2010).
776 46. van der Kolk, B. Posttraumatic stress disorder and the nature of trauma. *Dialogues Clin.*
777 *Neurosci.* **2**, 7–22 (2000).
778 47. Karin, O. *et al.* A new model for the HPA axis explains dysregulation of stress hormones on the
779 timescale of weeks. *Mol. Syst. Biol.* **16**, (2020).
780 48. Herman, J. P. *et al.* Regulation of the hypothalamic-pituitary-adrenocortical stress response.
781 *Compr. Physiol.* **6**, 603–621 (2016).
782 49. Holsboer, F. The Corticosteroid Receptor Hypothesis of Depression. *Neuropsychopharmacology*
783 **23**, 477–501 (2000).
784 50. Arborelius, L., Owens, M. J., Plotsky, P. M. & Nemeroff, C. B. The role of corticotropin-releasing
785 factor in depression and anxiety disorders. *J. Endocrinol.* **160**, 1–12 (1999).
786 51. Yehuda, R. Status of Glucocorticoid Alterations in Post-traumatic Stress Disorder. *Ann. N. Y.*
787 *Acad. Sci.* **1179**, 56–69 (2009).
788 52. Sheng, J. A. *et al.* The Hypothalamic-Pituitary-Adrenal Axis: Development, Programming Actions
789 of Hormones, and Maternal-Fetal Interactions. *Front. Behav. Neurosci.* **14**, (2021).
790 53. Notaras, M. & van den Buuse, M. Neurobiology of BDNF in fear memory, sensitivity to stress,
791 and stress-related disorders. *Mol. Psychiatry* **25**, 2251–2274 (2020).

792 54. Berton, O. *et al.* Essential Role of BDNF in the Mesolimbic Dopamine Pathway in Social Defeat
793 Stress. *Science* **311**, 864–868 (2006).
794 55. Eckert, A. *et al.* The link between sleep, stress and BDNF. *Eur. Psychiatry* **41**, S282 (2017).
795 56. Fenner, B. M. Truncated TrkB: Beyond a dominant negative receptor. *Cytokine Growth Factor*
796 *Rev.* **23**, 15–24 (2012).
797 57. Cazorla, M. *et al.* Identification of a low–molecular weight TrkB antagonist with anxiolytic and
798 antidepressant activity in mice. *J. Clin. Invest.* **121**, 1846–1857 (2011).
799 58. Azogu, I. & Plamondon, H. Blockade of TrkB receptors in the nucleus accumbens prior to
800 heterotypic stress alters corticotropin-releasing hormone (CRH), vesicular glutamate transporter
801 2 (vGluT2) and glucocorticoid receptor (GR) within the mesolimbic pathway. *Horm. Behav.* **90**,
802 98–112 (2017).
803 59. Bollen, E. *et al.* 7,8-Dihydroxyflavone improves memory consolidation processes in rats and
804 mice. *Behav. Brain Res.* **257**, 8–12 (2013).
805 60. Haapasalo, A., Koponen, E., Hoppe, E., Wong, G. & Castrén, E. Truncated trkB.T1 Is Dominant
806 Negative Inhibitor of trkB.TK+-Mediated Cell Survival. *Biochem. Biophys. Res. Commun.* **280**,
807 1352–1358 (2001).
808 61. Maussion, G. *et al.* Regulation of a truncated form of tropomyosin-related kinase B (TrkB) by
809 Hsa-miR-185* in frontal cortex of suicide completers. *PloS One* **7**, e39301 (2012).
810 62. Koolhaas, J. M., de Boer, S. F., Coppens, C. M. & Buwalda, B. Neuroendocrinology of coping
811 styles: Towards understanding the biology of individual variation. *Front. Neuroendocrinol.* **31**,
812 307–321 (2010).
813 63. Keatinge, M. *et al.* CRISPR gRNA phenotypic screening in zebrafish reveals pro-regenerative
814 genes in spinal cord injury. *PLOS Genet.* **17**, e1009515 (2021).
815 64. Dobrzycki, T., Krecsmarik, M. & Monteiro, R. Genotyping and Quantification of In Situ
816 Hybridization Staining in Zebrafish. *JoVE J. Vis. Exp.* e59956 (2020) doi:10.3791/59956.

817 65. Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **9**,
818 676–682 (2012).
819 66. Wullimann, M. F., Rupp, B. & Reichert, H. The brain of the zebrafish Danio rerio: a
820 neuroanatomical atlas. in *Neuroanatomy of the Zebrafish Brain: A Topological Atlas* (eds.
821 Wullimann, M. F., Rupp, B. & Reichert, H.) 19–87 (Birkhäuser, Basel, 1996). doi:10.1007/978-3-
822 0348-8979-7_5.
823 67. García-González, J., de Quadros, B., Havelange, W., Brock, A. J. & Brennan, C. H. Behavioral
824 Effects of Developmental Exposure to JWH-018 in Wild-Type and Disrupted in Schizophrenia 1
825 (disc1) Mutant Zebrafish. *Biomolecules* **11**, 319 (2021).
826 68. R Development Core Team. R: A language and environment for statistical computing.
827 https://stat.ethz.ch/pipermail/r-help/2008-May/161481.html (2008).
828 69. Gutiérrez, H. C. *et al.* Screening for drugs to reduce zebrafish aggression identifies caffeine and
829 sildenafil. *Eur. Neuropsychopharmacol.* **30**, 17–29 (2020).
830 70. Parker, M. O., Brock, A. J., Sudwarts, A. & Brennan, C. H. Atomoxetine reduces anticipatory
831 responding in a 5-choice serial reaction time task for adult zebrafish. *Psychopharmacology*
832 *(Berl.)* **231**, 2671–2679 (2014).
833 71. Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic*
834 *Acids Res.* **29**, e45 (2001).
835 72. Dean, R. B. & Dixon, W. J. Simplified Statistics for Small Numbers of Observations. *Anal. Chem.*
836 **23**, 636–638 (1951).

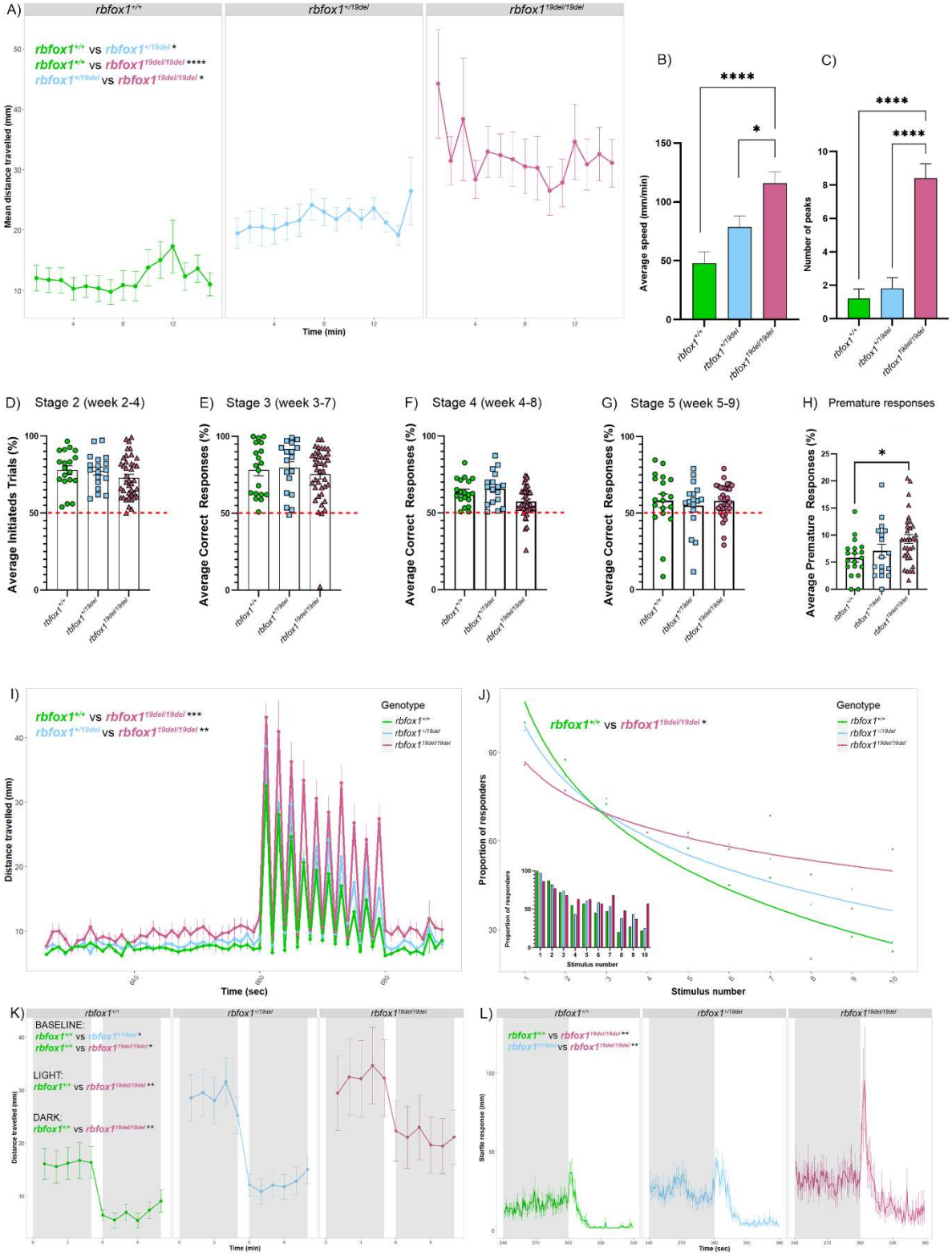

Figure 1. *rbfox1* mutant fish show hyperactivity, impulsivity and hyperarousal behaviour. A-C) Locomotion assay in 5 days post fertilization (dpf) zebrafish larvae (*rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$, *rbfox1*$^{19del/19del}$): **A)** distance travelled during 15 min; **B)** average speed; **C)** number of peaks/swimming burst (acceleration events when the fish travelled > 5 mm in < 12 s); N (A-C) = *rbfox1*$^{+/+}$ 24; *rbfox1*$^{+/19del}$ 24; *rbfox1*$^{19del/19del}$ 24. **D-H)** 5-choice serial reaction time task in adult (7 months old) zebrafish (*rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$, *rbfox1*$^{19del/19del}$): **D)** number of initiated trials during stage 2 (weeks 2-4); all

845 *rbfox1*$^{+/+}$ (19), all *rbfox1*$^{+/19del}$ (18) and all *rbfox1*$^{19del/19del}$ (40) moved to the next stage; **E-G)** average
846 correct responses for stages 3-5 (weeks 3-9); **E)** at the end of stage 3 (weeks 3-7), all *rbfox1*$^{+/+}$ (19), 17
847 *rbfox1*$^{+/19del}$ and 39 *rbfox1*$^{19del/19del}$ moved to the next stage; **F)** at the end of stage 4 (weeks 4-8), all
848 *rbfox1*$^{+/+}$ (19), all *rbfox1*$^{+/19del}$ (17) and 33 *rbfox1*$^{19del/19del}$ moved to the next stage; **F)** at the end of stage
849 5 (weeks 5-9), 15 *rbfox1*$^{+/+}$, 13 *rbfox1*$^{+/19del}$ and all *rbfox1*$^{19del/19del}$ (33) showed ≥ 50% correct responses;
850 **H)** premature responses during stage 5 (weeks 5-9). Each dot, square or triangle in D-H) represents a
851 single adult zebrafish *rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$ and *rbfox1*$^{19del/19del}$ respectively; N = *rbfox1*$^{+/+}$ 19;
852 *rbfox1*$^{+/19del}$ 18; *rbfox1*$^{19del/19del}$ 40. **I-J)** Response and habituation to acoustic startle assay in 5dpf
853 zebrafish larvae (*rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$, *rbfox1*$^{19del/19del}$): **I)** mean distance travelled during the assay; **J)**
854 rate of habituation over time/stimuli; N = *rbfox1*$^{+/+}$ 40; *rbfox1*$^{+/19del}$ 39; *rbfox1*$^{19del/19del}$ 35. **K-L)** Forced
855 light-dark transition assay in 5dpf zebrafish larvae (*rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$, *rbfox1*$^{19del/19del}$): **K)** distance
856 travelled during the 10 min assay; **I)** 1 sec time bin resolution plots of the dark-light transition; N =
857 *rbfox1*$^{+/+}$ 43; *rbfox1*$^{+/19del}$ 34; *rbfox1*$^{19del/19del}$ 37. All larvae employed in behavioural experiments were
858 progeny of *rbfox1*$^{+/19del}$ in-cross and were genotyped after experiments and prior to data analysis. In
859 all graphs: bars represent standard error of the mean (SEM); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$;
860 **** $p < 0.0001$.

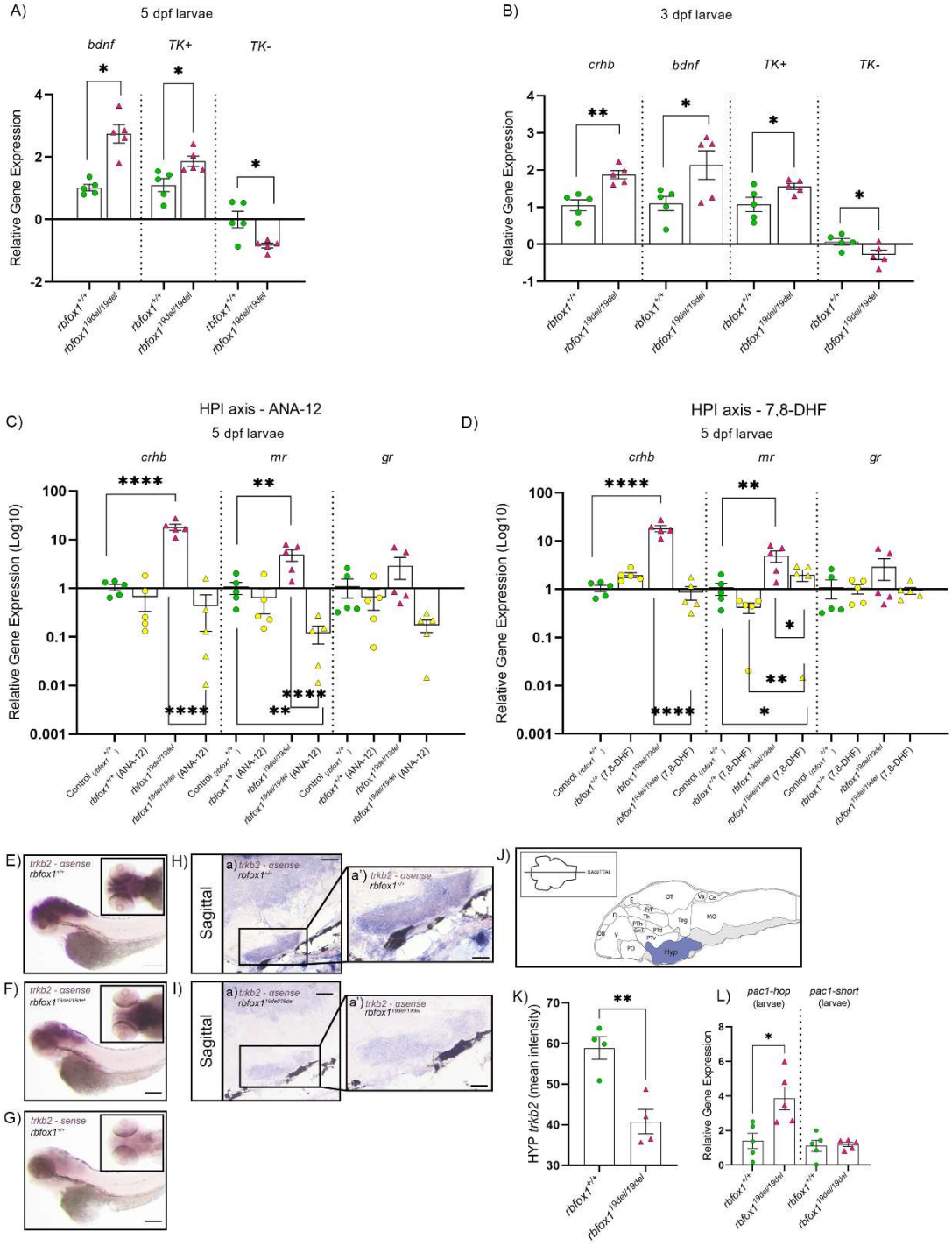

Figure 2. *rbfox1* LoF disrupts zebrafish larvae HPI axis, *bdnf/trkb2* pathway and *pac1a* expression. TRKB modulation restore HPI gene expression. A) Expression levels of *bdnf* and *trkb2* full-length (*TK+*) *and* truncated (*TK-*) in 5 days post fertilisation (dpf) larvae and of **B)** *crhb*, *bdnf*, *TK+* and *TK-* in 3dpf larvae (*rbfox1*$^{+/+}$ and *rbfox1*$^{19del/19del}$). **C-D)** Expression levels of HPI axis genes *crhb*, *mr* and *gr* in 5dpf larvae (*rbfox1*$^{+/+}$ and *rbfox1*$^{19del/19del}$) in presence or absence of the TRKB selective **C)** antagonist ANA-12 and **D)** agonist 7,8-DHF. **E-G)** Whole mount *in situ* hybridisation (ISH) for *trkb2* anti-sense riboprobe in **E)** *rbfox1*$^{+/+}$ and **F)** *rbfox1*$^{19del/19del}$ and **G)** *trkb2* sense riboprobe in *rbfox1*$^{+/+}$ 5dpf larvae (lateral view in the main boxes and dorsal view in the smaller boxes on the top right corner). **H, I)** Sagittal cryosection of *trkb2* ISH in **H)** *rbfox1*$^{+/+}$ and **I)** *rbfox1*$^{19del/19del}$ 5 dpf larvae. Black boxes in **H-a)** and **I-a)** represent the region of the brain showed in higher magnification panels in **H-a')** and **I-a')**. Scale bars:

200 µm in E), F) and G); 100 µm in F-a) and I-a); 50 µm in F-a) and I-a). **J)** Schematic depiction (sagittal) of zebrafish larval brain indicating position of levels illustrated by ISH on sagittal cryosections. **K)** *trkb2* ISH intensity mean in the hypothalamus of 5dpf zebrafish larvae, *rbfox1*$^{+/+}$ versus *rbfox1*$^{19del/19del}$. N = 4 larvae x genotype. **L)** Expression levels of *pac1-hop* and *pac1-short* in *rbfox1*$^{+/+}$ and *rbfox1*$^{19del/19del}$ 5 dpf larvae. For qPCR experiments, reference genes were actin – β 2 (*actb2*) and ribosomal protein L13a (*rpl13a*). Each green dot/pink triangle in A-D, K-L) represents a pool of 15 larval heads (eyes and jaw removed), while yellow ones represent larvae exposed to TRKB drugs. Where indicated, we used Log_{10} transformation to normalize the data facilitating a clearer visualization of trends within the dataset. All larvae employed were progeny of *rbfox1*$^{+/19del}$ in-cross and were genotyped after experiments and prior to data analysis. In all graphs: bars represent standard error of the mean (SEM); * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

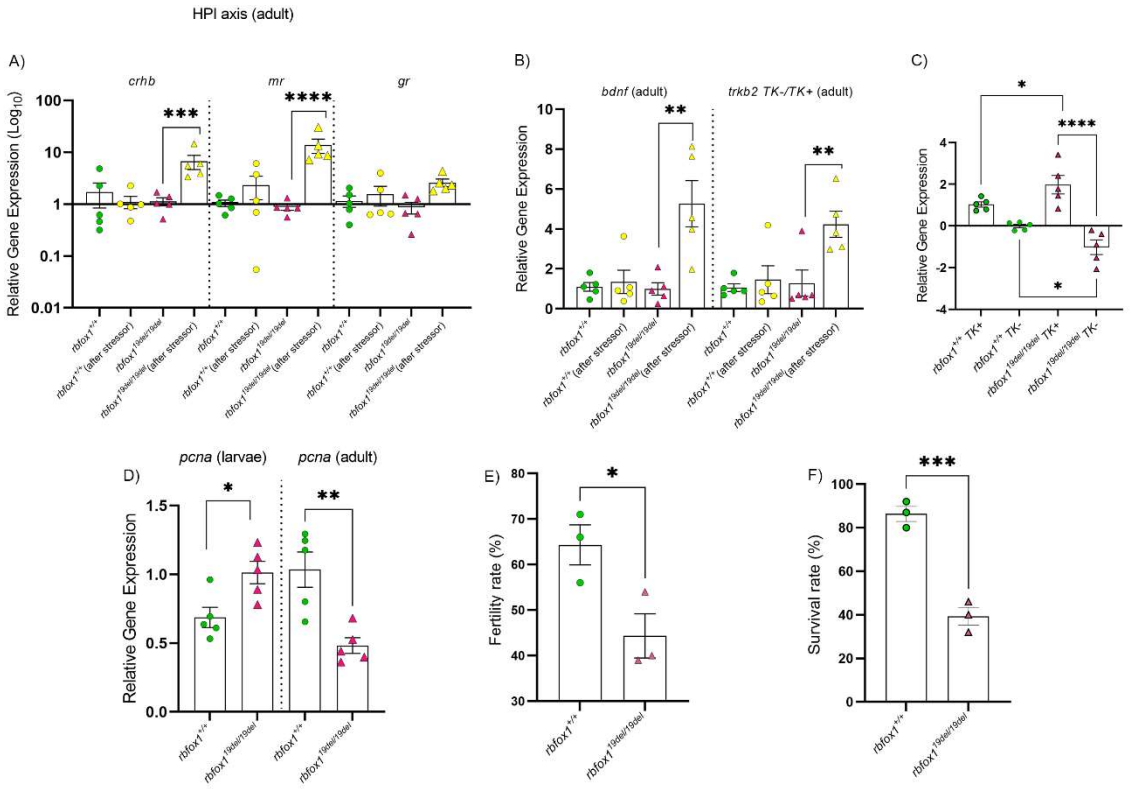

Figure 3. *rbfox1* mutants undergo adaptive mechanisms and allostatic overload during development. Expression levels of **A)** HPI axis genes corticotropin releasing hormone b (*crhb*), mineral corticoid receptor (*mr*) and glucocorticoid receptor (*gr*) and of **B)** *bdnf* and *trkb2* truncated/full-length (*TK-/TK+*) (common primers) in adult zebrafish brain (*rbfox1*$^{+/+}$ and *rbfox1*$^{19del/19del}$) in normal resting conditions and after exposure to a stressor (novel tank diving). **C)** Expression levels of the *trkb2 TK+* and *TK-* in adult zebrafish brain (*rbfox1*$^{+/+}$ and *rbfox1*$^{19del/19del}$) after exposure to a stressor. **D)** Expression levels of proliferating cell nuclear antigen (*pcna*) in 5 days post fertilisation zebrafish larvae and adult zebrafish brain (*rbfox1*$^{+/+}$ and *rbfox1*$^{19del/19del}$) in normal resting conditions. **E)** Fertility rate and **F)** survival rate of *rbfox1*$^{+/+}$ and *rbfox1*$^{19del/19del}$. Each green dot/pink triangle in A-B) represents a single adult *rbfox1*$^{+/+}$ or *rbfox1*$^{19del/19del}$ brain under resting physiological conditions respectively, while

897 yellow dots/triangles represent *rbfox1*$^{+/+}$ or *rbfox1*$^{19del/19del}$ brain after stress exposure respectively. In
898 B) each green dot/pink triangle represents single adult *rbfox1*$^{+/+}$ or *rbfox1*$^{19del/19del}$ brain after stress
899 exposure respectively. In D) for larvae each green dot/pink triangle represents a pool of *rbfox1*$^{+/+}$ or
900 *rbfox1*$^{19del/19del}$ 15 larval heads (eyes and jaw removed) respectively; for adults each green dot/pink
901 triangle represents a single adult *rbfox1*$^{+/+}$ or *rbfox1*$^{19del/19del}$ brain respectively. In E) each dot/triangle
902 represents average fertility of 3-5 trios (1 male and 2 females) assessed over 2-3 petri dish (50 embryos
903 per dish) per trio. Each trio belonged to a different tank (for each genotype for each batch) to avoid
904 tank effect). In F) each dot/triangle represents the percentage of survival of a single fish stock
905 comprising 50 larvae. For qPCR experiments, reference genes were actin – β 2 (*actb2*) and ribosomal
906 protein L13a (*rpl13a*). Where indicated, we used Log_{10} transformation to normalize the data
907 facilitating a clearer visualization of trends within the dataset. All larvae employed were progeny of
908 *rbfox1*$^{+/19del}$ in-cross and were genotyped after experiments and prior to data analysis. In all graphs:
909 bars represent standard error of the mean (SEM); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p <$
910 0.0001.

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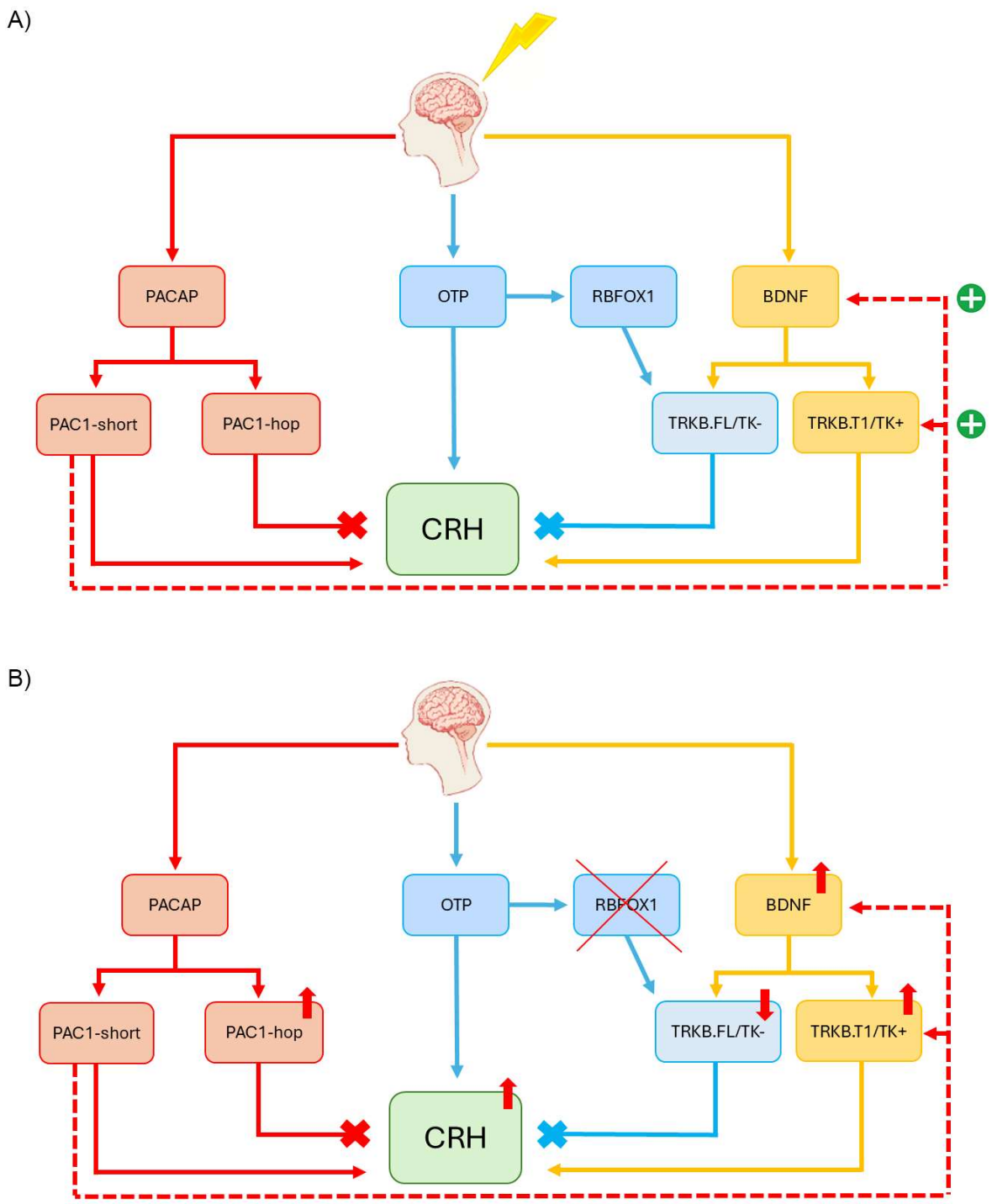

Figure 4. RBFOX1 role in stress resilience and chronic stress recovery: proposed mechanism of action. A) In response to stressful challenges, BDNF expression levels increase, stimulating *CRH* transcription via TRKB.FL/TK+ (yellow pathway). At the same time, stress triggers also *PACAP* transcription (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 (red dashed line) and enhances TRKB.FL/TK+ activity (red dashed line). RBFOX1 functions as a "switch" maintaining the balance between full-length/truncated TRKB isoforms (promoting TRKB mRNA stability and/or alternative splicing): during the stress recovery phase, *RBFOX1* switches the balances in support of *TRKB.T1/TK-*

923 isoform, decreasing *CRH* levels and turning off the HPA axis. **B)** *RBFOX1* deletion leads to an increase
924 in *BDNF* expression levels and an unbalance between *TRKB.FL/TK+* (increased) and *TRKB.T1/TK-*
925 (decreased) isoforms. Such dysregulation ultimately leads to an increase in *CRH* transcription and
926 hyperactivation of the HPA axis. Our data suggest that RBFOX1-mediated regulation of *TRKB.T1/TK-*
927 mRNA stability and/or alternative splicing is necessary to promote neuroplasticity and stress
928 resilience.
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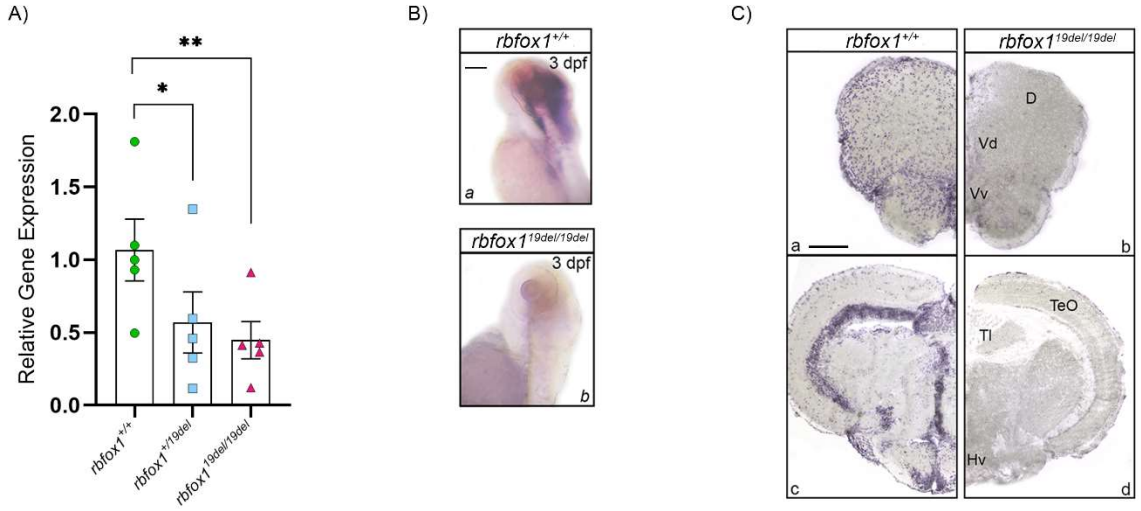

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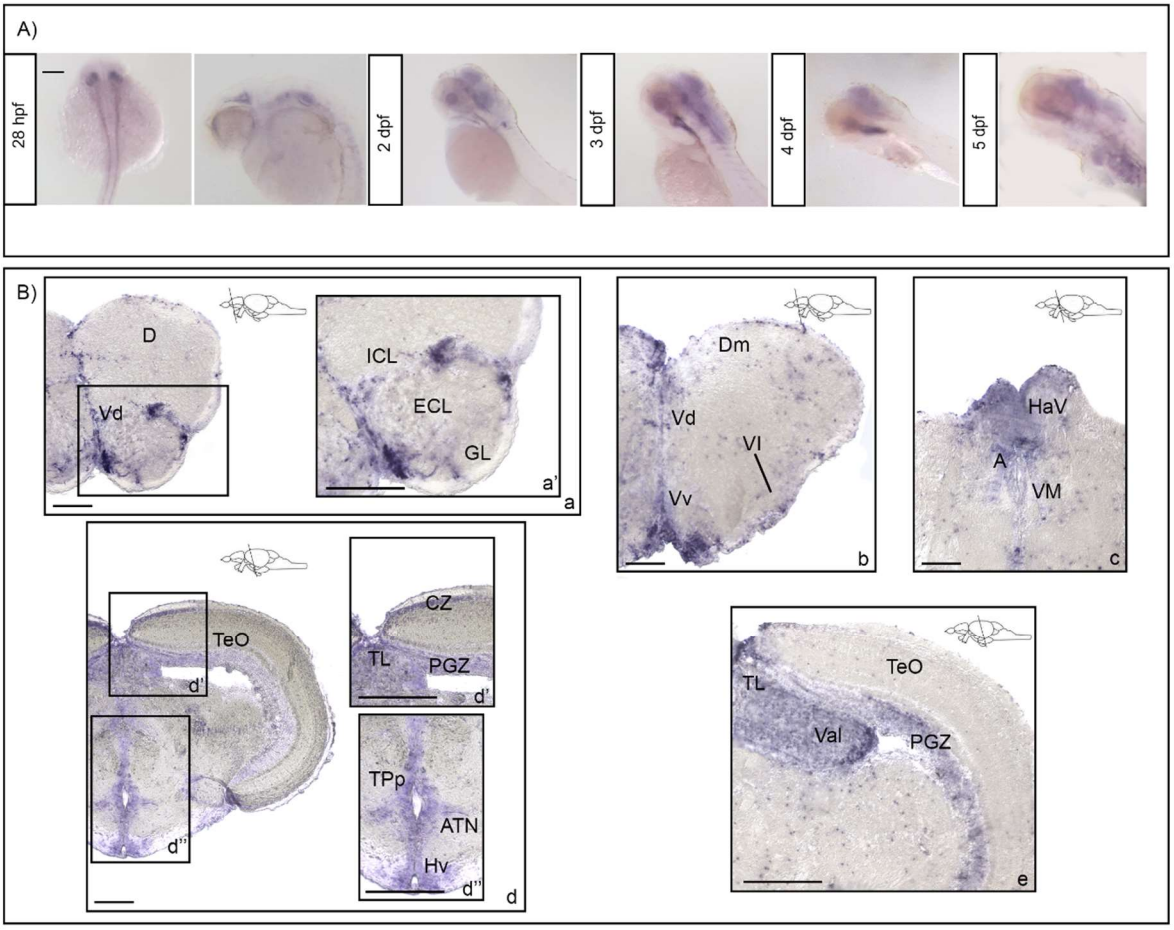
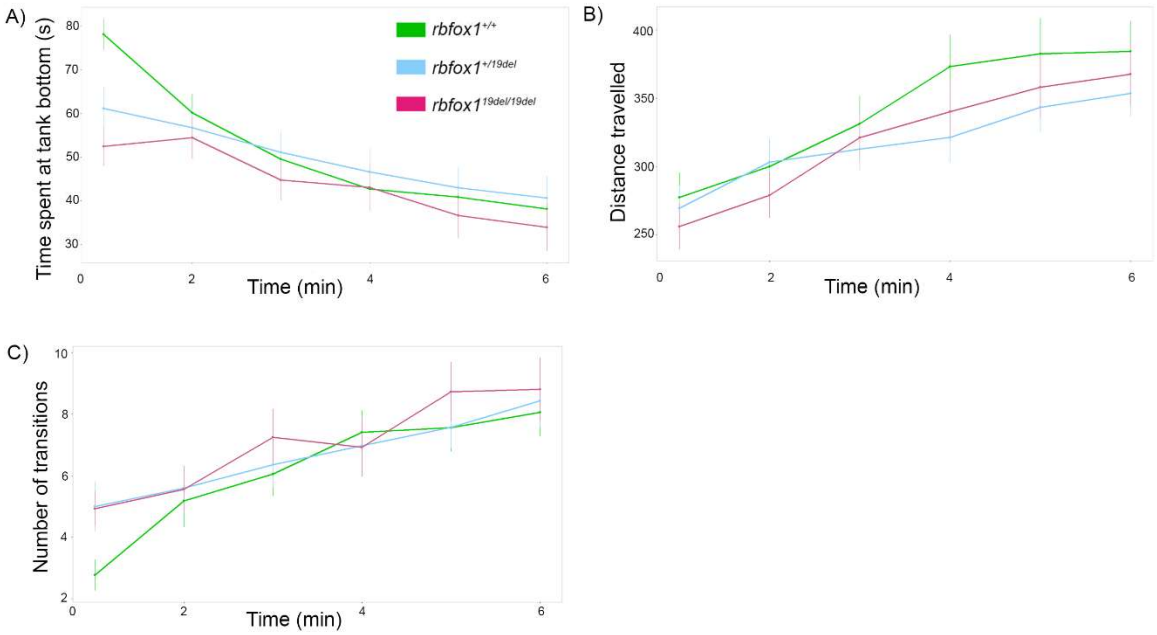
934 **Supplementary Figure 1. Generation of a *rbfox1* loss of function line. A)** *rbfox1* mRNA expression
935 in *rbfox1*$^{+/+}$, *rbfox1*$^{+/19del}$ and *rbfox1*$^{19del/19del}$ 5 days post fertilisation (dpf) larvae. *In situ* hybridisation
936 on **B)** whole mount zebrafish larvae at 3dpf, a) *rbfox1*$^{+/+}$ and b) *rbfox1*$^{19del/19del}$ and **C)** adult transverse
937 sections of *rbfox1*$^{+/+}$ (a) fore- and (c) mid- brain and *rbfox1*$^{19del/19del}$ (b) fore- and (d) mid-brain. In A)
938 each dot/square/triangle represents a pool of *rbfox1*$^{+/+}$ or *rbfox1*$^{19del/19del}$ 15 larval heads (eyes and jaw
939 removed) respectively. For qPCR experiments, reference genes were actin – β 2 (*actb2*) and ribosomal
940 protein L13a (*rpl13a*). All larvae employed were progeny of *rbfox1*$^{+/19del}$ in-cross and were genotyped
941 after experiments and prior to data analysis. In A): bars represent standard error of the mean (SEM);
942 * $p < 0.05$; ** $p < 0.01$. Scale bars: 200 µm in B); 100 µm in C). Abbreviations: D, dorsal area of dorsal
943 telencephalon; Hv, ventral hypothalamus; TeO, optic tectum; Tl, longitudinal tori; Vd, ventral area of
944 dorsal telencephalon; Vv, ventral area of ventral telencephalon.



Supplementary Figure 2. *rbfox1* is expressed in regions of the brain associated with 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. Abbreviations: A, anterior tuberal nucleus; ATN, anterior thalamic nucleus; CZ, central zone of the TeO; D, dorsal area of dorsal telencephalon; Dm, dorsal area of medial telencephalon; ECL, external cellular layer; GL, glomerular layer; HaV, ventral area of the habenula; Hv, ventral hypothalamus; ICL, internal cellular layer; PGZ, periventricular gray zone; TeO, optic tectum; TL, longitudinal tori; TPp, periventricular nucleus of the posterior tuberculum; Val, valvula cerebelli; Vd, ventral area of dorsal telencephalon; Vl, central area of lateral telencephalon; VM, ventromedial nucleus; Vv, ventral area of ventral telencephalon.



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

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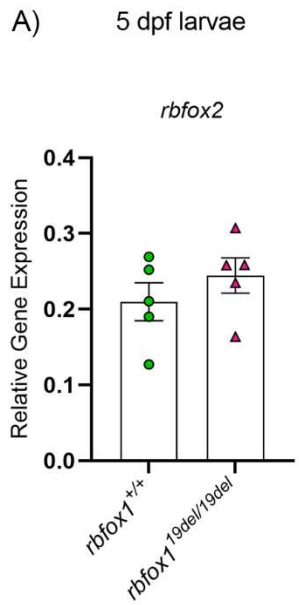

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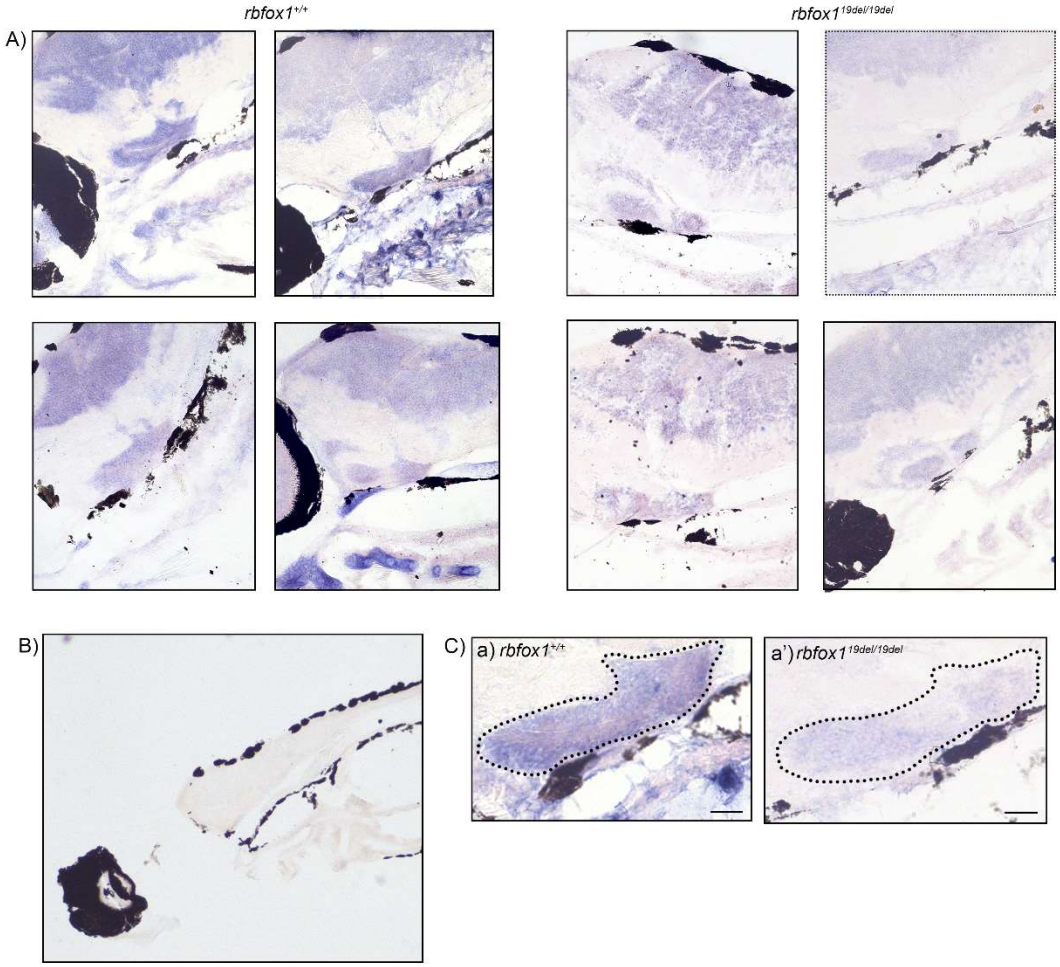
967 **Supplementary Figure 4. A) *rbfox2* expression levels in 5 days old larvae, *rbfox1*$^{+/+}$ and**
968 ***rbfox1*$^{19del/19del}$**. Actin – β 2 (*actb2*) and ribosomal protein L13a (*rpl13a*) were used as reference genes.
969 Dots/triangles represent pool of 15 larval heads (eyes and jaw removed). Bars represent standard
970 error of the mean (SEM). N = *rbfox1*$^{+/+}$ 5; *rbfox1*$^{19del/19del}$ 5.



Supplementary Figure 5. Details of *in situ* hybridisation experiments for *trkb2* anti-sense riboprobe on 5 days post fertilisation zebrafish larvae, sagittal sections. **A)** Overview of larval sagittal sections, *rbfox1*$^{+/+}$ (four panels on the left) and *rbfox1*$^{19del/19del}$ (four panels on the right). Each panel corresponds to a different animal. N = 4 per genotype. Sections have been placed on the same slide and were stained under the same conditions. **B)** *In situ* hybridisation for *trkb2* sense riboprobe on 5 days post fertilisation zebrafish larva, sagittal section. **C)** Example of areas selected (areas enclosed by the black dotted lines) to calculate *trkb2* mean intensity in the hypothalamus of zebrafish *rbfox1*$^{+/+}$ (left panel) and *rbfox1*$^{19del/19del}$ (right panel) larval sections.

990 **Supplementary Table 1.** List of primers used for qPCR experiments.
991

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: AACAGCATACAGGCGGTCTC	110
			Reverse: GGCTGCGTTTTGATTTCTGT	
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.TK+/TK-*	NM_001197161.2	Forward: CGCATACCTGACGTGGATCA	130
			Reverse: AAGCAACCCCAGCTATTCCC	
neurotrophic tyrosine kinase, receptor, type 2b (tyrosine kinase domain primers)	*trkb2.TK+*	NM_001197161.2	Forward: TGAAAGTGGGCGAGCAGATT	173
			Reverse: CATCTGGACCATGAGCCCTG	
adenylate cyclase activating	*pac1a-hop*	NM_001142926.1	Forward: CAGAAATGCTTCAGTGAGCCC	82

polypeptide 1a (pituitary) receptor type I (adcyap1r1a) transcript variants 1 and 2		NM_001013444.2	Reverse: CCAGACGTAGCGTGATGGTA	
adenylate cyclase activating polypeptide 1a (pituitary) receptor type I (adcyap1r1a), transcript variant 3	*pac1a-short*	NM_001142925.1	Forward: GATTCTGGCTGCTGGGACAT Reverse: GCGCGCCAGACGTAGATAAA	183
RNA binding fox-1 homolog 2	*rbfox2*	NM_214746.2	Forward: CCGTATGCTAACGGGGACAG Reverse: ACCCTGGAACTGCGTAGAAC	106

* These primers amplify all *Danio rerio rbfox1* isoforms available on NCBI: XM_068216713.1, XM_068216714.1, XM_017354041.3, XM_068216717.1, XM_068216718.1, XM_017354043.3, XM_017354044.3, XM_068216722.1, XM_017354045.3, XM_068216724.1, XM_068216725.1, XM_068216726.1, XM_068216728.1, XM_017354049.3 XM_068216731.1.

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 is released. All fish are moved to the next stage of training after 1 week.
Stage 2	Initiator training	Initiator 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. The initiator training is repeated for 30 trials. 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 whilst the light is on, 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. The stimulus light training is repeated for 30 trials. Fish move to the next stage when

		both 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. The 5-CSRTT/no-delay stage is repeated for 30 trials. Fish move to the next stage when the average of both initiated trials to total trials and correct responses to initiated trials are > 50%.
Stage 5	5-CSRTT/variable-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 variable pre-stimulus interval of 0-8 sec, one of five choice lights is illuminated for 20 sec. This is followed by 10 sec ITI. If during the pre-stimulus interval, the fish swims to any unilluminated choice area, a premature response is recorded. The average of premature responses to initiated trials over four days is reported as a measure of impulsive action.

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