Splice-specific deficiency of the PTSD-associated gene PAC1 leads to a paradoxical age-dependent stress behavior

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Abstract

The pituitary adenylate cyclase-activating polypeptide receptor (PAC1, also known as ADCYAP1R1) is associated with post-traumatic stress disorder and modulation of stress response in general. Alternative splicing of PAC1 results in multiple gene products, which differ in their mode of signalling and tissue distribution. However, the roles of distinct splice variants in the regulation of stress behavior is poorly understood. Alternative splicing of a short exon, which is known as the "hop cassette", occurs during brain development and in response to stressful challenges. To examine the function of this variant, we generated a splice-specific zebrafish mutant lacking the hop cassette, which we designated '*hopless'*. We show that *hopless* mutant larvae display increased anxiety-like behavior, including reduced dark exploration and impaired habituation to dark exposure. Conversely, adult *hopless* mutants displayed superior ability to rebound from an acute stressor, as they exhibited reduced anxiety-like responses to an ensuing novelty stress. We propose that the developmental loss of a specific PAC1 splice variant mimics prolonged mild stress exposure, which in the long term, predisposes the organism's stress response towards a resilient phenotype. Our study presents a unique genetic model demonstrating how early-life state of anxiety paradoxically correlates with reduced stress susceptibility in adulthood.

53 **Introduction**

54 PAC1 (a.k.a. Adcyap1r1) is a G-protein coupled receptor (GPCR) that serves as the
55 high-affinity receptor for pituitary adenylate cyclase-activating polypeptide (PACAP).
56 PAC1 has pleiotropic functions and was demonstrated to be involved in the regulation
57 of several homeostatic processes including metabolic rate and food consumption[1,2],
58 circadian rhythm[3] and, in particular, stress response[4,5]. Intracerebroventricular injection
59 of PACAP increased phosphorylated cyclic AMP response element binding protein
60 (pCREB) and CRH immunoreactivity in the rat paraventricular nucleus[6]. PACAP
61 knockout mice display blunted hypothalamic CRH levels in response to restraint
62 challenge[7]. PACAP/PAC1 signaling was also associated with hypothalamo-pituitary-
63 adrenal activity and stress-related behaviors in humans and rodents[5,8,9]. Furthermore,
64 this pathway was correlated with stress-related risky behaviors in human and
65 rodents[10,11]. Overall, these findings support positive stress regulation by PAC1; yet,
66 some data suggest that it may also act to suppress stress phenotypes[4,12].

67 It has been suggested that genetic vulnerability to post-traumatic stress disorder
68 (PTSD) may depend on PAC1 expression and single-nucleotide polymorphism (SNP)
69 in the PAC1 gene. Ressler et al. demonstrated that a specific PAC1 genotype is strongly
70 correlated with susceptibility to PTSD in women, probably due to perturbed expression
71 of PAC1 resulting in impaired stress responses[13]. The same PAC1 SNP was associated
72 with PTSD in African-American females, emotional numbing in traumatized
73 earthquake Chinese survivors, dark-enhanced startle response in children[14-16], and with
74 impaired hippocampal and amygdalar activation in response to fearful stimuli and
75 contextual fear conditioning in non-traumatized individuals[17,18]. Moreover, PTSD was
76 correlated with altered expression of PAC1 in the human cortex[13].

77 These accumulating data have associated PAC1 to PTSD based on SNPs in the
78 PAC1 gene promoter, global PAC1 expression or both. However, PAC1 mRNA
79 undergoes extensive alternative splicing, resulting in the generation of several protein
80 isoforms that differ in their ligand affinity and signal transduction cascades[19-21]. There
81 are at least 17 known PAC1 splice isoforms. The predominant brain isoforms are
82 PAC1-hop, which contains an alternatively spliced short exon termed the "hop
83 cassette", and PAC1-short that lacks it[22-24]. PAC1-hop encodes 28 amino acids of the
84 third intracellular loop of this GPCR. PAC1-hop is expressed in neuroendocrine cells

85 in mammals and it is widely expressed in the brain and gonad tissues[21,22,25]. The splicing
86 of the hop cassette occurs during brain development and in response to stressful
87 challenges. However, the specific roles of distinct splice variants in the regulation of
88 stress-related behaviors are yet to be uncovered.

89 Neuroendocrine systems mediating homeostatic response to stressful challenges are
90 well conserved between mammals and fish; indeed, PAC1 splice variants have been
91 identified in zebrafish[21,26]. The zebrafish genome contains two PAC1 paralogs, namely
92 *pac1a* and *pac1b;* however, only the *pac1a* gene contains the hop exon. We have
93 previously demonstrated that alternative splicing of PAC1 is induced by an acute stress
94 challenge to mice, which suggested that the ratio between PAC1-hop and PAC1-null
95 isoforms may be involved in the adaptive response to stress[4]. We further demonstrated
96 that zebrafish larvae injected with an antisense oligonucleotide that prevents the
97 inclusion of the hop cassette displayed prolonged increase of *corticotropin-releasing*
98 *hormone* (*crh*) transcription and impaired anxiety-like dark avoidance behavior[4]. In the
99 present study, we examined the short- and long-term behavioral consequences of this
100 splice-specific PAC1 deficiency by generating a germline-transmitted zebrafish mutant
101 lacking the hop cassette, which we designated '*hopless'*. We show that while *hopless*
102 larvae display increased anxiety-like and stress related behaviors, adult *hopless* mutants
103 exhibit increased resilience to acute stress. These findings suggest that PAC1-hop splice
104 isoform is involved in the developmental establishment of stress responsiveness.

105 **Results**

106 **Generation of zebrafish mutant with germline deletion of the hop-coding exon**

107 As mentioned, while PAC1 is known to be involved in modulation of stress
108 response, the functions of each of its many splice variants are poorly understood. To
109 examine the specific role of PAC1-hop splice variant, we have generated a zebrafish
110 mutant that harbors a deletion of the alternatively-spliced hop exon of *pac1a* gene (**Fig.**
111 **1A**). To generate this mutant, we used the CRISPR/Cas9 system to simultaneously
112 target two intragenic sites around exon 10 of *pac1a* (**Fig. 1A**). To avoid introduction of
113 a phantom exon due to partial intronic retention, gRNA binding sites were designed at
114 least 600 bp upstream and downstream to the hop cassette, thus including its predicted
115 cis-regulatory splice sites. Mosaic F_0 fish identified for carrying large genomic

116 deletions were outbred with *Tüpfel long fin* (*TL*) wild-type (WT) strain. Their offspring
117 were screened for individuals carrying at least 1,600 bp deletion in their genomic DNA.
118 Four individual F_1 fish were identified as carriers of the same genomic ~1,600 bp indel.
119 The resulting germline-transmitting mutant allele lacked the *hop* exon (**Fig. 1B**) as did
120 the *pac1a* mRNA gene product (**Fig. 1C**); hence, we named this mutant '*hopless*'.
121 Sequencing of *pac1a* cDNA that was derived from the *hopless* mutant line confirmed
122 the in-frame deletion of exon 10 in the mature mRNA gene product, without perturbing
123 the amino acid reading frame of the flanking exons 9 and 11.

124 Finally, homozygous *hopless* fish were viable and fertile and displayed no detectable
125 morphological abnormalities compared to their WT siblings. Therefore, the generated
126 mutant allowed us to assess the behavioral effects of the deleted *hop* exon in both larval
127 and adult stages.

128 ***hopless* mutant larvae display heightened anxiety-like behaviors**

129 To measure the behavioral effect of *hop* exon deficiency, we first used the light-dark
130 preference test (**Fig. 2A**). In this assay, a larvae that is placed in a half lit, half dark
131 arena displays a strong preference to the lit region[4,27]. The time spent in the dark is
132 increased following exposure to anxiolytic drugs and, therefore, this behavioral assay
133 is considered a measure of larval anxiety-like state[4,27]. We performed the test on 6 days
134 post-fertilization (dpf) *hopless* larvae and genetically matched WT larvae. As
135 previously demonstrated for larvae exposed to the anxiogenic caffeine[27], we found that
136 *hopless* larvae displayed significant reduction in the time spent in the dark zone, as well
137 as in the number of entries to the dark zone (**Fig. 2B,C**). The observed phenotype was
138 not due to locomotion defects, as the distance and speed of swimming of *hopless* larvae
139 was similar to those of WT larvae throughout the test (**Fig. 2D,E**).

140 We next performed the light-dark transition assay, which measures the locomotion
141 response of larvae to a sequence of sudden transitions between bright light and total
142 darkness. Larvae tend to respond to sudden darkness by a short burst of vigorous
143 swimming, the intensity and duration of which are increased under stressful
144 conditions[28]. We analyzed larval locomotion using a 30 minute dark/light alternating
145 periods[29], as we found that this interval enables complete rebound from the stressful
146 transitions (**Fig. 3A**). Results showed that immediately after light-to-dark transitions,

147 WT larvae displayed darting behavior followed by a gradual decrease in locomotion,
148 presumably due to acclimatization to the darkened environment (**Fig. 3A**). Conversely,
149 dark-to-light transitions induced a short freezing episode, which was followed by a
150 locomotion rebound (**Fig. 3A**). Moreover, the intensity of larval locomotion response
151 to the above transitions was reduced during repeated light-dark cycles, indicating that
152 they habituated to the sudden changes in the environment. To test whether the light-to-
153 dark transition measures anxiety-like response, WT larvae were treated with diazepam,
154 which was shown to be anxiolytic in both larvae and adult zebrafish[27,30]. Diazepam-
155 treated larvae displayed reduced darting activity during the transition from light to dark,
156 suggesting that light-to-dark sensory challenge is anxiogenic (**Fig. 3F,G**).

157 In contrast to the behavioral pattern observed in WT fish, *hopless* larvae failed to
158 habituate to repeated light-to-dark transitions, as their darting response did not change
159 between the first and third transitions (**Fig. 3B**). Interestingly, their habituation to
160 sudden dark-to-light transitions was similar to that of the control, suggesting that this
161 challenge may be less stressful to the larvae (**Fig. 3C**). Finally, we observed that *hopless*
162 larvae exhibited heightened locomotion in both light and dark phases. This may indicate
163 an impaired response of this mutant to the novel environment of the behavioral arena
164 (**Fig. 3D,E**).

165 Taken together, our results indicate that *hopless* mutant larvae, which lack the
166 alternatively spliced *hop* exon, display heightened anxiety-like responses to sensory-
167 based light-dark stress stimuli.

168 **Adult *hopless* zebrafish display impaired behavioral response to novel**
169 **environment**

170 To assess whether the stress-related behavioral phenotypes of the *hopless* mutant
171 persist to adulthood, we utilized the novel tank diving and open field assays. When
172 adult zebrafish are introduced to a novel tank, they initially dive to the bottom half of
173 the tank and, over time, they gradually begin to explore its top half[31]. This stereotypical
174 behavior is commonly used to measure stress and anxiety-like response of zebrafish to
175 a novel environment[32,33]. When we introduced adult WT or *hopless* zebrafish to the
176 novel tank arena with no prior stressful challenge, both genotypes displayed similar
177 behaviors, as reflected by similar swimming velocity and distance (Fig. 4A,B), as well

as by the similar number of entries and time spent in the top half of the tank (**Fig. 4C,D**). Likewise, no significant differences between WT and *hopless* zebrafish were found in open field behavioral parameters, including locomotion, place preference towards center versus the walls, and freezing, which are also considered anxiety-like responses (**Supp. Fig. 2**). These results indicate that naïve adult *hopless* mutants do not display differential response in novelty-based behavioral assays.

We next examined the behavioral responses of *hopless* adults in novelty-based assays following an acute stressful challenge, namely a short restraint in a confined space. Wild-type and *hopless* siblings were restrained for 2 minutes in a 50 ml conical tube containing 5 ml of water, such that the fish was covered with water allowing normal breathing, but in a highly confined space. Thereafter, both genotypes were analyzed in the novel tank diving and open field assays. Novel tank diving assay duration was extended to 14 minutes, in order to allow the restrained fish to recover from the stressful restraint challenge.

When placed in the novel tank arena, stressed fish of both genotypes covered the same distance with a similar swim velocity (**Fig. 5A,B**). However, *hopless* mutants displayed more entries to the top half of the tank, indicative of reduced stress behavior (**Fig. 5C**). *hopless* mutants also spent more time and covered more distance in the top half of the arena (**Fig. 5D-F**). When analyzed in the open field arena, stressed *hopless* mutants covered more distance with higher average velocity than stressed WT zebrafish (**Fig. 6A,B**). This effect was probably due to the reduced freezing of *hopless* mutants (**Fig. 6C**). Although no significant difference was found in the time spent in the center of the arena, *hopless* mutants performed significantly more visits to the center and showed a tendency to spend less time per visit, indicative of increased arena exploration (**Fig. 6D-F**). It is noteworthy that following restraint, WT fish froze also in the center of the arena (data not shown), which may explain, at least in part, the lack of difference in time spent in the center. We conclude that adult *hopless* mutants display improved behavioral recovery following an acute stress, suggesting that they are more resilient to stressful challenges.

Discussion

PAC1 is an important player in the regulation of physiological and behavioral stress responses. Perturbation of its expression was associated with PTSD and impaired stress responses[34,35]. Yet, although PAC1 undergoes extensive alternative splicing, the specific roles of its isoforms in the regulation of stress response have not been elucidated. In the present study, we have generated a unique genetic model, which lacks the *hop* exon, and used this mutant to examine the role of PAC1-hop splice variant in the regulation of stress-related behaviors throughout life. Our results revealed an intriguing phenomenon of an age-dependent switch in stress-related behaviors. As larvae, *hopless* mutants display increased anxiety-like responses. Conversely, adult *hopless* fish rebounded better from a major stressor (restraint), displaying reduced anxiety-like responses to a subsequent milder stressor (introduction into novel environment), which indicates improved resilience to stress during adulthood. We suggest that the developmental loss of a specific PAC1 splice variant mimics a heightened anxiety state that, in the long-term, renders an improved ability to cope with stressful challenges.

PAC1 splice isoforms are known to mediate distinct signal transduction pathways due to differential coupling to G-proteins[21,36,37]. The PAC1-null isoform, which does not contain the hop exon, displays very low or no activation of PLCβ, whereas PAC1-hop1 switches its mode of signaling from AC to PLCβ[38-42]. PAC1-hop1 was also shown to regulate Ca^{2+} mobilization and neurosecretion[43-46]. Such differential signal transduction could affect behavioral phenotypes, as previously demonstrated for other GPCRs[47-50]. Accordingly, we have previously shown in adult mice that acute foot-shock stressor induces a change in the ratio between PAC1-hop and PAC1-null splice isoforms in the hypothalamic paraventricular nucleus[4].

Using an antisense morpholino oligonucleotide in larval fish, we also showed that interference with PAC1a-hop alternative splicing leads to impaired *crh* transcriptional dynamics, as well as adaptation to a stressful challenge[4]. However, due to the transient nature of this gene-knockdown approach, the long-term consequences of developmental deficiency of PAC1-hop on adult stress responses were not examined. In the present study, we utilized the CRISPR/Cas9 method to generate a germline

240 mutation resulting in the loss of PAC1-hop isoform mRNA without affecting the
241 generation of the other PAC1 isoforms, allowing functional investigation of PAC1-hop.

242 Early life stress exposure may have negative or positive impact on adult
243 psychological well-being. For example, in humans early life stress is known to increase
244 the risk for psychopathologies in adulthood[51,52]. In particular, PTSD was recently
245 correlated with childhood anxiety of both military and civilian men[52]. Nonetheless,
246 more than 60 years ago, Levine demonstrated that mild stress exposure can lead to an
247 inoculation effect in the adult rat, making it more resilient to stressors[53]. These findings
248 were later validated in mice and squirrel monkeys[54,55]. In line with this notion, in our
249 study *hopless* larvae exhibited increased anxiety-like behavior in the light-dark
250 preference test and reduced habituation to repeated light-to-dark transitions. However,
251 pre-challenged adult mutants displayed a blunted stress response of increased
252 exploration and reduced freezing in both novel tank diving and open field assays,
253 indicating enhanced resilience to novelty stressors.

254 As mentioned above, in various animal models, PAC1 signaling has been mainly
255 associated with activation of physiological and behavioral stress responses, including
256 PTSD[10,13,56-58]. Infusion of PACAP, the high-affinity PAC1 ligand, to the bed nucleus
257 of stria terminalis (BNST) potentiated anxiogenic response in pre-stressed but not in
258 naïve male rats, which was likely conveyed by PAC1 signaling[9,59]. PAC1-KO mice
259 display blunted elevation in corticosterone following acute restraint stress, but not
260 under naïve conditions[5]. However, our current study highlights the need for better
261 understanding of the physiological and psychological functions of specific PAC1 splice
262 isoforms. Generation of additional site- and splice-specific models is needed to further
263 elucidate mechanisms underlying splicing-based regulation of the stress response.

264 Although the underlying mechanism for the switch in stress responsiveness of
265 *hopless* mutants studied herein is still unknown, some studies may provide hints. A
266 dynamic shift in the ratio of PAC1-hop and PAC1-short isoforms was shown to regulate
267 pro- and anti-mitogenic activity of PACAP in the developing brain[60]. The ratio of
268 PAC1-hop2 mRNA to other PAC1 variants in the ventromedial nucleus of the
269 hypothalamus of female rats appears to be dynamic, depending on different steroid
270 environments[61]. This study suggests that steroid-regulated changes in the expression of
271 PAC1 splice variant in the hypothalamus contribute to the effect of PACAP on female

272 receptivity[61,62]. We have previously suggested that alternative splicing of the hop
273 cassette serves as an ON/OFF stress switch[21], in line with our finding that stress-
274 induced alternative splicing of PAC1-hop is involved in the termination of *crh*
275 transcription and adaptive anxiety-like larval behavior. Larvae with depleted PAC1-
276 hop1 display delayed dark-avoidance recovery following a stressful challenge
277 response[4].

278 Taken together, our findings suggest that PAC1-hop is involved in the
279 developmental establishment of stress responsiveness. Thus, in our current study,
280 *hopless* larvae likely experienced an impaired ability to terminate their stress response,
281 which presumably led to prolonged and possibly heightened anxiety-like states during
282 early life stages. These states could occur in response to any of the very mild stressful
283 events that the animals experience during rearing in an enclosed laboratory setting, for
284 example, the sudden daily switch from dark to light, encounter with human caretakers,
285 tank cleaning, etc. We submit that in the case of the *hopless* mutant, this early life state
286 of anxiety paradoxically correlates with reduced stress susceptibility in adulthood,
287 reminiscent to the early-life stress inoculation proposed by Levin [53] and others.

288

289 **Materials and methods**

290 **Animal care and maintenance**

291 Zebrafish were bred and reared at 28.5°C under 14 h/10 h light/dark cycle, according
292 to standard protocols. Embryos were raised at 28.5°C in 30% Danieau's medium (0.17
293 mM NaCl, 0.21 mM KCl, 0.12 mM $MgSO_4$, 0.18 mM $Ca(NO_3)_2$, 0.15 mM HEPES, pH
294 7.4) supplemented with 0.01 mg/L methylene blue. All experimental procedures were
295 approved by and conducted in accordance with the Weizmann Institute's Institutional
296 Animal Care and Use Committee (IACUC).

297 **Genome editing using CRISPR/Cas9**

298 To generate the *hopless*^{wz18} allele (ZFIN ID: ZDB-ALT-191028-3) we employed
299 genome editing as previously described [63,64], with slight modifications. Cas9 protein
300 was produced by the Weizmann Institute of Science Protein Purification Unit using the

301 pET-28b-Cas9-His (Alex Schier Lab Plasmids, Addgene, Cambridge, MA, United
302 States) as a template. CRISPR sgRNAs (Supplementary Table S1) were designed using
303 CHOPCHOP [65]. Oligonucleotide containing the SP6 promoter sequence upstream of
304 specific target sites was annealed with a constant oligonucleotide bearing Cas9 binding
305 site. sgRNA were generated by *in vitro* transcription using a SP6 RNA polymerase
306 MEGA script SP6 kit (Life Technologies, United States) and purified using miRNeasy
307 kit (Qiagen, Germantown, MD, United States). Cas9 protein (600 ng) and sgRNAs
308 (1500-2000 ng) were co-injected to *Tupfel long fin* (TL) zygotes at the one-cell stage.
309 PCR analysis of ten embryos was performed to evaluate genomic mutations and
310 occurrence of large deletions induced by multiple gRNAs injection. Injected siblings
311 were raised to adulthood and screened for large deletions in genomic-DNA (gDNA)
312 extracted from tail clips. Fish that were found to be positive for large genomic deletions
313 were crossed with TL zebrafish to identify germline transmission. Germline
314 heterozygotes were genotyped using Sanger sequencing and fish carrying the *hopless*
315 allele were crossed to generate homozygous mutants.

316 **DNA extraction and genotyping**

317 gDNA was extracted from tail fin clips of adult fish or whole embryo at 24 hours
318 post-fertilization. DNA was extracted according to [66]. Briefly, samples were digested
319 at 50°C with 1% SDS and proteinase K solution (20 mg/ml). Protein precipitation was
320 performed by adding 5 M NaCl solution and centrifugation. Supernatant was
321 transferred to a clean tube, followed by DNA precipitation using ice-cold isopropanol.
322 DNA pellet was isolated by centrifugation and washed with 70% ethanol. DNA pellets
323 were air-dried, dissolved in 50 µl double distilled water and stored at -20°C until further
324 analysis. The genomic region of *hop* cassette was amplified by using Taq DNA
325 Polymerase Master Mix Red 2x reaction mix (Ampliqon, Odense, Denmark).
326 Amplified DNA region was detected by 1-1.5% agarose gel electrophoresis, then
327 extracted and purified by using the NucleoSpin® Gel and PCR Clean-up (Macherey-
328 Nagel, Düren, Germany). Purified DNA fragments were sequenced by the Biological
329 Services Unit at the Weizmann Institute of Science. Results were analyzed by ApE-A
330 software (version 2.0, by M. Wayne Davis, University of Utah). See
331 Supplementary Table S1 for oligonucleotide sequences.
332

333 **Light/dark preference test**

334 Anxiety-like behavior of zebrafish larvae was measured by recording the preference
335 of 6-day-old zebrafish larvae for either side of a custom-made light-dark arena. The test
336 was generally performed according to [4] with slight modifications. Briefly, larval
337 activity was recorded for a 10 min time period by high resolution infrared video camera
338 (Flare 2M360-CL, I/O industries, London Ontario), with an image acquisition Sapera
339 LT-development package (Teledyne Dalsa, Waterloo, Ontario) and recorded with
340 Stream5 software (IO Industries, London, Ontario). The resulting videos were analyzed
341 using EthoVision XT ((Noldus Information Technology, Wageningen, Netherlands).
342 *Hopless* larvae and matched TL control larvae were randomly assigned into separate
343 arenas with bottom lighting. Each arena was divided into two equal compartments in
344 which the top, bottom and sides of the dark half were covered with Optical Cast Infrared
345 (IR) Longpass Filters, allowing penetration of infrared light (Edmund Optics,
346 Barrington, NJ, USA). The total distance swum in the whole arena and in each
347 compartment was calculated and compared for each genotype. Animals that stopped
348 swimming for periods of 60 s or more were removed from the analysis.

349 **Larval light/dark transitions test**

350 Larval responses to sudden transitions from light to dark and from dark to light were
351 monitored using the Noldus DanioVision tracking system (Noldus Information
352 Technology, Wageningen, Netherlands), as previously described [29]. For each
353 experiment, individual larvae were placed in 48-well plates, which were put in the
354 DanioVision system with light on for 1 h prior to the beginning of the trial. Larvae were
355 subjected to three cycles of 30 min light/30 min dark followed by 10 min light. The
356 experiment was repeated three times, each with a new batch of 6 dpf *hopless* and TL
357 larvae. Total swimming distance and velocity were calculated using EthoVision XT
358 Software (Noldus Information Technology). For the diazepam treatment experiment,
359 larvae were exposed to 5 μM diazepam (Renaudin, France) for 2 h prior to the start of
360 the behavioral testing. Control larvae were kept in similar conditions during this period.
361 At the end of the incubation, larvae were transferred into individual wells of a 48-well
362 plate and subjected to 30 min light/30 min dark cycles as described above.
363

364 **Open field**

365 Behavioral phenotyping in open field arena was performed using a custom-made
366 apparatus, as was previously described [67]. Fish were placed in a circular arena of 23 cm
367 in diameter filled to a height of 5 cm with regular system water. The arena was placed
368 in a custom-built enclosure that restricted all visual cues from the laboratory frame, and
369 was top-lit with ambient LED light. Videos were acquired with a 2M360-CL camera
370 (IO Industries, London, Ontario), with an image acquisition Sapera LT-development
371 package (Teledyne Dalsa, Waterloo, Ontario) and recorded with Stream5 software (IO
372 Industries, London, Ontario). Tracking analysis was performed using EthoVision video
373 tracking system (Noldus Information Technologies, Wageningen, Netherlands).

374 **Novel tank diving assay**

375 Novel tank diving assay was performed as was previously described [4]. The assay
376 was conducted within the same enclosure as the open field assay and using the same
377 recording equipment.

378 **Restraint challenge**

379 Mild stress was induced in adult fish by placing individual fish in a standard 50 ml
380 conical tube with 5 ml of system water for 2 min. The tube was placed in an upright
381 position such that the fish could not swim, but was completely covered with water to
382 prevent dehydration or loss of oxygen. Following the 2 min restraint, the fish were
383 introduced to the open field arena or novel tank diving arena for behavioral tracking.

384 **Statistical analysis**

385 Data are presented as mean ± standard error of the mean (SEM) and were analyzed
386 using GraphPad Prism 7.01. All data sets were tested for departures from normality
387 with Shapiro-Wilks test. Student's *t*-test was used for all comparisons between two
388 groups. 2-Way ANOVA was used for comparing multiple variables. Single asterisks
389 indicates $p<0.05$, double asterisks indicates <0.01, triple asterisks indicates $p<0.001$
390 and quadruple asterisks indicates $p<0.0001$.

391

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632 **Authors' contributions**

633 J.B. and G.L. planned the project. J.B. and M.G. and I.S. performed the behavioral
634 experiments and analyzed the data. A.S. performed the diazepam experiment. L.A.
635 and T.L.D. contributed to the initial establishment of the light-dark transition assay.
636 J.B. prepared the figures. J.B. and G.L. wrote the manuscript. All authors reviewed
637 the manuscript's text.

638

639 **Competing interests**

640 The authors declare that no competing interests exist.

641

642 **Legends to Figures**

643 **Figure 1. Establishment of *hopless*^{wz18} zebrafish mutant.** A pair of gRNAs
644 (indicated by scissors) were designed to target the upstream and downstream genomic
645 regions flanking the zebrafish hop cassette (**A**). PCR amplification of the genomic
646 hop-cassette site demonstrate a single truncated product in the mutant genome (**B**).
647 Amplification of PAC1-hop region in the zebrafish PAC1 mRNA shows loss of the
648 hop isoform in cDNA produced from 6 dpf larvae (**C**). Plus sign represents wild-type
649 allele; minus sign indicates *hopless* allele. Source images for the DNA gels
650 electrophoresis are provided in Supplementary Figure 1.

651 **Figure 2. *Hopless* larvae display increased light preference in light-dark**
652 **preference test.** Larvae were placed on the lit side of a half lit-half dark rectangular
653 arena and their behavior was recorded during 10 minutes (A). Analysis of locomotor
654 activity showed that *hopless* mutants covered significantly less distance in the dark
655 zone (**B**) and exhibited less entries into the dark (**C**) relative to WT larvae.
656 Nonetheless, 6 dpf *hopless* larvae (n=42) did not differ from WT controls (n=35) in
657 total distance swam (**D**) or average velocity (**E**). Data are presented as mean±SEM of
658 1 minute time bins. Data were analyzed using 2-way ANOVA. For all analyzed
659 parameters, a significant effect of time ($p<0.0001$) was observed. Quadruple asterisks
660 indicate significant difference between genotypes ($p<0.0001$). No significant
661 interaction was found for time*genotype.

662 **Figure 3. *Hopless* larvae display impaired stress-related behavior in light-dark**
663 **transitions assay.** Mean total locomotor activity of 6 dpf wild type (WT; n=48) and
664 *hopless* mutant (n=41) larvae was measured during 3 h of 30 min alternating light-
665 dark cycles (**A**). Mean total activity of each genotype was calculated for the middle 10
666 min interval of each phase (black squares in **A**) or as the difference in activity
667 between 5 min post-transition and 5 min pre-transition (dashed rectangles in **A**).
668 Analysis of larval activity during light-to-dark transitions revealed that WT larvae
669 gradually decreased their dark-enhanced activity with each cycle, representing
670 habituation to the challenge, whereas *hopless* larvae failed to display such habituation
671 (**B**). During the opposite dark-to-light transitions no differential responses between
672 genotypes were observed (**C**). In general, *hopless* larvae exhibited significantly
673 increased activity under both light and dark conditions (**D** and **E**, respectively). WT
674 larvae (n=24) that were treated with 5 µM diazepam displayed similar activity pattern
675 to non-treated siblings (n=24; **F**); yet, treated larvae displayed reduced dark-enhanced
676 activity (**G**). Data were analyzed using 2-way ANOVA. Single asterisks indicate
677 $p<0.05$; double asterisks indicate $p<0.01$; triple asterisks indicate $p<0.001$. No
678 significant interaction was found for time*genotype.

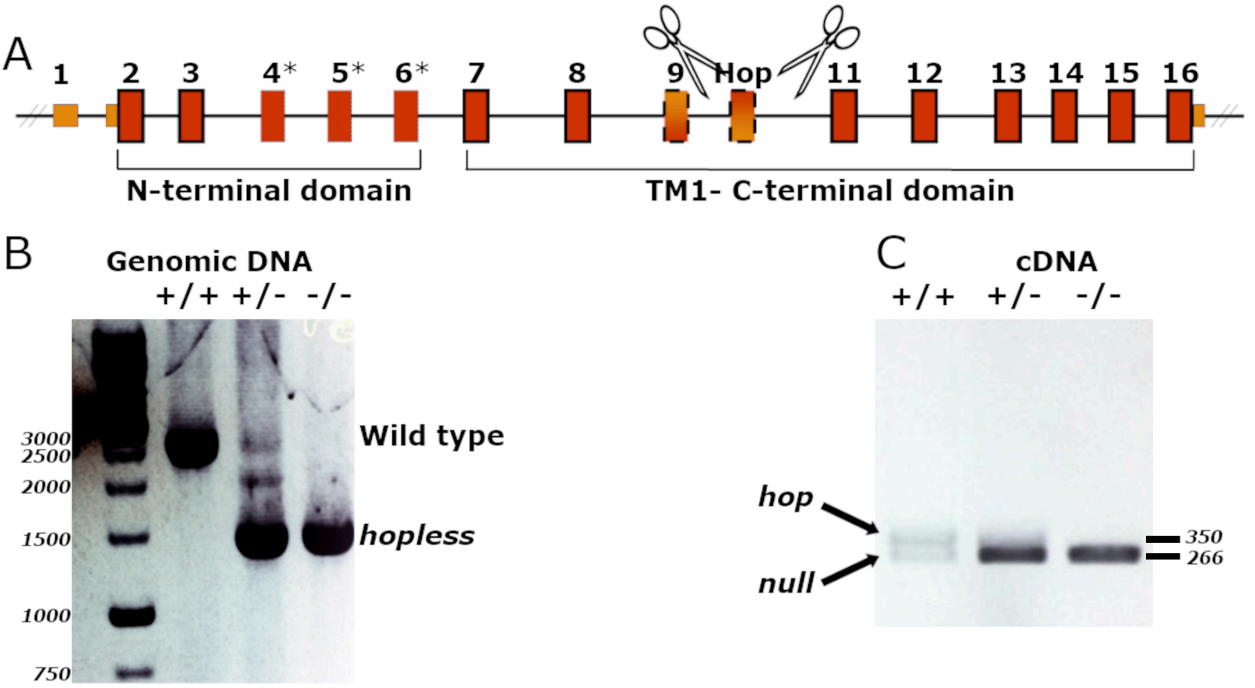
679 **Figure 4. Naïve *hopless* adults display normal behavior in novel tank diving**
680 **assay.** The behavioral response of adult *hopless* mutants (n=12) and WT siblings
681 (n=11) to a novel tank was measured . The two genotypes exhibited a similar activity
682 level, as seen in their total swimming distance swum and average swimming velocity
683 during the 10 min test (**A** and **B**). Both genotypes displayed similar exploration of the
684 top half of the tank (**C** and **D**). Data was analyzed using students-t test.

685 **Figure 5. A preceding stressful challenge leads to blunted stress-related**
686 **behavior of *hopless* adults in the novel tank assay.** The behavioral response of adult
687 *hopless* mutants (n=11) and WT siblings (n=13) to a novel tank was measured
688 following 2 min restraint in a confined space. The two genotypes exhibited similar
689 activity, as seen in their mean swimming distance and velocity (**A** and **B**).
690 Nonetheless, when subjected to a preceding restraint stressfull challenge we observed
691 significantly different behaviors between genotypes, namely that *hopless* mutants
692 entered more times to the top half of the tank, spent more time and covered more
693 distance there, even when normalized to distance swum at the bottom of the tank (**C**-

694 **F**). Data were analyzed using 2-way ANOVA. Single asterisks indicate $p<0.05$;
695 double asterisks indicate $p<0.01$; quadruple asterisks indicate $p<0.0001$.

696 **Figure 6. A preceding stressful challenge leads to blunted stress-related**
697 **behavior of adult *hopless* zebrafish in the open field assay.** Following 2 min
698 restraint in a confined space, adult *hopless* mutants (n=10) and WT siblings (n=13)
699 behavior was measured in a novel open field arena. A significant reduction is seen in
700 activity level of WT, but not of *hopless* mutant zebrafish (**A** and **B**). This effect was
701 probably due to the increased freezing of WT adults (**C**). No significant difference
702 was seen in the time spent in the center of the open field arena (**D**); however, *hopless*
703 mutants displayed significantly increased number of visits to the arena center and a
704 tendency ($p=0.09$) to spend less time in each visit to center (**E** and **F**). Data were
705 analyzed using 2-way ANOVA. Double asterisks indicate $p<0.01$; triple asterisks
706 indicate $p<0.001$; quadruple asterisks indicate $p<0.0001$.

707



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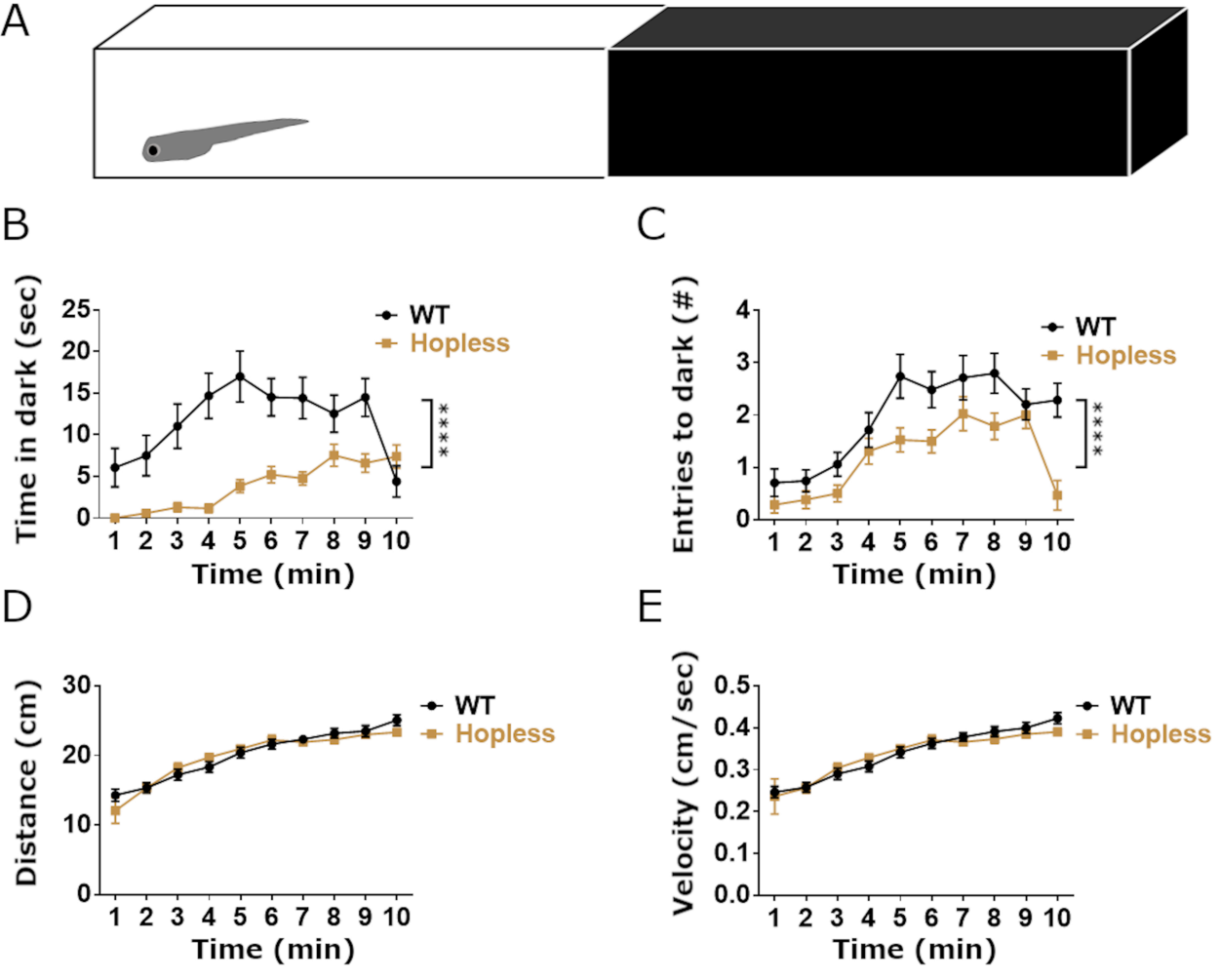

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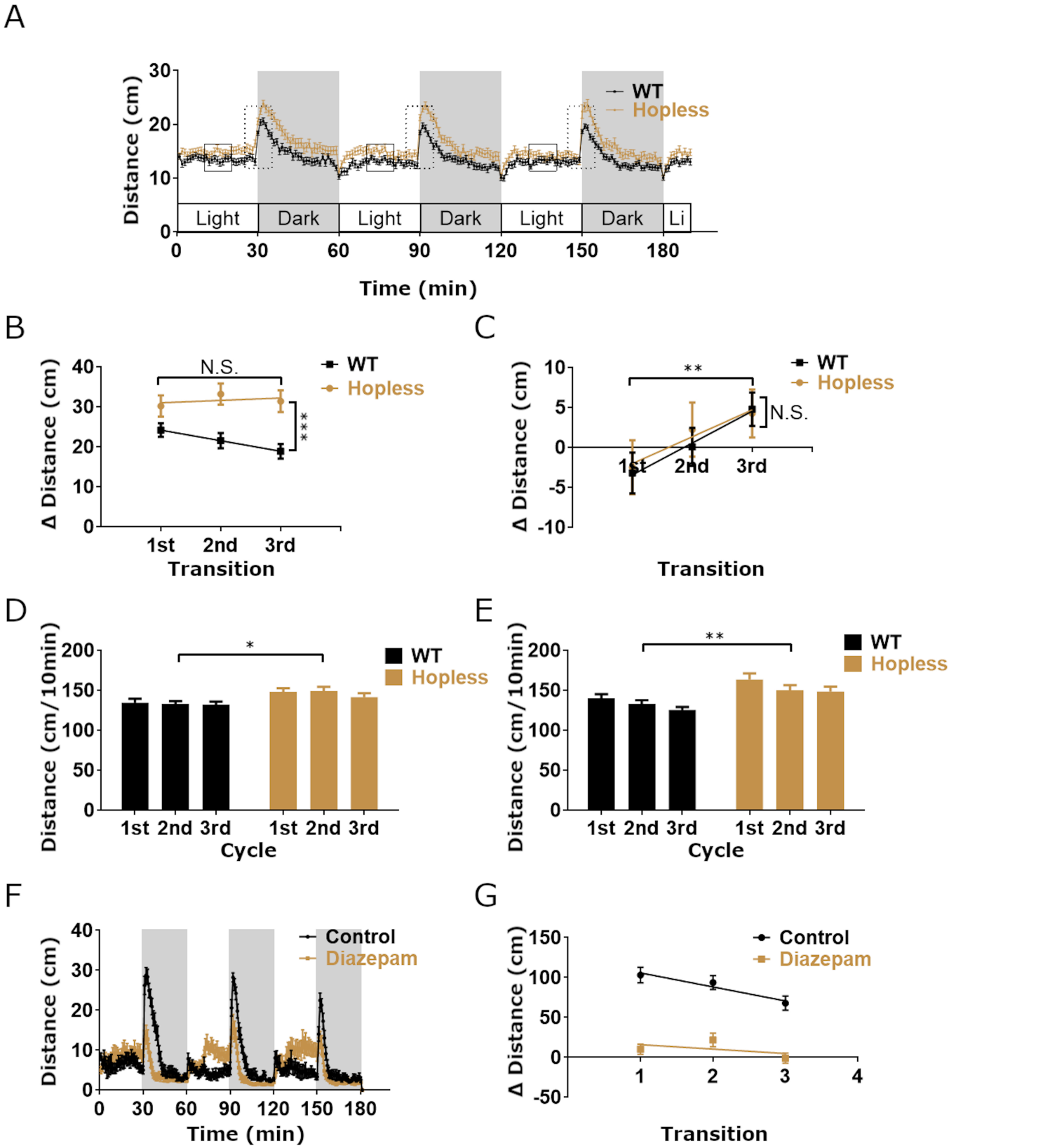

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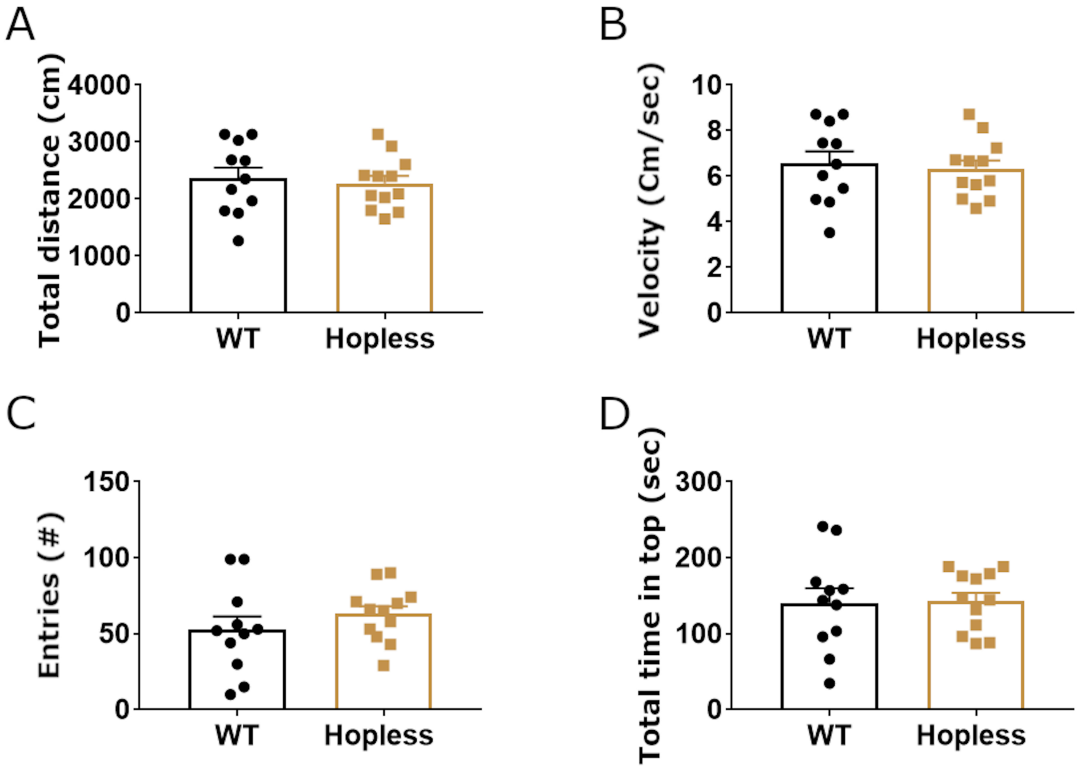

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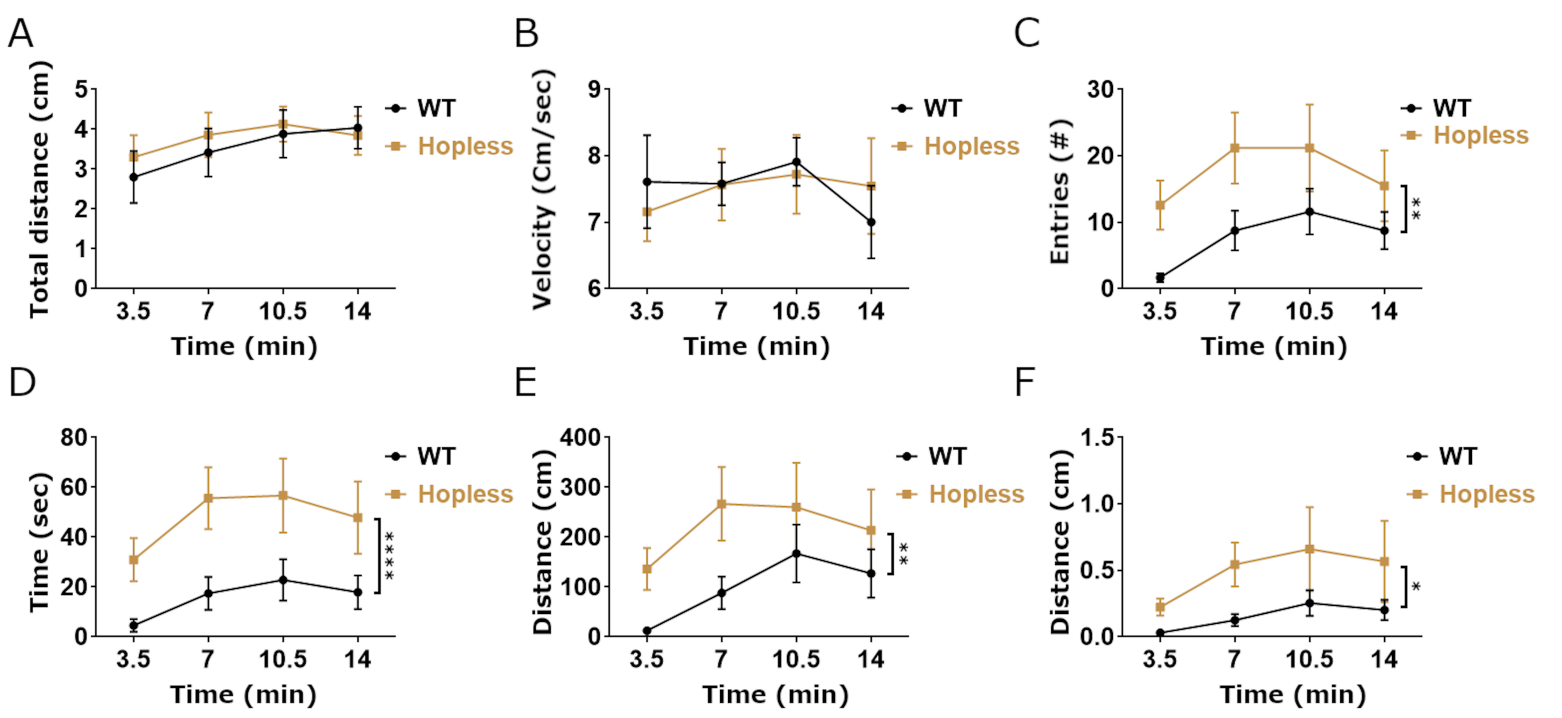

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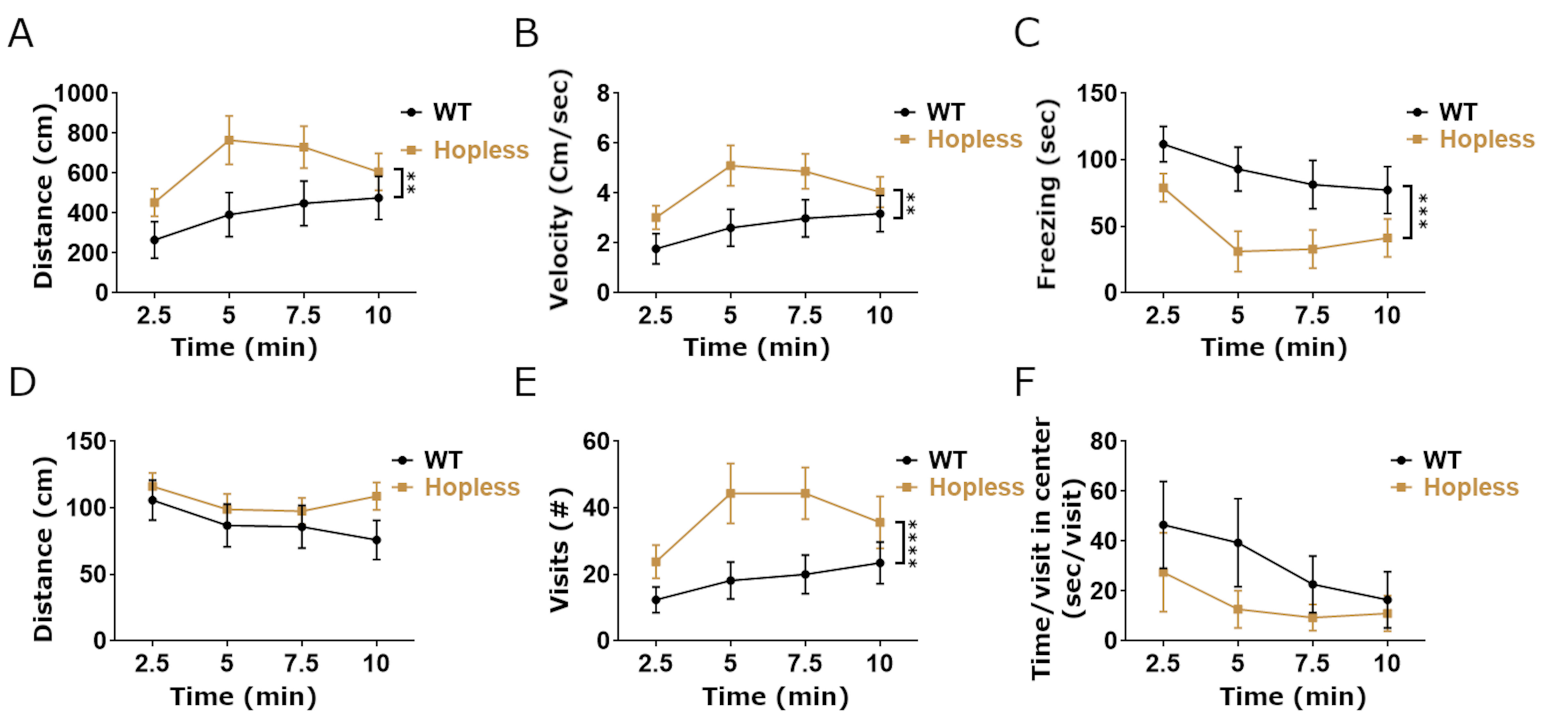

Biran et al Figure 6

708

709 **Supplementary Information**

710

711 Splice-specific deficiency of the PTSD-associated gene PAC1 leads to 712 a paradoxical age-dependent stress behavior

713

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724

725 **Legends to Supplementary Figures**

726 **Supplementary Figure 1.** Source uncropped gel electrophoresis images of
727 genomic DNA (**A**) and cDNA (**B**) in Figure 1. Images were cropped at the yellow
728 dashed rectangle and black & white color was inverted for better contrast.

729 **Supplementary Figure 2. Naïve adult *hopless* mutants display normal**
730 **behavior in the open field assay.** The behavioral response of adult *hopless* mutants
731 (n=11) and WT siblings (n=13) in a novel open field arena was measured. Similarly to
732 the novel tank diving assay, the two genotypes exhibited a similar activity level, as
733 seen in their mean swimming distance and velocity, which significantly increased
734 with time (**A** and **B**). In addition, both genotypes displayed significantly decreased
735 freezing, distance from walls and time spent in the center of the arena (**C-E**) and a
736 slight but significant increase in their visits to the center (**F**), suggesting increased
737 exploration activity over time. Data were analyzed using Student's *t*-test.

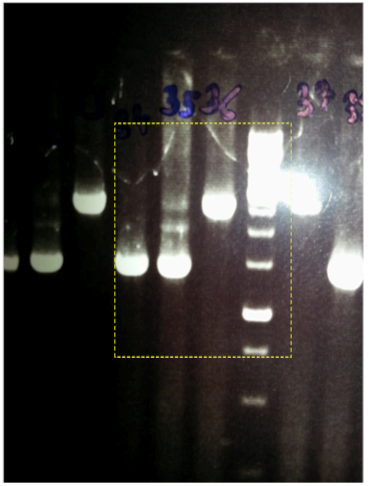
738

739 **Supplementary Table 1. Oligonucleotides used for gRNA transcription and RT-**
740 **PCRs**

Primer	Sequence	Length (nt)
Hop genotyping 1F	CAAAGGTTGTTGGCGTCATA	20
Hop genotyping 2697R	ATTCCCCACGTTTGTCTCAG	20
Upstream hop gDNA	ATTTAGGTGACACTATAGTGTGAAAAGGAACAGTGTGTGTTTTAGAGCTAGAAATAGCAAG	60
Downstream hop gDNA	ATTTAGGTGACACTATATGGTGTGGACATTAAAGGAGGTTTTAGAGCTAGAAATAGCAAG	60
Constant gDNA	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC	80
PAC1 cDNA – F	TGATAACACTGCCCTCTGGTG	21
PAC1 cDNA –R	CCAGGCCGAGCTCAAAGACC	20

741

A



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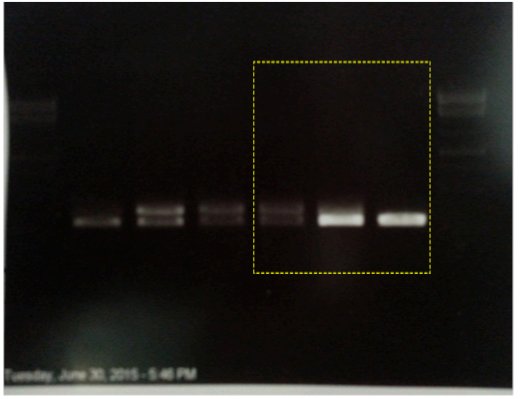
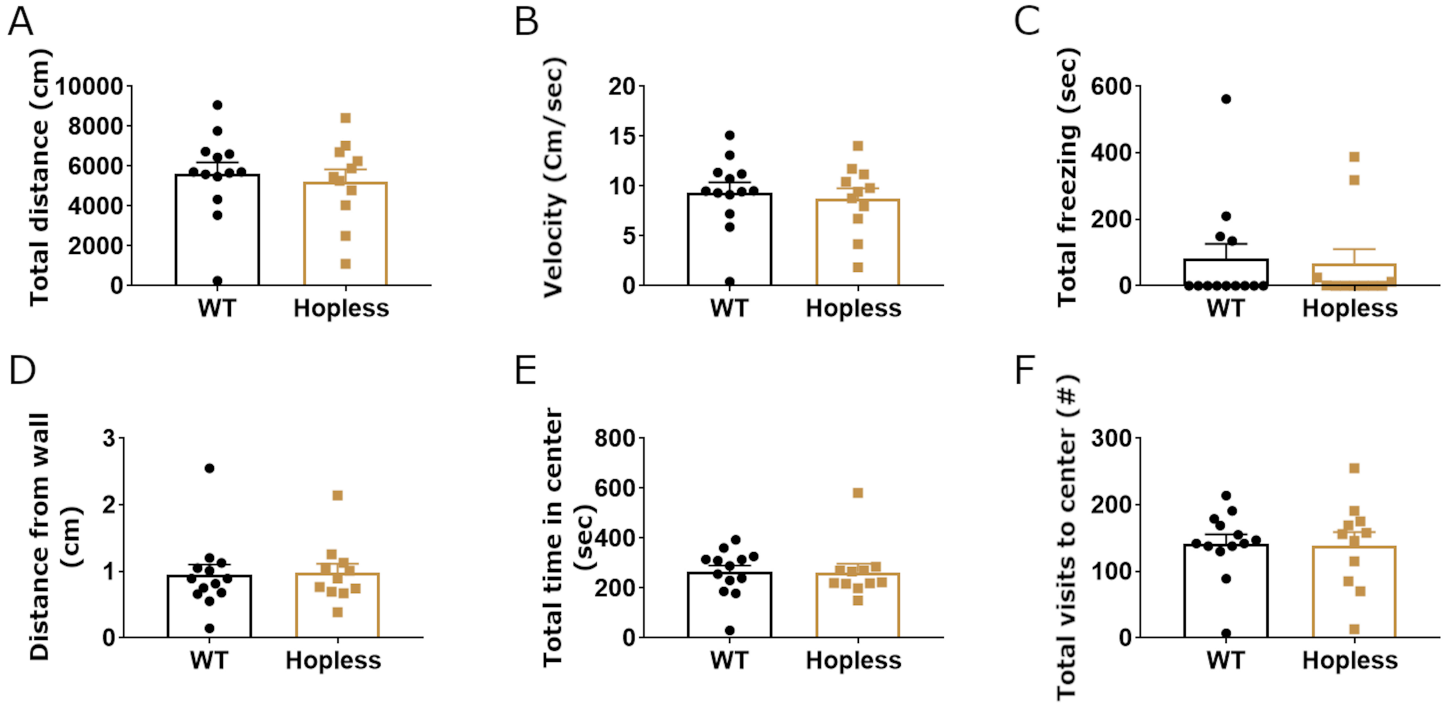


Biran et al Supplementary Figure 1



Biran et al Supplementary Figure 2