Effects of chronic prazosin, an alpha-1 adrenergic antagonist, on anxiety-like behavior and cortisol levels in a chronic unpredictable stress model in zebrafish (*Danio rerio*)

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Post-traumatic stress disorder (PTSD) is often associated with significant neuroendocrine dysfunction and a variety of other symptoms. Today, there are limited efficacious treatment options for PTSD, none of which directly target the dysfunction observed with the hypothalamic-pituitary-adrenal (HPA) axis. The development of new pharmacological treatments is expensive and time consuming; thus, there is utility in repurposing compounds already approved for use in other conditions. One medication in particular that has shown promise for the alleviation of PTSD symptoms is prazosin, an alpha-1 adrenergic receptor antagonist used to treat hypertension. While there have been many studies indicating the efficacy of prazosin in the treatment of PTSD symptoms, no studies fully elucidate mechanisms elicited by this treatment, nor is it clear if prazosin normalizes neuroendocrine dysfunction associated with trauma exposure. The use of zebrafish (*Danio rerio*) has been growing in popularity, in part, due to the homology of the stress response system with mammals. In this study, the zebrafish model was utilized to determine behavioral and biological changes induced by chronic unpredictable stress (CUS) and how these effects could be modulated by chronic prazosin treatment. The results indicated that 7d of CUS increased anxiety-like behavior in the novel tank test and decreased basal levels of cortisol. Chronic (7d) prazosin treatment decreased anxiety-like behaviors overall but did not appear to affect CUS-induced changes in behavior and basal cortisol levels. This suggests that the clinical effectiveness of prazosin may not normalize dysregulated stress responses prevalent in many patients with PTSD, but that prazosin-induced relief from anxiety in stress-related conditions may involve an alternative mechanism other than by normalizing neuroendocrine dysfunction.

1 **Effects of chronic prazosin, an alpha-1 adrenergic antagonist, on anxiety-like**

2 **behavior and cortisol levels in a chronic unpredictable stress model in**

3 **zebrafish (*Danio rerio*)**

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23 **Abstract**

24 Post-traumatic stress disorder (PTSD) is often associated with significant neuroendocrine

25 dysfunction and a variety of other symptoms. Today, there are limited efficacious treatment

26 options for PTSD, none of which directly target the dysfunction observed with the hypothalamic-

27 pituitary-adrenal (HPA) axis. The development of new pharmacological treatments is expensive

28 and time consuming; thus, there is utility in repurposing compounds already approved for use in

29 other conditions. One medication in particular that has shown promise for the alleviation of

30 PTSD symptoms is prazosin, an alpha-1 adrenergic receptor antagonist used to treat

31 hypertension. While there have been many studies indicating the efficacy of prazosin in the

32 treatment of PTSD symptoms, no studies fully elucidate mechanisms elicited by this treatment,

33 nor is it clear if prazosin normalizes neuroendocrine dysfunction associated with trauma

34 exposure. The use of zebrafish (*Danio rerio*) has been growing in popularity, in part, due to the

35 homology of the stress response system with mammals. In this study, the zebrafish model was

36 utilized to determine behavioral and biological changes induced by chronic unpredictable stress

37 (CUS) and how these effects could be modulated by chronic prazosin treatment. The results

38 indicated that 7d of CUS increased anxiety-like behavior in the novel tank test and decreased

39 basal levels of cortisol. Chronic (7d) prazosin treatment decreased anxiety-like behaviors overall

40 but did not appear to affect CUS-induced changes in behavior and basal cortisol levels. This

41 suggests that the clinical effectiveness of prazosin may not normalize dysregulated stress

42 responses prevalent in many patients with PTSD, but that prazosin-induced relief from anxiety in

43 stress-related conditions may involve an alternative mechanism other than by normalizing

44 neuroendocrine dysfunction.

Introduction

47 Post-traumatic stress disorder (PTSD) is a disorder that inhibits day-to-day functionality

48 due to a plethora of disrupting symptoms including flashbacks, vivid nightmares, mood

49 alterations, and hypervigilance (Bisson, Cosgrove, Lewis, & Robert, 2015). According to the

50 World Health Organization (WHO) World Mental Health Surveys, the overall lifetime

51 prevalence of PTSD is estimated to be around 3.9%, increasing to about 5.6% of trauma-exposed

52 individuals (Koenen et al., 2017). The most common traumas associated with PTSD are

53 interpersonal traumas, including rape and other types of sexual assault (Kessler et al., 2017).

54 Current evidence-based treatment plans for PTSD include cognitive behavioral therapy and

55 pharmacological options, mainly serotonin reuptake inhibitors (Lancaster, Teeters, Gros, &

56 Back, 2016); however, many cases are resilient to or respond ineffectively to first-line treatments

57 (Foa, Keane, Friedman, & Cohen, 2009). It appears that there is a growing trend of prescribing

58 benzodiazepines for the management of PTSD symptoms, at least in U.S. active duty service

59 members (Loeffler, Coller, Tracy, & Derderian, 2018); however, this practice is associated with

60 concerning outcomes, such as increased suicide risk (Deka et al., 2018). In addition, it is well

61 known that chronic use of benzodiazepines is associated with physiological dependence and

62 subsequent withdrawal symptoms upon treatment discontinuation (Pétursson, 1994).

63 Due to the resilience to treatment and concerning growth in use of sedative prescription

64 medications, the need for novel treatments for PTSD has become apparent. Because of the

65 exorbitant cost of effective novel drug synthesis and testing, there has been growing interest in

66 the repurposing of compounds already approved by the Food and Drug Administration in the

67 treatment of other conditions (Papapetropoulos & Szabo, 2018). Prazosin, an alpha-1 adrenergic

68 receptor antagonist originally utilized in the treatment of hypertension, has been shown to

69   alleviate clinical symptoms of PTSD (Ahmadpanah et al., 2014; De Berardis et al., 2015; Green,

70   2014; Koola, Varghese, & Fawcett, 2014; Simon & Rousseau, 2017; B. Singh, Hughes, Mehta,

71   Erwin, & Parsaik, 2016; Writer, Meyer, & Schillerstrom, 2014). However, there have been few

72   investigations into the mechanisms behind the clinical efficacy of this compound, particularly

73   involving physiological symptoms associated with chronic stress exposure.

74   The physiological stress response in mammals is largely controlled by the hypothalamic-

75   pituitary-adrenal (HPA) axis. The HPA axis acts in a negative feedback loop, wherein the

76   hypothalamus signals the pituitary gland via corticotropin-releasing hormone (CRH) to stimulate

77   the release of adrenocorticotropic hormone (ACTH) into the bloodstream. ACTH then acts

78   peripherally to stimulate the release of glucocorticoids (e.g., cortisol, corticosterone) from the

79   adrenal glands to mobilize the body's resources to deal with a stressor. Then, cortisol binds to

80   glucocorticoid receptors in the hypothalamus, pituitary gland, and other upstream brain

81   structures to attenuate the stress response. Studies indicate that the HPA axis is dysregulated in

82   patients with PTSD (Mason, Giller, Kosten, Ostroff, & Podd, 1986; Pervanidou & Chrousos,

83   2010; Wichmann, Kirschbaum, Böhme, & Petrowski, 2017; Yehuda et al., 1990). There is

84   evidence that prazosin helps to normalize HPA dysfunction in subjects in the early stages of

85   alcohol withdrawal (Fox et al., 2012); however, it is unknown if prazosin would similarly

86   alleviate HPA dysfunction associated with chronic stress exposure. Furthermore, the exact

87   direction of chronic stress-induced dysregulation (i.e., upregulation or downregulation) is

88   complex and likely dependent on a number of factors, such as biological sex and early life stress

89   exposure (Dunlop & Wong, 2019). Thus, a more complete understanding of individual factors

90   affecting stress-induced alterations in HPA functioning can be examined with animal models.

91   The stress response is heavily conserved amongst vertebrates. The zebrafish (*Danio*

92   *rerio*) has been asserted as a viable model for stress related research because of the similarities in

93   the physiological stress response (Clark, Boczek, & Ekker, 2011). The hypothalamic-pituitary-

94   interrenal (HPI) axis is considered to be the zebrafish analogue to the mammalian HPA axis

95   (Nesan & Vijayan, 2013; Wendelaar Bonga, 1997). In addition, zebrafish have been growing in

96   popularity for translational research due to genetic and physiological similarities to mammals in

97   stress and anxiety-like behavioral responses (Caramillo, Khan, Collier, & Echevarria, 2015).

98   Zebrafish exposed to chronic unpredictable stress (CUS) may facilitate a better understanding of

99   factors that may convey vulnerability to behavioral disorders that affect humans, such as major

100   depressive disorder (Fulcher, Tran, Shams, Chatterjee, & Gerlai, 2017) and PTSD (Caramillo et

101   al., 2015; Stewart, Yang, Nguyen, & Kalueff, 2014). The stressors that are utilized in the CUS

102   zebrafish model vary in intensity, duration of stress, and type of stress and include mechanical,

103   chemical, and temperature changes. The stressors are randomized and are administered at

104   different times. Several studies have indicated that CUS modeling in zebrafish elicits anxiety-

105   like behaviors in a variety of testing paradigms (Chakravarty et al., 2013; Fulcher et al., 2017;

106   Marcon et al., 2016, 2018; Piato et al., 2011; Song et al., 2018). CUS also increases whole-body

107   cortisol levels (Manuel et al., 2014; Marcon et al., 2016, 2018; Piato et al., 2011; Song et al.,

108   2018), although in one report, CUS was shown to increase basal cortisol levels only in male fish

109   but did not significantly alter basal cortisol levels in female fish (Rambo et al., 2017). Thus, the

110   CUS model in zebrafish could be used to examine the efficacy of repurposed compounds and

111   clarify the mechanisms by which these compounds could alleviate physiological dysfunction

112   associated with stress-related conditions.

113   The current study utilized the seven-day chronic unpredictable stress (CUS) model as

114   reported in the literature (Manuel et al., 2014; Marcon et al., 2016, 2018; Piato et al., 2011;

115   Rambo et al., 2017) to replicate the effects of the protocol on anxiety-like behavior and basal

116   cortisol levels. Then, in another experiment, chronic treatment with either prazosin or vehicle

117   followed a week of CUS to examine whether prazosin would reverse any CUS-induced changes

118   in hormones or behavior. It was hypothesized that CUS would increase basal levels of cortisol

119   and elicit increases in anxiety-like behavior, as evidenced by increased freezing and decreased

120   exploratory behavior in the novel tank test. It was also expected that chronic prazosin treatment

121   would normalize both neuroendocrine and behavioral alterations observed after CUS exposure.

122   The results from the current study could provide evidence for the mechanism of prazosin's

123   clinical efficacy and suggest that it may be a viable treatment option for individuals with HPA

124   dysfunction associated with stress-related conditions, such as PTSD.

125

126   **Methods and Materials**

127   *Animals and housing*

128   Wild-type, adult mixed-sex zebrafish (total N = 122) were purchased from Carolina

129   Biological Supply (Burlington, NC). Upon delivery, zebrafish were randomly placed into

130   housing tanks and allowed to acclimate to the facility for at least one week before any

131   experimental procedures were initiated (Dhanasiri, Fernandes, & Kiron, 2013). Zebrafish were

132   housed in a stocking density of 5-7 fish per liter in 1.8L tanks and maintained in a two-shelf,

133   stand-alone zebrafish housing rack purchased from Aquaneering (San Diego, CA). Fish were

134   maintained on a 14h:10h light:dark cycle, with water kept at $27 \pm 1°C$ and pH of approximately

135   7.2. Other water quality parameters were measured biweekly, such as ammonia, nitrates, nitrites,

136 alkalinity, and hardness, and were kept constant throughout the experiments. Fish were fed once

137 per day with flake food and once per day with dried shrimp ground to a powder with a mortar

138 and pestle. Feeding commenced around 9 a.m. each day, before any stress or drug procedures

139 were conducted, except for on days of data collection. The fish were not fed prior to the

140 behavioral testing. The total food weight given per day per fish approximated 4% of the average

141 fish body weight. All procedures were carried out by following established recommendations

142 (Harper & Lawrence, 2011; National Research Council, 2011; Westerfield, 2000).

143 *Drugs and materials*

144   Prazosin hydrochloride was manufactured by TCI America and purchased from VWR

145 International (Radnor, PA). N-N-dimethylacetamide was manufactured by Frontier Scientific

146 and purchased from Fisher Scientific (Hampton, NH).

147 *Experiment 1: Chronic unpredictable stress on anxiety-like behavior and basal cortisol levels*

148   Upon arrival to the facility, fifty zebrafish were randomly allocated into four separate

149 1.8L housing tanks. After at least 7 days of acclimation to the facility, two tanks of fish were

150 randomly selected and subsequently exposed to the chronic unpredictable stress model for seven

151 days and the other two tanks of fish served as untreated, unstressed controls. On the day after the

152 completion of the chronic stress paradigm, fish from both control and stressed groups were

153 placed in the novel tank test one at a time to assess anxiety-like behavior. Fish were immediately

154 euthanized after the behavioral assessment and decapitated to assess basal levels of trunk

155 cortisol. At the end of the experiment, N = 25 were exposed to chronic unpredictable stress and

156 N = 25 were untreated for 7 days.

157

158   *Experiment 2: Chronic unpredictable stress and chronic prazosin treatment on anxiety-like*

159   *behavior and basal cortisol levels*

160   Upon arrival to the facility, seventy-two zebrafish were randomly allocated into eight

161   separate 1.8L housing tanks. After at least 7 days of acclimation to the facility, four tanks of fish

162   were randomly selected and subsequently exposed to the chronic unpredictable stress model for

163   seven days and the other four tanks of fish were not stressed (left unhandled) for seven days.

164   Then, two tanks of the stressed fish and two tanks of the non-stressed fish were treated with

165   prazosin for 30 minutes per day for the seven days following the CUS treatment. The other four

166   tanks were exposed to vehicle treatment for 30 minutes per day for seven days. On the day after

167   the completion of the drug or vehicle treatment, fish from all groups were placed in the novel

168   tank test one at a time to assess anxiety-like behavior. Fish were immediately euthanized after

169   the behavioral assessment and decapitated to assess basal levels of trunk cortisol. A total of three

170   fish died during the course of the procedures (N = 2 from the unstressed/vehicle-treated group

171   and N = 1 from the stressed/vehicle-treated group). At the end of the experiment, N = 18 were

172   exposed to 7d chronic unpredictable stress followed by chronic (7d) prazosin, N = 17 were

173   exposed to 7d chronic unpredictable stress followed by 7d vehicle, N = 18 were untreated for 7

174   days and then exposed to chronic (7d) prazosin, and N = 16 were untreated for 7 days and then

175   exposed to vehicle for 7 days.

176   *Chronic unpredictable stress (CUS) model*

177   The chronic unpredictable stress (CUS) model was adapted from previously published

178   procedures (Marcon et al., 2016, 2018; Piato et al., 2011; Rambo et al., 2017). Seven different

179   types of stressors were ordered at random. Fish in the stressed group were exposed to two

180   stressors per day for seven days at random times between 9 a.m. and 4 p.m. (see Table 1 for

181   schedule). The stressors included (1) tank changes (three times) in rapid succession, (2) cooling

182   home tank water abruptly to 23°C and maintaining that temperature with chilled system water for

183   30 minutes before placing tank back on the system, (3) heating home tank water abruptly to 33°C

184   and maintaining that temperature with heated system water for 30 minutes before placing tank

185   back on the system, (4) lowered water (1 cm depth for 15 minutes), (5) net chasing in home tank

186   (8 minutes chase, 15 minute rest, 8 minute chase), (6) crowding all fish from one home tank in

187   200 ml system water (9-13 fish total, density of 45-65 fish per liter) in a 250 ml beaker for 60

188   minutes, and (7) social isolation (individual fish were placed in 200 ml system water in 250 ml

189   beakers separated by opaque dividers). Control fish were not stressed (not handled) and were not

190   removed from the system for 7 days.

191   *Drug treatment*

192   Fish undergoing chronic administration of prazosin were gently netted from the home

193   tank and individually placed into a 100 mL beaker containing 2 mg prazosin dissolved in 100

194   microliters of N,N-dimethylacetamide and 50 mL of system water for 30 minutes (A. Singh,

195   Subhashini, Sharma, & Mallick, 2013). This concentration was chosen due to the observed

196   anxiolytic behavior in the light-dark test displayed by the fish acutely exposed to prazosin (A.

197   Singh et al., 2013). In the current experiment, the drug exposure was repeated once daily for

198   seven days; fresh drug solution was prepared for each fish for each exposure from a concentrated

199   prazosin solution prepared at the beginning of the experiment. Subjects in the control group were

200   subjected to similar handling and conditions although only exposed to the vehicle (100

201   microliters of N,N-dimethylacetamide in 50 mL system water in a 100 mL beaker for 30 minutes

202   per day). Fresh vehicle solution was prepared each day. All fish were returned to their respective

203   home tanks after the treatment and placed back on the system between daily treatment sessions.

204   *Novel tank test (NTT)*

205   On the day of the experiment (the day after the last episode of chronic stress for fish in

206   Experiment 1 and the day after the last drug treatment for fish in Experiment 2), home tanks

207   were removed from the system and moved into the experiment room adjacent to the housing

208   facility. To minimize the impact of the stress of moving the tanks, fish were left to acclimate for

209   at least 30 minutes before assessing behavior. The experimental room had the same lighting and

210   temperature conditions as the housing room. Fish were individually netted and placed into a

211   trapezoidal novel tank, the same size and dimensions as the home tanks (15.2 cm height × 27.9

212   cm top × 22.5 cm bottom × 7.1 cm width), for six minutes. The novel tank was filled with water

213   from the system and was changed on each new day of data collection. The behavior of each fish

214   was recorded and subsequently analyzed with BehaviorCloud motion-tracking software (Alia &

215   Petrunich-Rutherford, 2019; Aponte & Petrunich-Rutherford, 2019; Pilehvar, Town, & Blust,

216   2020). Total distance traveled (cm) and mean ambulatory speed (cm/sec) were measured as

217   markers of general motor activity; immobility duration (sec), the number of entries to the top of

218   tank, time spent in top (sec), and distance traveled in the top (cm) and were used as markers of

219   anxiety-like behavior (Cachat et al., 2010; Egan et al., 2009; Wong et al., 2010). The top of the

220   tank was defined as the top 50% (approximately 7 cm) of the water column (total approximately

221   14 cm). Behavioral data collection and euthanasia of the subjects occurred between 9:30 a.m.

222   and 2:30 p.m.

223   *Euthanasia*

224   Immediately after the novel tank test, fish were netted from the novel tank and placed

225   individually in a 50 mL beaker with approximately 30 mL 0.1% (100 mg/L) clove oil in system

226   water. Death occurred within 5 to 10 seconds of introduction to the solution and was determined

227   upon visual examination for cessation of opercular (gill) movement and nonresponse to tactile

228   stimulation (Davis et al., 2015). The fish were then decapitated. The trunk samples were frozen

229   in individual 1.5 ml tubes and stored at -20°C for cortisol analysis.

230   *Cortisol extraction and assay*

231   The cortisol extraction and assay was done by slightly modifying previously published

232   procedures (Cachat et al., 2010; Canavello et al., 2011). In brief, trunk samples were thawed and

233   weighed, and subsequently homogenized with phosphate-buffered saline (PBS). Diethyl ether

234   was added to the homogenates and centrifuged for 15 minutes at 4°C. The ether layer containing

235   cortisol was isolated in a separate tube. The addition of ether, centrifugation, and ether isolation

236   was repeated for a total of three times, collecting all three ether layers in one tube for each

237   sample. The ether was then dried under a light stream of air until only a yellow oil containing the

238   cortisol remained in each tube. The oil in each tube was reconstituted with PBS and refrigerated

239   overnight (4°C). Cortisol was quantified via an enzyme-linked immunosorbent assay (ELISA) as

240   per the manufacturer's instructions (Salimetrics, State College, PA).

241   *Data analysis*

242   *A priori* sample size calculations were conducted using G*Power software (Faul,

243   Erdfelder, Lang, & Buchner, 2007) using the following parameters: $d = 0.95$, $\alpha = 0.05$, power =

244   0.95. Effect size was based on the effects of chronic unpredictable stress and prazosin on anxiety

245   measures in previously published studies (Marcon et al., 2016; A. Singh et al., 2013).

246   Upon selection from the home tank on the day of the experiment, each fish was given a

247   sample number. Behavioral and cortisol analyses were conducted; sample numbers were then

248   matched with the treatment(s) and analyzed by group. Data are presented as the means and

249   standard errors of the mean (SEM) for each group. Raw data (see supplemental file 1) was

250   processed using JASP software (University of Amsterdam, Amsterdam, The Netherlands,

251   https://jasp-stats.org/). For Experiment 1, overall (6-minute) behavioral variables, cortisol, and

252   trunk weights were compared by independent sample t-tests (with stress condition as the

253   independent variable) and one-minute bin data for behavioral variables were compared by

254   repeated-measures ANOVA. For Experiment 2, overall (6-minute) behavioral variables, cortisol,

255   and trunk weights were compared by two-way ANOVA analyses (with stress condition and drug

256   treatment as the independent variables) and one-minute bin data for behavioral variables were

257   compared by repeated-measures ANOVA. Tukey post-hoc analyses were conducted when

258   appropriate and Greenhouse-Geisser sphericity correction was made if Mauchly's test of

259   sphericity indicated a violation of the sphericity assumption for the repeated-measures ANOVA

260   tests. A significance level of $p < 0.05$ was used as the criterion for results to reach statistical

261   significance.

262

263   **Results**

264   *Experiment 1: Effects of seven days of chronic unpredictable stress (CUS) treatment on*

265   *behavioral measures in the novel tank test (NTT), basal levels of cortisol, and body weights in*

266   *adult zebrafish.*

267   **Motor activity in the novel tank test**

268   A t-test for independent means indicated no significant effect of chronic unpredictable

269   stress on either the total distance traveled ($t(48) = 1.274$, $p = 0.209$) or mean ambulatory speed

270   ($t(48) = 1.077$, $p = 0.287$) for the entire 6 minutes of the novel tank test (Table 2). When the total

271   distance data was broken down into six 60-second bins (Figure 1A) and analyzed with a

272   repeated-measures ANOVA, there was no effect of stress ($F(1,48) = 1.551$, $p = 0.219$), a

273   significant effect of time $(F(3.877,186.085) = 7.403, p < 0.001)$, but no interaction between stress

274   and time $(F(3.877,186.085) = 0.452, p = 0.765)$. For the mean ambulatory speed (Figure 1B),

275   again, there was no effect of stress $(F(1,48) = 0.934, p = 0.339)$, a significant effect of time

276    $(F(4.077,195.709) = 16.657, p < 0.001)$, but no interaction between stress and time

277    $(F(4.077,195.709) = 1.006, p = 0.406)$. These results show that the fish appeared to habituate

278   after introduction to the novel tank, as the total distance per minute and mean ambulatory speed

279   gradually increased across the duration of the novel tank test, but there was no effect of treatment

280   on these measures.

281   **Freezing behavior in the novel tank test**

282   A t-test for independent means indicated no significant effect of chronic unpredictable

283   stress on the total immobility time $(t(48) = 0.544, p = 0.589)$ in the novel tank test (Table 2).

284   When the immobility data was broken down into six 60-second bins (Figure 2) and analyzed

285   with a repeated-measures ANOVA, there was no effect of stress $(F(1,48) = 0.359, p = 0.552)$, no

286   effect of time $(F(2.191,105.178) = 1.359, p = 0.262)$, and no interaction between stress and time

287    $(F(2.191,105.178) = 1.114, p = 0.336)$. Thus, neither time nor treatment significantly altered

288   freezing behavior across the six minutes of the novel tank test.

289   **Exploratory behavior in the novel tank test**

290   A t-test for independent means indicated a marginally significant effect of chronic

291   unpredictable stress for the number of entries to the top zone $(t(48) = -1.920, p = 0.061$; CUS <

292   untreated), a significant effect on the total time spent in the top zone $(t(48) = -2.129, p = 0.038$;

293   CUS < untreated), but no significant difference in the distance traveled in the top zone $(t(48) = -$

294    $1.309, p = 0.197$; Table 2). When the number of entries to the top zone was broken down into six

295   60-second bins (Figure 3A) and analyzed with a repeated-measures ANOVA, there was a

296   marginal effect of stress (F(1,48) = 3.454, p = 0.069), a significant effect of time

297   (F(3.855,185.031) = 6.701, p < 0.001), but no interaction between stress and time

298   (F(3.855,185.031) = 0.935, p = 0.442). For the time spent in the top zone (Figure 3B), there was

299   a significant effect of stress (F(1,48) = 4.530, p = 0.038), a significant effect of time

300   (F(3.540,169.936) = 13.317, p < 0.001), but no interaction between stress and time

301   (F(3.540,169.936) = 1.318, p = 0.268). For the distance traveled in the top zone (Figure 3C),

302   there was no effect of stress (F(1,48) = 1.635, p = 0.207), a significant effect of time

303   (F(3.631,174.291) = 12.430, p < 0.001), but no interaction between stress and time

304   (F(3.631,174.291) = 1.408, p = 0.237). Similar to the motor measures, the fish appeared to

305   habituate and explore more of the top zone of the novel tank over time, but the fish that were

306   chronically stressed generally explored the top zone less than the fish that were left untreated for

307   seven days.

308   **Trunk cortisol**

309   A t-test for independent means indicated that fish exposed to seven days of chronic

310   unpredictable stress (CUS) had decreased basal levels of trunk cortisol compared to untreated

311   control fish (t(48) = -3.130, p = 0.003; Figure 4A). This finding suggests that seven days of

312   chronic unpredictable stress decreases basal levels of cortisol in zebrafish compared to fish that

313   were untreated.

314   **Trunk weights**

315   A t-test for independent means indicated that fish exposed to 7 days of chronic

316   unpredictable stress (CUS) had similar trunk weights as fish that were left untreated for 7 days

317   (t(48) = -1.111, p = 0.272; Figure 4B). This finding suggests that 7 days of chronic unpredictable

318   stress does not appear to alter factors involved with body weight regulation, such as feeding, in

319   zebrafish.

320   *Experiment 2: Effects of seven days of chronic unpredictable stress (CUS) treatment and seven*

321   *days of prazosin treatment on behavioral measures in the novel tank test (NTT), basal levels of*

322   *cortisol, and body weights in adult zebrafish.*

323   **Motor activity in the novel tank test**

324   A two-way ANOVA indicated that there was no effect of stress ($F_{(1,65)} = 0.358$, $p =$

325   0.552), no effect of drug treatment ($F_{(1,65)} = 0.139$, $p = 0.710$), and no interaction between

326   stress and drug ($F_{(1,65)} = 0.103$, $p = 0.750$) on the total distance traveled in the novel tank test

327   (Table 3). A two-way ANOVA indicated that there was no effect of stress ($F_{(1,65)} = 0.657$, $p =$

328   0.421), no effect of drug treatment ($F_{(1,65)} = 1.186$, $p = 0.280$), and no interaction between

329   stress and drug ($F_{(1,65)} = 0.355$, $p = 0.553$) on the mean ambulatory speed of fish in the novel

330   tank test (Table 3). The total distance (Figure 5A) and mean ambulatory speed (Figure 5B) data

331   was also broken down into six 60-second bins and analyzed with a repeated-measures ANOVA

332   (see Table 4 for statistical analyses). These results indicate that, similar to the results from

333   Experiment 1, fish appear to habituate to the novel tank across the duration of the test, but that

334   there is no effect of stress or drug treatment on these measures of motor activity.

335   **Freezing behavior in the novel tank test**

336   A two-way ANOVA indicated that there was a marginal effect of stress ($F_{(1,65)} = 3.050$,

337    $p = 0.085$; CUS < control), a marginal effect of drug treatment ($F_{(1,65)} = 3.050$, $p = 0.085$;

338   prazosin < vehicle), but no interaction between stress and drug ($F_{(1,65)} = 2.087$, $p = 0.153$) on

339   total immobility time in the novel tank test (Table 3). The immobility data (Figure 6) was also

340   broken down into six 60-second bins and analyzed with a repeated-measures ANOVA (see Table

341   4 for statistical analyses). These results indicate that immobility generally decreases across the

342   duration of the novel tank test, but that there is no significant effect of stress or drug treatment on

343   this behavioral measure.

344   **Exploratory behavior in the novel tank test**

345   A two-way ANOVA indicated that there was a significant effect of stress ($F_{(1,65)}$ =

346   5.939, p = 0.018; CUS > control), a marginally significant effect of drug treatment ($F_{(1,65)}$ =

347   2.903, p = 0.093; prazosin > vehicle), but no interaction between stress and drug ($F_{(1,65)}$ =

348   1.404, p = 0.240) on the number of entries to the top zone (Table 3). A two-way ANOVA

349   indicated that there was no effect of stress ($F_{(1,65)}$ = 2.769, p = 0.101), a significant effect of

350   drug treatment ($F_{(1,65)}$ = 4.061, p = 0.048; prazosin > vehicle), but no interaction between stress

351   and drug ($F_{(1,65)}$ = 0.956, p = 0.332) on the total amount of time spent in the novel tank test

352   (Table 3). A two-way ANOVA indicated that there was a significant effect of stress ($F_{(1,65)}$ =

353   4.876, p = 0.031; CUS > control), no effect of drug treatment ($F_{(1,65)}$ = 0.745, p = 0.391), but

354   no interaction between stress and drug ($F_{(1,65)}$ = 1.947, p = 0.168) on the distance traveled in

355   the top zone of the novel tank (Table 3). The number of entries to the top (Figure 7A), time spent

356   in the top zone (Figure 7B), and distance traveled in the top zone (Figure 7C) were also broken

357   down into six 60-second bins and analyzed with a repeated-measures ANOVA (see Table 4 for

358   statistical analyses). These results suggest that allowing additional time (seven days) to elapse

359   between the chronic stress paradigm and testing in the novel tank perhaps reverses the deficits in

360   top zone exploration elicited by CUS observed in Experiment 1. In addition, these results suggest

361   that chronic prazosin increases exploration in the novel tank test in the absence of stress, but

362   does not appear to alter any stress-induced effects on top zone exploration in the novel tank when

363   prazosin treatment follows chronic unpredictable stress.

364 **Trunk cortisol**

365   A two-way ANOVA indicated that there was no effect of stress ($F_{(1,65)} = 2.511$, $p =$

366 0.118), no effect of drug treatment ($F_{(1,65)} = 0.624$, $p = 0.432$), and no interaction between

367 stress and drug ($F_{(1,65)} = 0.636$, $p = 0.428$) on basal levels of cortisol (Figure 8A). Although

368 these results did not reach statistical significance, the fish subjected to chronic unpredictable

369 stress and subsequently were vehicle-treated had lower levels of cortisol than untreated-vehicle

370 controls, which is similar to the pattern of results observed in Experiment 1. Prazosin treated-fish

371 also had lower levels of basal cortisol compared to unstressed/vehicle-treated fish; however, this

372 finding did not reach statistical significance.

373 **Trunk weights**

374   A two-way ANOVA indicated that there was no effect of stress ($F_{(1,65)} = 0.057$, $p =$

375 0.811), no effect of drug treatment ($F_{(1,65)} = 0.173$, $p = 0.679$), and no interaction between

376 stress and drug ($F_{(1,65)} = 0.467$, $p = 0.497$) on subject trunk weights (Figure 8B). Similar to the

377 results from Experiment 1, these results indicate that neither stress treatment nor chronic drug

378 treatment altered factors involved with body weight regulation.

379

380 **Discussion**

381   The noradrenergic system is critical for the regulation of several functions, including the

382 regulation of stress responses. The locus coeruleus, the major noradrenergic nucleus of the brain,

383 supplies norepinephrine both systemically and directly to regions throughout the brain including

384 the amygdala, hypothalamus, and the medial prefrontal cortex (mPFC), all areas involved with

385 regulating responses to stress. Noradrenergic dysfunction has been hypothesized to be involved

386 with the neuropathology associated with PTSD (Hendrickson & Raskind, 2016; O'Donnell,

387 Hegadoren, & Coupland, 2004; Southwick, Bremner, et al., 1999; Southwick, Paige, et al., 1999;

388 Strawn & Geracioti, 2008). Dysregulation of the noradrenergic system may ultimately contribute

389 to the alterations in the function of the physiological stress axis observed in patients and animal

390 models. Thus, pharmacological agents that target the norepinephrine regulation of stress

391 responses have the potential to normalize neuroendocrine dysfunction associated with stress-

392 related conditions.

393   The hypothesis of the current study was that the zebrafish model of CUS would increase

394 basal levels of cortisol and increase anxiety-like behavior in the novel tank test, and that chronic

395 prazosin treatment would reverse alterations induced by chronic stress. In support of this

396 hypothesis, CUS slightly increased anxiety-like behavior in the novel tank test; however, this

397 change in behavior was associated with significantly lower levels of basal cortisol. In addition,

398 prazosin appeared to decrease levels of anxiety-like behavior in the absence of CUS. However, it

399 also appeared that CUS-induced decreases in exploratory behavior and basal cortisol levels

400 started to normalize (and in the case of the behavioral variables, reversed) in the 7d interim when

401 vehicle and drug treatment were being administered between CUS and dependent variable

402 assessments.

403   The study design and timing of the assessment of the dependent variables should be

404 considered when interpreting the results of the current study. In the first experiment, behavioral

405 and neuroendocrine measurements were assessed immediately after the seven days of chronic

406 stress. In the second experiment, subjects were exposed to seven days of chronic stress followed

407 by a week of chronic drug treatment administered in the absence of unpredictable stressors. It is

408 possible that any stress-induced effects may have been blunted or reversed by the time the

409 measurements were assessed. For example, in Experiment 1, fish that were exposed to CUS had

410   lower levels of basal cortisol compared to unstressed fish. In Experiment 2, fish that were

411   chronically stressed but subsequently treated with vehicle for seven days still had lower basal

412   cortisol levels than non-stressed, vehicle treated subjects; however, this difference did not reach

413   the criterion for statistical significance (see Figure 8A). Alternatively, in Experiment 2, the

414   additional week of handling necessary to administer the drug/vehicle treatment may have

415   triggered adaptive mechanisms in animals previously exposed to the CUS paradigm, which could

416   have increased the elevated exploratory behavior observed in the novel tank test compared to

417   non-stressed, vehicle-treated controls (see Figure 7). Baseline measures of anxiety could have

418   been increased with the 7 additional days of handling; this supposition is supported by the fact

419   that unstressed/vehicle-treated fish in Experiment 2 generally displayed more anxiety-like

420   behavior (increased immobility and decreased top zone exploration) compared to the unstressed

421   fish from Experiment 1 (see group means in Tables 2 and 3). Future studies should address the

422   duration or persistence of long-term neuroendocrine and behavioral effects of 7d CUS and

423   whether chronic prazosin treatment administered during the same period as the stressors would

424   circumvent any possible effects of allowing previously stressed animals to adapt back to non-

425   stressed conditions. Other studies have examined the effects of anxiolytic compounds

426   administered concurrent with chronic stress paradigms; for example, in a previous study,

427   zebrafish were exposed to chronic unpredictable stress for five weeks but then were treated with

428   the antidepressant fluoxetine during the last 8 days of the stressor paradigm (Song et al., 2018).

429   Another study exposed zebrafish to chronic unpredictable stress for 14 days but treated with the

430   putative anxiolytic N-acetylcysteine concurrent with the last 7 days of the stress (Mocelin et al.,

431   2019). Thus, it would be interesting to see whether continuing the chronic unpredictable stress

432 exposure during treatment or by conducting the stress exposure during the dark phase (Manuel et

433 al., 2014) would make prazosin-induced effects more obvious.

434   In the current report, prazosin treatment alone appeared to enhance exploratory behavior

435 in the novel tank test but did not affect CUS-induced changes in anxiety-like behavior. In

436 addition, prazosin-treated groups had lower levels of cortisol than non-stressed, vehicle-treated

437 subjects, although this decrease did not reach statistical significance. Prazosin treatment may

438 function to generally prevent reactions to stress (Rasmussen, Kincaid, & Froehlich, 2017); thus,

439 further studies should also determine whether preventative prazosin treatment is effective at

440 blocking any chronic or acute stress effects on anxiety-like behavior or neuroendocrine

441 dysfunction. These studies would help clarify whether prazosin would be clinically efficacious,

442 not by normalizing the effects of trauma or stress, but by preventing any responses to further

443 triggering stimuli that may elicit PTSD symptomology. As prazosin acts by putatively blocking

444 α1 receptors, antagonism of alpha-1 receptors could possibly preventing further stress or trauma

445 from triggering the norepinephrine-mediated stimulation of stress axis reactivity (Ma & Morilak,

446 2005). Thus, prazosin may work better as a prophylactic in treating stress-related conditions, as

447 has been observed for other medications (Roque, 2015), although much more clinical work

448 would be necessary to establish this as a potential option for therapy.

449   It is also interesting to note that, although it was expected that seven days of CUS

450 treatment would elicit increases in basal cortisol levels based on previously published reports

451 (Manuel et al., 2014; Marcon et al., 2016, 2018; Piato et al., 2011), the results from the first

452 experiment indicate that the CUS protocol can elicit hypocortisolic responses. There are several

453 factors that could explain the different results between laboratories, such as the source, strain,

454 previous stress exposure, or age of the subjects. For example, one previous report indicated that

455   there are possible sex-specific differences in basal cortisol levels after exposure to chronic stress,

456   with male zebrafish exhibiting increases in cortisol compared to untreated controls, while the

457   levels of basal cortisol did not change in females relative to untreated controls (Rambo et al.,

458   2017). Thus, the impact of both prazosin and chronic stress on our dependent measures may be

459   masked by including both sexes in the analyses. The current results support a recent call for

460   much more research into the housing, breeding, and other husbandry conditions that may be

461   contributing factors to differences in experimental results between laboratories (Lidster,

462   Readman, Prescott, & Owen, 2017; Tsang et al., 2017; Varga, Ekker, & Lawrence, 2018).

463   Another factor that could play a role in our findings is the binding profile of prazosin to

464   α1 adrenergic receptors in the zebrafish brain. Although the binding of prazosin in brain has been

465   extensively studied in rodent and other mammalian models (Dashwood, 1982; Greengrass &

466   Bremner, 1979; Mignot, Bowersox, Maddaluno, Dement, & Ciaranello, 1989; Morrow,

467   Battaglia, Norman, & Creese, 1985; Morrow & Creese, 1986; Rainbow & Biegon, 1983), the

468   same cannot be said about prazosin binding in zebrafish brain. Studies using preparations of

469   codfish brain indicate that prazosin binding may in fact be different in fish brains compared to

470   rodent brains (Bergström & Wikberg, 1986a, 1986b). Perhaps more importantly, although

471   zebrafish α2 and β receptor binding and distribution has been explored (Ampatzis & Dermon,

472   2010; Ampatzis, Kentouri, & Dermon, 2008; Ruuskanen, Laurila, et al., 2005; Ruuskanen,

473   Peitsaro, Kaslin, Panula, & Scheinin, 2005; Wang et al., 2009), little comparable information on

474   zebrafish α1 receptor binding and distribution is available in the extant literature. Thus, more

475   work is necessary for a complete understanding of noradrenergic modulation of stress responses

476   in the zebrafish model.

477   In sum, this study suggests that the clinical efficacy of prazosin in reducing symptoms of

478 stress-related conditions like PTSD does not involve the normalization of physiological stress

479 axis dysfunction. Rather, the clinical effectiveness of prazosin likely involves other mechanisms

480 of altering stress regulation. In addition, this study also highlights the importance of considering

481 methodological and husbandry factors when interpreting results across several vertebrate animal

482 studies, which will ultimately contribute to a better understanding of the complex nature of the

483 regulation and expression of stress responses.

484

485            **Conclusions**

486   This study demonstrated that seven days of chronic unpredictable stress exposure in

487 zebrafish increased the expression of anxiety-like behavior and decreased basal levels of cortisol.

488 When prazosin, a putative alpha-1 receptor antagonist, was chronically administered to subjects

489 after the stress exposure, stress-related effects on behavior and hormones were not reversed;

490 however, prazosin appeared to decrease anxiety-like behaviors in the novel tank test in the

491 absence of stress exposure. Further studies are necessary to determine the longevity of chronic

492 stress-induced responses and whether effects on stress-induced alterations in stress responses are

493 dependent on the timing of drug treatment. These studies also suggest that normalization of

494 neuroendocrine dysfunction may not be involved with the clinical efficacy of prazosin in the

495 treatment of PTSD, although human studies are needed to confirm this finding.

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Figure 1

Motor measures of zebrafish in the novel tank test (Experiment 1)

There was a significant effect of time on (A) the total distance traveled per minute and (B) the mean ambulatory speed in the novel tank test (6 minutes). All fish generally swam longer distances at faster speeds due to habituation to the novel tank, but there was no effect of 7 days of chronic unpredictable stress and no interaction between stress and time on motor measures (N = 25 in each group).

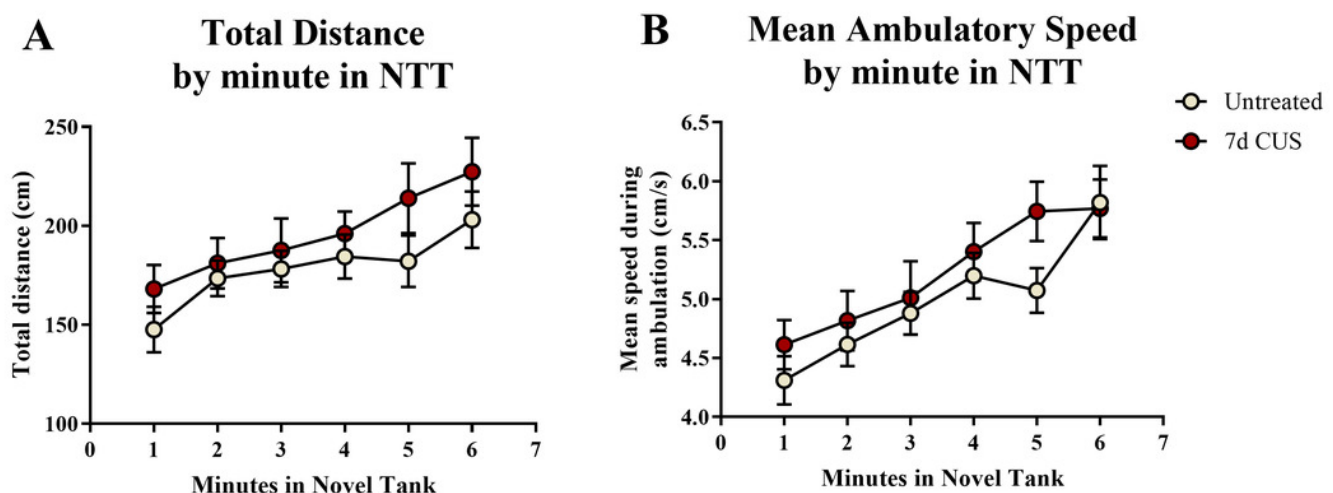



Figure 2

Freezing behavior of zebrafish in the novel tank test (Experiment 1)

There was no effect of treatment or time on the amount of time zebrafish spent immobile in the novel tank test (6 minutes, N = 25 in each group).

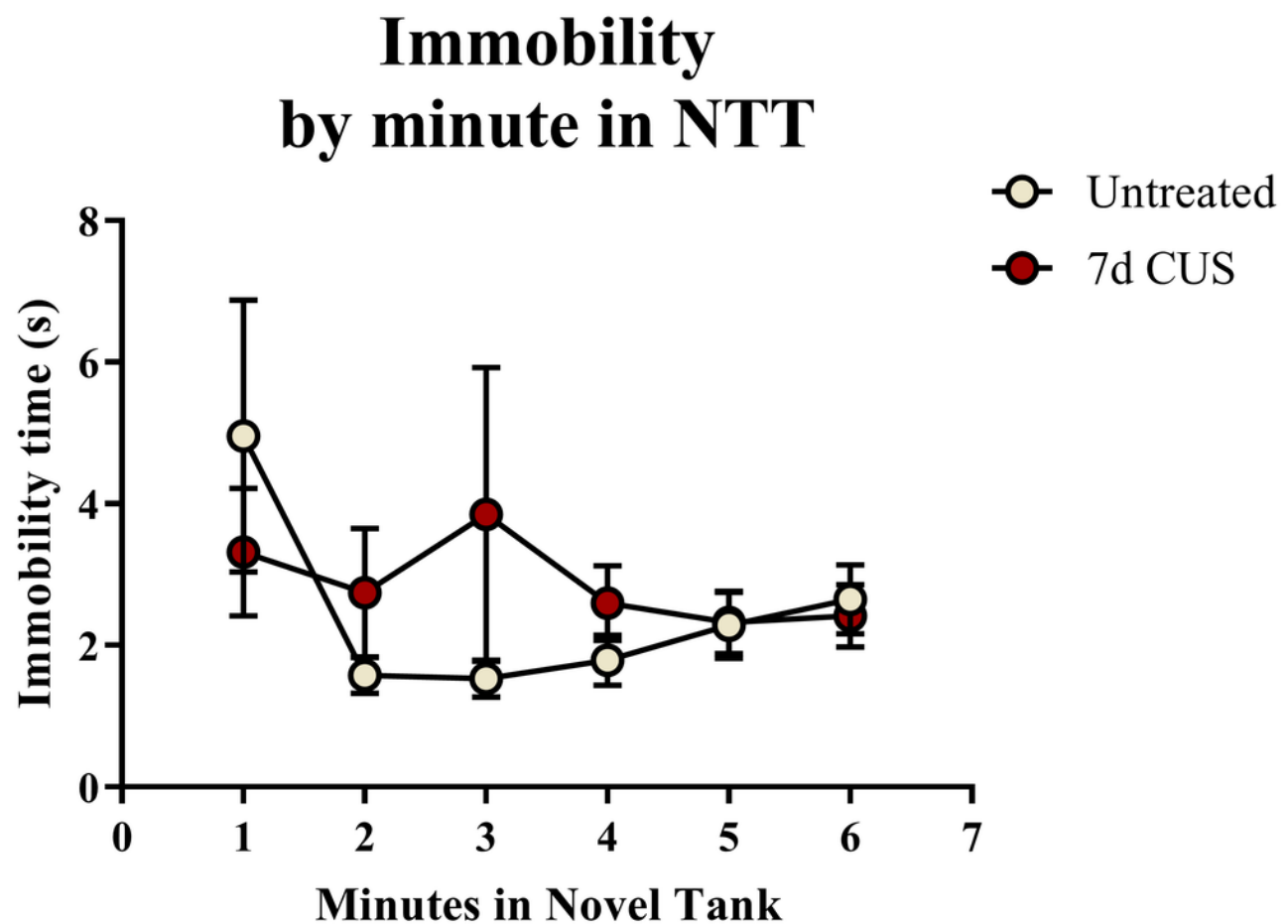



Figure 3

Exploratory measures of zebrafish in the novel tank test (Experiment 1)

There was a significant effect of time on all top measures in the novel tank test. Additionally, 7 days of chronic unpredictable stress (CUS) (A) marginally decreased the number of times zebrafish entered the top zone, (B) significantly decreased the amount of time zebrafish explored the top zone, and (C) slightly (but non-significantly) decreased the distance traveled in the top of the novel tank test over 6 minutes. Thus, all zebrafish tended to explore the top zone more across the duration of the test; however, chronically stressed fish explored less than non-stressed fish (N = 25 in each group).

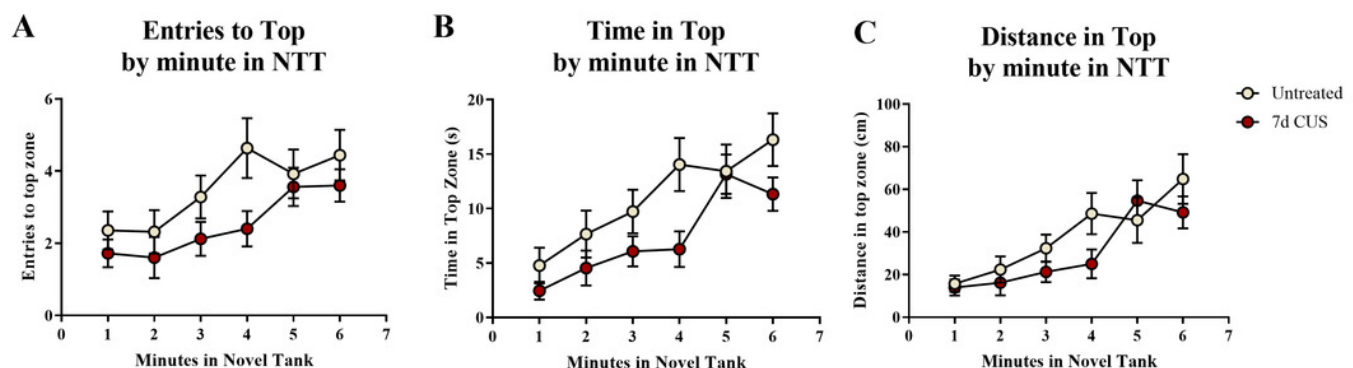



Figure 4

Cortisol and body weight measures of zebrafish (Experiment 1)

Seven days of chronic unpredictable stress (CUS) significantly decreased (**p < 0.01) basal levels of trunk cortisol (A) but did not alter the body weight (B) of zebrafish compared to fish that were left untreated (N = 25 in each group).

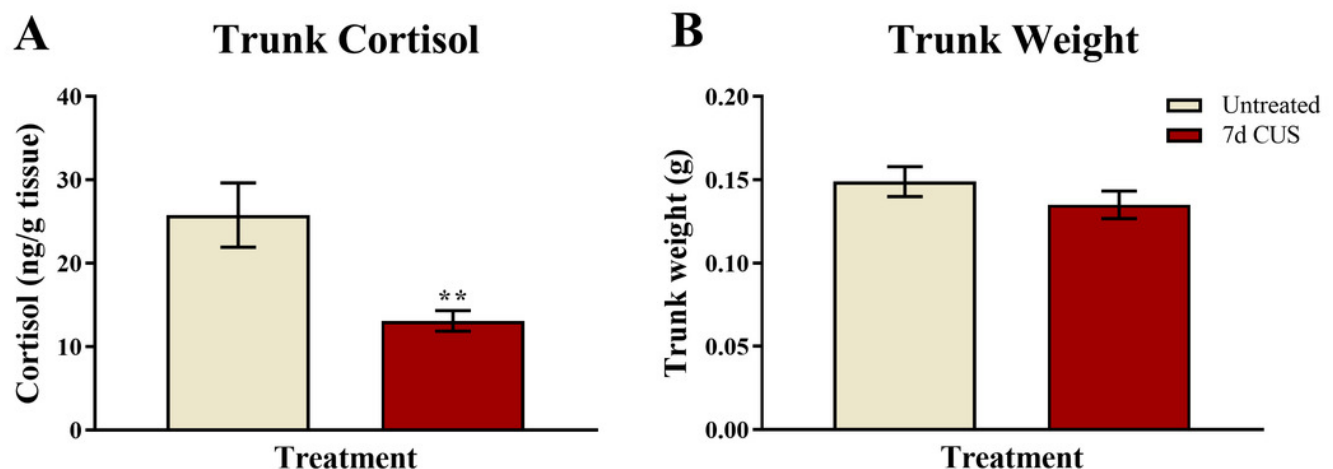



Figure 5

Motor measures of zebrafish in the novel tank test (Experiment 2)

In general, zebrafish gradually increased the total distance traveled per minute (A) and demonstrated increased ambulatory speeds (B) across the duration of the novel tank test (6 minutes), but there was no effect of chronic stress or drug treatment on these measures (N = 16-18 in each group).

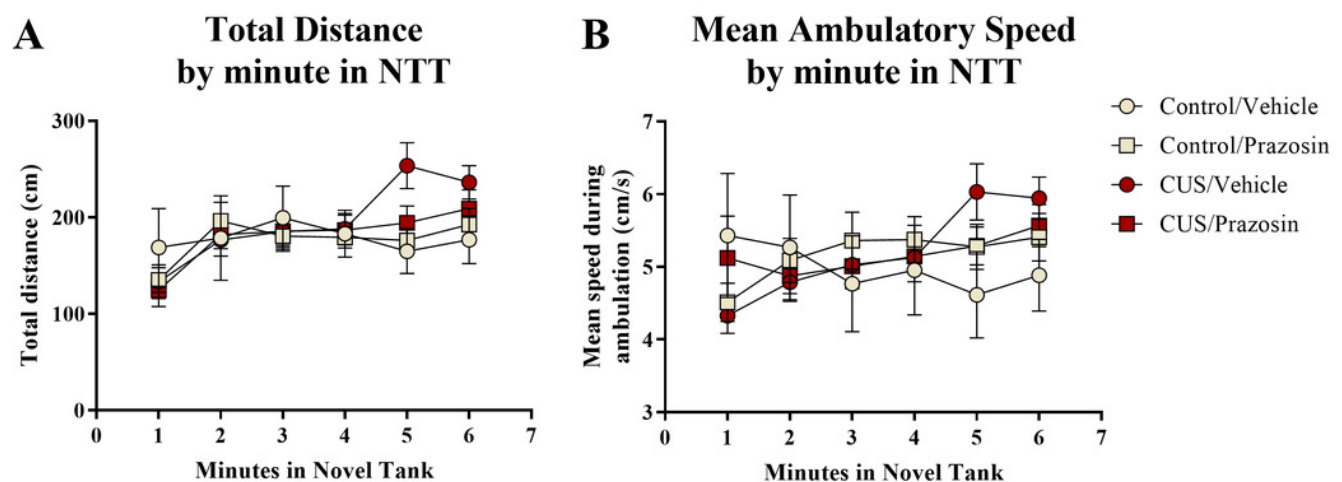



Figure 6

Freezing behavior of zebrafish in the novel tank test (Experiment 2)

In general, zebrafish spent less time immobile across the novel tank test (6 minutes), but there was no significant effect of chronic stress or drug treatment on immobility (N = 16-18 in each group).

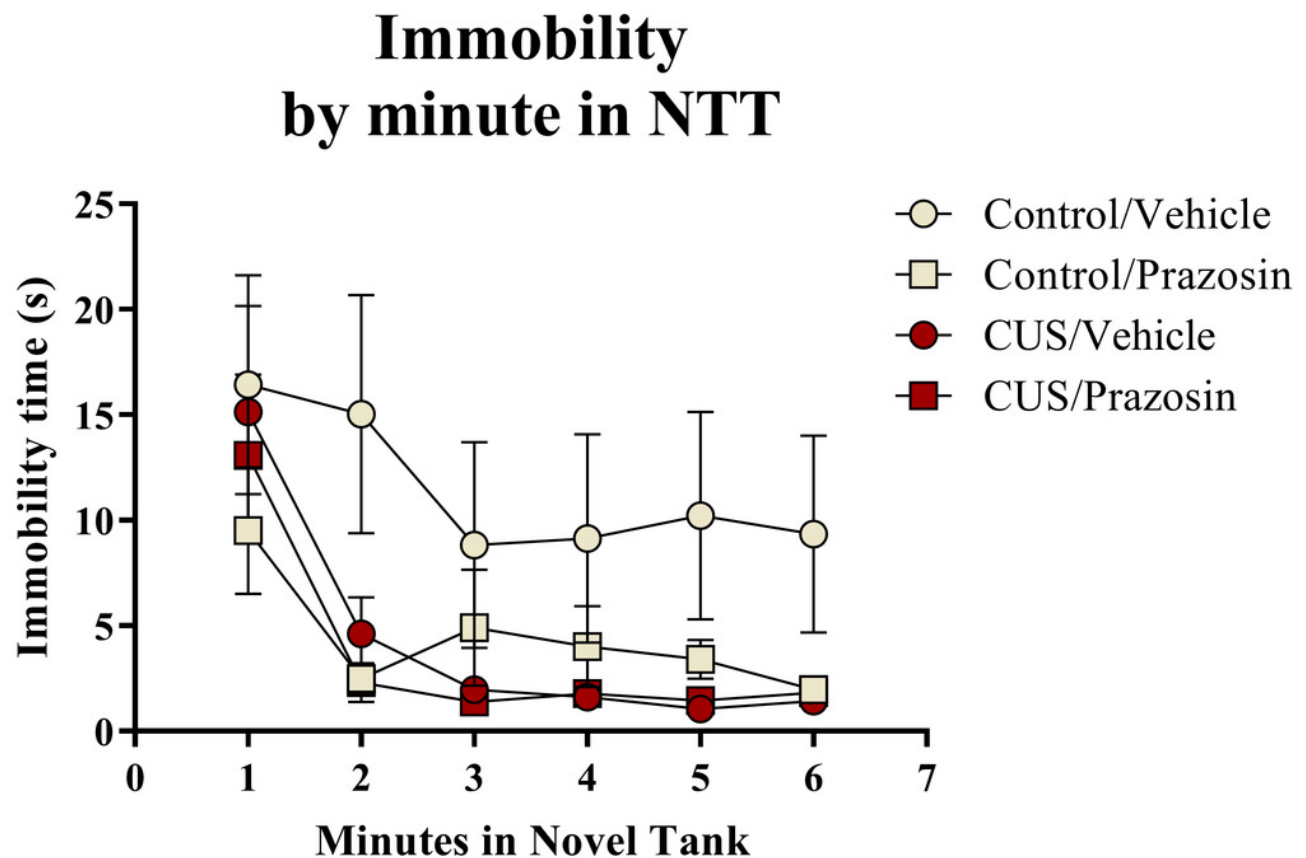



Figure 7

Exploratory measures of zebrafish in the novel tank test (Experiment 2)

In general, zebrafish explored the top of the novel tank more across the duration of the novel tank test (6 minutes) by (A) entering the top zone more frequently, (B) spending more time in the top zone, and (C) traveling a longer distance in the top zone. Zebrafish exposed to chronic unpredictable stress (CUS) for 7 days before drug or vehicle treatments entered the top zone significantly more times and traveled a significantly longer distance in the top compared to non-stressed subjects. If fish were chronically treated with prazosin, they spent significantly more time in the top of the tank compared to vehicle-treated fish (N = 16-18 in each group).

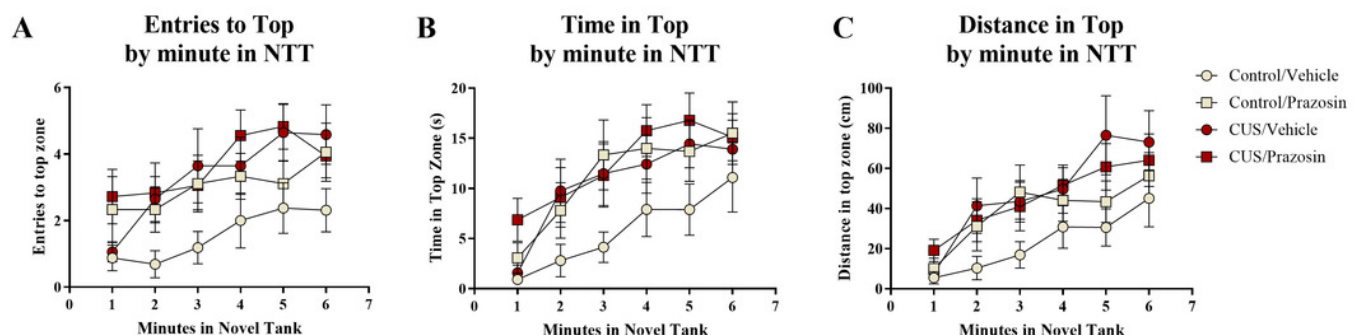
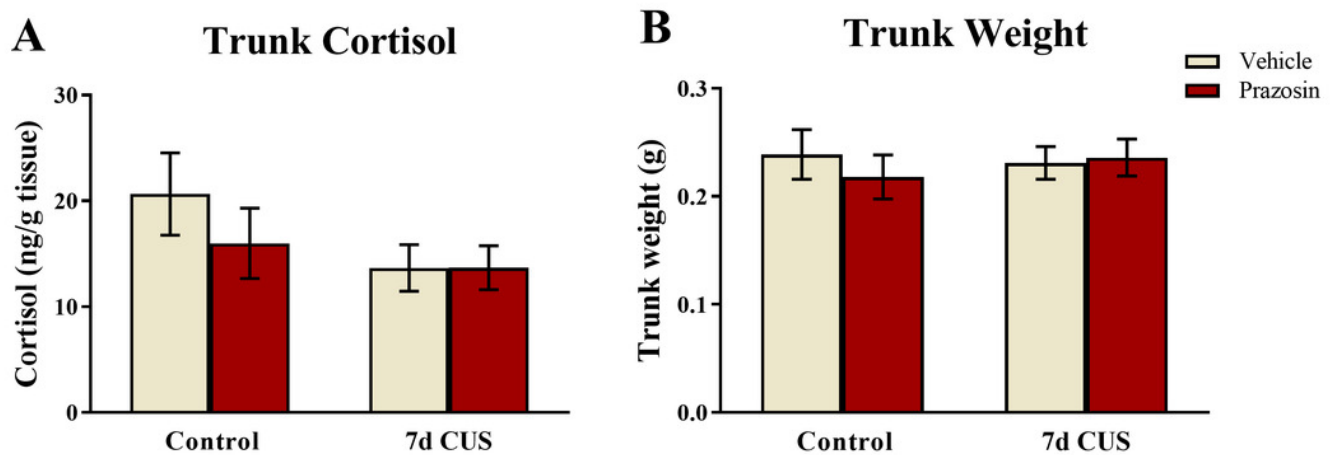



Figure 8

Cortisol and body weight measures of zebrafish (Experiment 2)

Neither chronic unpredictable stress treatment (CUS) and nor drug treatment significantly altered basal levels of trunk cortisol (A) or body weight (B) of zebrafish (N = 16-18 in each group).

Chronic unpredictable stress schedule for Experiment 1 and Experiment 2.

Seven stressors were randomized; fish in the stressed group were exposed to the cycle of stressors twice over 7 days. Subjects were exposed to stressors twice a day at random times during the light period between the times of 9 a.m. and 4 p.m.

1 **Table 1:** Chronic unpredictable stress schedule for Experiment 1 and Experiment 2.

2 Seven stressors were randomized; fish in the stressed group were exposed to the cycle of

3 stressors twice over 7 days. Subjects were exposed to stressors twice a day at random times

4 during the light period between the times of 9 a.m. and 4 p.m.

Experiment Day	Stressor	Time of day (Experiment 1)	Time of day (Experiment 2)
1	Tank changes (3 times)	11 a.m.	12 p.m.
1	Cooling (23°C, 30 min)	1 p.m.	1 p.m.
2	Lowered water (15 min)	3 p.m.	2 p.m.
2	Net chase (8 min + 15 min rest + 8 min)	4 p.m.	3 p.m.
3	Crowding (250 ml beaker, 60 min)	10 a.m.	10 a.m.
3	Heating (33°C, 30 min)	2 p.m.	4 p.m.
4	Social isolation (250 ml individual beakers, 45 min)	11 a.m.	9 a.m.
4	Tank changes (3 times)	12 p.m.	11 a.m.
5	Cooling (23°C, 30 min)	3 p.m.	11 a.m.
5	Lowered water (15 min)	4 p.m.	4 p.m.
6	Net chase (8 min + 15 min rest + 8 min)	1 p.m.	11 a.m.
6	Crowding (250 ml beaker, 60 min)	2 p.m.	4 p.m.
7	Heating (33°C, 30 min)	10 a.m.	10 a.m.
7	Social isolation (250 ml individual beakers, 45 min)	12 p.m.	1 p.m.

Overall behavioral measures of zebrafish in the novel tank test (Experiment 1)

Exposure to 7 days of chronic unpredictable stress (CUS) decreased the time spent in the top and marginally decreased the number of times fish entered the top zone of the novel tank in adult zebrafish compared to unstressed (control) fish (N = 25 in each group).

1   **Table 2:** Overall behavioral measures of zebrafish in the novel tank test (Experiment 1)

2   Exposure to 7 days of chronic unpredictable stress (CUS) decreased the time spent in the top and

3   marginally decreased the number of times fish entered the top zone of the novel tank in adult

4   zebrafish compared to unstressed (control) fish (N = 25 in each group).

	Control		7d CUS					
Variable	M	SD	M	SD	t	df	p	Cohen's d
Total distance moved (cm)	1082.44	225.84	1191.60	364.03	1.274	48	0.209	0.360
Mean ambulatory speed (cm/s)	5.03	0.81	5.30	0.96	1.077	48	0.287	0.305
Time immobile (s)	13.26	9.99	15.42	17.11	0.544	48	0.589	0.154
Number of entries to top	20.00	13.12	14.12	7.90	-1.920	48	0.061	-0.543
Total time in top (s)	65.96	44.75	43.82	26.49	-2.129	48	0.038	-0.602
Distance in top (cm)	233.58	160.16	182.32	112.61	-1.309	48	0.197	-0.370

Overall behavioral measures of zebrafish in the novel tank test (Experiment 2)

In adult zebrafish, exposure to 7 days of chronic unpredictable stress (CUS) increased the distance traveled in the top and the number of top zone entries whereas 7 days of chronic prazosin treatment increased the time spent in the top of zone of the novel tank test (N = 16-18 in each group). See text for results of significance testing.

1 **Table 3:** Overall behavioral measures of zebrafish in the novel tank test (Experiment 2)

2 In adult zebrafish, exposure to 7 days of chronic unpredictable stress (CUS) increased the

3 distance traveled in the top and the number of top zone entries whereas 7 days of chronic

4 prazosin treatment increased the time spent in the top of zone of the novel tank test (N = 16-18 in

5 each group). See text for results of significance testing.

Variable	Control/Vehicle N = 16		Control/Prazosin N = 18		CUS/Vehicle N = 17		CUS/Prazosin N = 18	
	M	SD	M	SD	M	SD	M	SD
Total distance moved (cm)	1075.90	611.65	1070.93	206.33	1162.93	349.64	1097.22	320.95
Mean ambulatory speed (cm/s)	5.83	2.60	5.21	1.07	5.31	1.05	5.13	0.96
Time immobile (s)	67.71	108.90	24.05	28.06	24.06	27.47	19.93	18.79
Number of entries to top	9.19	9.68	17.39	10.49	19.47	13.07	20.94	13.30
Total time in top (s)	34.78	40.07	67.42	44.41	63.59	45.87	74.91	49.61
Distance in top (cm)	140.69	160.39	236.92	173.72	294.23	207.33	271.54	161.64

Results of repeated measures ANOVA (Experiment 2)

Adult zebrafish from all treatment groups generally habituated to the novel tank across the duration of the test (6 minutes), but there was no significant interaction between time and drug treatment or stress on any of the behavioral measures of anxiety (N = 16-18 in each group). Significance for all dependent variables were determined with a repeated-measures ANOVA with Greenhouse-Geisser correction.

1 **Table 4:** Results of repeated measures ANOVA (Experiment 2)

2 Adult zebrafish from all treatment groups generally habituated to the novel tank across the

3 duration of the test (6 minutes), but there was no significant interaction between time and drug

4 treatment or stress on any of the behavioral measures of anxiety (N = 16-18 in each group).

5 Significance for all dependent variables were determined with a repeated-measures ANOVA

6 with Greenhouse-Geisser correction.

	Total distance (cm)		Mean ambulatory speed (cm/s)		Time immobile (s)		Number of entries to top		Total time in top (s)		Distance in top (cm)	
	F	**p**	**F**	**p**	**F**	**p**	**F**	**p**	**F**	**p**	**F**	**p**
Within-subjects Effects												
Time	9.795	**<0.001**	2.022	0.133	15.136	**<0.001**	7.112	**<0.001**	15.075	**<0.001**	13.679	**<0.001**
Time* Stress	4.262	**0.005**	2.134	0.118	1.705	0.190	0.766	0.545	0.375	0.822	0.898	0.452
Time* Drug	0.573	0.644	0.240	0.802	0.948	0.381	0.425	0.785	0.215	0.927	0.350	0.811
Time* Stress* Drug	1.811	0.136	3.226	**0.039**	0.392	0.651	0.771	0.541	0.920	0.451	0.620	0.620
Between-subjects Effects												
Stress	0.308	0.581	0.118	0.732	3.050	0.085	5.886	**0.018**	2.768	0.101	5.100	**0.027**
Drug	0.191	0.663	0.050	0.824	2.931	0.092	3.131	0.082	4.062	**0.048**	0.750	0.390
Stress* Drug	0.100	0.753	0.123	0.727	2.018	0.160	1.431	0.236	0.956	0.332	1.876	0.176