1   Nicotine prevents anxiety-like behavioral responses in zebrafish

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Abstract

Anxiety-related disorders are severe psychiatric conditions that involve complex physiological and behavioral maladaptive responses. The use of conspecific alarm substance (CAS) for inducing anxiety-like behaviors in fish species provides important translational insights of how aversive conditions modulate neurobehavioral functions. Because nicotine may elicit anxiolytic-like responses, here we investigated whether acute nicotine exposure prevents CAS-induced anxiogenic-like behaviors in zebrafish. We used both novel tank and light-dark tests as two well-established paradigms for measuring anxiety-like behavior. Fish were individually exposed to 1 mg/L nicotine or non-chlorinated water for 3 min and then transferred to other tanks in the absence or presence of 3.5 mL/L CAS for 5 min. Later, the behavior of fish was tested in the novel tank test or in the light-dark preference test. As expected, CAS triggered aversive behaviors by increasing bottom dwelling, freezing, erratic movements, scototaxis, and risk assessment episodes. Nicotine alone elicited anxiolytic-like behaviors, since it increased the time spent in top area, as well as the average duration of entry in the lit compartment. Moreover, nicotine pretreatment prevented CAS-induced aversive responses without changing locomotion, suggesting that anxiolysis could play a role, at least in part, to the behavioral effects of nicotine observed here. Overall, these novel findings show beneficial effects of nicotine on anxiogenic responses in zebrafish. We also reinforce the practical advantages of this aquatic species to explore the relieving properties of nicotine, as well as to understand the neurobiological bases involved in anxiety-related disorders and associated therapeutic targets.

Keywords: anxiety-related disorders; conspecific alarm substance; nicotine; aversive behaviors; zebrafish.

1. Introduction

Anxiety-related disorders are one of the most prevalent psychiatric conditions worldwide, with multiple genetic and environmental determinants (Bartlett et al., 2017; Bishop, 2007). Psychological and physical symptoms include muscular tension, irritability, agitation, concentration problems, palpitations, dizziness, and epigastric discomfort (Peres et al., 2017; Shoham et al., 2018). Mounting evidence suggests that different neurotransmitter systems, including the cholinergic neurotransmission, modulate anxiety-like behaviors (File et al., 2000b; Zarrindast and Khakpai, 2015).

Nicotine influences a large number of physiological processes, such as learning and memory, locomotion, and anxiety (Jones et al., 1999; Zarrindast and Khakpai, 2018; Ziani et al., 2018). This molecule is an alkaloid extracted from the tobacco plant (Powledge, 2004) composed of pyridine and a pyrrolidine ring [1-methyl-2-(3-pyridyl) pyrrolidine], which binds to nicotinic acetylcholine receptors (nAChRs) (Lester et al., 2009). As an agonist of nAChRs, nicotine elicits various effects on the central nervous system (CNS) depending on site and receptor composition (Bencan and Levin, 2008; File et al., 2000a). A dualistic role of nicotine has been shown in experimental models, where it promotes anxiolysis or anxiogenic-like effects following acute and chronic exposure, respectively (Falco and Bevins, 2015; Singer et al., 2016; Stewart et al., 2015).

Although rodents have been widely used to assess the effects of nicotine on anxiety-related behaviors, there is a growing utility of zebrafish (*Danio rerio*) models in translational neuropsychiatric research (Fontana et al., 2018; Stewart et al., 2014). The advantages that make zebrafish an excellent tool include the efficient rate of absorption of substances through water (de Abreu et al., 2018), their evolutionarily conserved neurotransmitter systems when compared to the mammalian counterparts (Rico et al., 2011), their well-characterized behaviors (Kalueff et al., 2013), and the pharmacological

80   sensitivity to anxiolytic and anxiogenic drugs (Egan et al., 2009; Mezzomo et al., 2018;

81   Rosemberg et al., 2012). Moreover, the presence of lateral pallium and habenula,

82   analogous brain structures to mammalian hippocampus and amygdala, respectively,

83   makes zebrafish a suitable model organism to investigate the neural bases of emotion and

84   aversive responses (Champagne et al., 2010; Fontana et al., 2018).

85   In zebrafish, the conspecific alarm substance (CAS) exposure elicits anxiety-like

86   behaviors (Canzian et al., 2017; Speedie and Gerlai, 2008). This substance is released

87   when epidermal club cells are injured, reflecting a potential predator risk (Quadros et al.,

88   2016). CAS triggers exacerbated defensive behaviors, as well as prolonged aversive

89   responses (Lima et al., 2016; Maximino et al., 2018). Two well-established models for

90   measuring anxiety-like behavior are the novel tank diving test (Egan et al., 2009; Levin

91   et al., 2007) and the light-dark preference test (Maximino et al., 2010). While in the first

92   task animals show a typical geotaxis and gradually habituate to novelty stress (Wong et

93   al., 2010), in the light-dark test, zebrafish usually prefers the dark compartment of the

94   apparatus, thereby facilitating crypsis (Maximino et al., 2010). Anxiolytic drugs increase

95   the exploration of the lit area, whereas anxiogenic compounds exacerbate dark preference

96   as a protective response (Mezzomo et al., 2016).

97   **Because nicotine acutely induces anxiolysis in zebrafish, (Klee et al., 2011;**

98   **Levin et al., 2007; Singer et al., 2016), we hypothesize the occurrence of relieving**

99   **properties of nicotine when fish are challenged with an acute chemical stressor.**

100   Thus, the goal of our study was to investigate whether a short-term nicotine exposure

101   prevents CAS-induced anxiogenic-like behaviors in zebrafish using the novel tank and

102   the light-dark preference tests.

103

104   **2. Methods**

105 *2.1. Animals and housing*

106   Subjects were adult zebrafish (*Danio rerio*, 3–4 months-old) from the short-fin

107 phenotype (~50:50 male:female ratio) obtained from a local distributor (Hobby Aquários,

108 RS, Brazil). Fish were kept in 40 L tanks at a maximum density of 2 fish per liter and

109 acclimatized in the laboratory for two weeks before the experiments. Tanks were

110 maintained under constant aeration and mechanical filtration and the water temperature

111 was set at 27 ± 1ºC, pH 7.0–7.2. Room illumination was provided by fluorescent light

112 tubes with photoperiod 14h light and 10h dark. Animals were fed thrice daily with

113 commercial flake fish food (Alcon BASIC™, Alcon, Brazil). After the experiments, fish

114 were anesthetized in water at 4°C and then euthanized by section of the spinal cord.

115 Animals were maintained in accordance with the National Institute of Health Guide for

116 Care and Use of Laboratory Animals. **Experiments were run in multiple days using**

117 **three independent batches to ensure data reproducibility.** All experimental protocols

118 were approved by the Ethics Commission on Animal Use of the Federal University of

119 Santa Maria (protocol number 6894010616).

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121 *2.2. Nicotine exposure*

122   S(-)-Nicotine (98%) was purchased from Sigma-Aldrich™ (St. Louis, MO, USA).

123 Fish were exposed to 1 mg/L of nicotine added in tank water for 3 min as described

124 elsewhere (Singer et al., 2016; Ziani et al., 2018). Later, animals were exposed to CAS

125 and behavioral tests were performed. Control group was handled in a similar manner but

126 kept in non-chlorinated water in the absence of nicotine.

127

128 *2.3. Conspecific alarm substance preparation*

129   Conspecific alarm substance was extracted from donor fish previously euthanized.

130 Briefly, fish were placed in a Petri dish kept on ice and CAS were obtained through 10-

131 15 superficial shallow cuts in epidermal cells with a razor blade (Egan et al., 2009; Lima

132 et al., 2016; Quadros et al., 2016). Animals were then washed on both sides with 10 mL

133 of distilled water per fish for preparing a CAS stock solution. The exposure was

134 performed using 3.5 mL/L of CAS solution for 5 min, which elicits robust defensive

135 responses in zebrafish (Canzian et al., 2017; Maximino et al., 2018; Speedie and Gerlai,

136 2008). For control group, only distilled water was added in the experimental tanks. **Fig.**

137 **1** summarizes all experimental procedures, behavioral tests, and the groups assessed.

138

139 *2.4. Behavioral analyses*

140 All behaviors were recorded for 6 min using a webcam connected to a laptop at

141 30 frames/s with appropriate video-tracking software (Any-Maze™, Stoelting, CO,

142 USA). **Importantly, all treatments described above were run in a randomized order**

143 **and fish were not originated from the same housing tank. Randomization was**

144 **performed using a computerized random number generator (www.random.org) and**

145 **a 50:50 male:female ratio was separated per group. Because the exposure tanks were**

146 **assigned by a code (performed by a researcher who did not participate in the**

147 **experiments), investigators were blind to the experimental conditions. Codification**

148 **was revealed only when data were analyzed. Two experimental apparatus were used**

149 **per group and since no tank effects or gender influence were observed (data not**

150 **shown), data were pooled for subsequent analyses.**

151

152 *2.4.1. Novel tank diving test*

153 After the exposure period, fish were individually transferred to the novel tank

154 apparatus (25 cm length x 15 cm height x 6 cm width), which was virtually divided in

155 two segments (bottom area and top area) and filled with 2 L home tank water. Locomotor

activity and vertical explorations were measured during habituation to novelty (Egan et al., 2009; Levin et al., 2007; Rosemberg et al., 2011) and the following endpoints were determined: distance traveled, absolute turn angle, maximum speed, transitions and time spent in top, latency to enter the top, number and duration of freezing, number and duration of erratic movements. **The absolute turn angle reports the sum of the absolute angle between each movement vector of the animal, with anti-clockwise movement being negative and clockwise movement being positive (*i.e.* the angle is from -180° to 180°).** Freezing was defined as a complete immobility of fish (≥ 2 s) with concomitant increased opercular beat rate, while erratic movements were defined as fast swimming bouts with sudden changes in direction (Kalueff et al., 2013). Both freezing and erratic movements were manually counted by two trained observers (inter-rater reliability $\geq$ 0.85) blinded to the experimental condition of fish.

2.4.2. Light-dark test

Following nicotine and CAS exposure period, fish were individually transferred to the light-dark tank based on the protocol described elsewhere (Maximino et al., 2010). The apparatus (25 cm length × 10 cm depth × 15 cm height) was divided into two equally sized compartments by opaque self-adhesive plastic in white and black colors, covering the floor and walls. The respective behaviors were assessed: time spent in lit area, shuttling, average duration of entry in the lit area, and number of risk assessments. A risk assessment episode was defined as a fast (≤ 1 s) or partial entry into the lit area with a subsequent return to the dark compartment, which reflects a conflict between the motivation to explore and avoid unknown environments (Kalueff et al., 2013). Risk assessment episodes were manually counted by two trained observers (inter-rater reliability $\geq$ 0.85) blinded to the experimental condition.

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182   *2.5. Statistical analyses*

183   **Sample sizes were estimated *a priori* based on pilot experiments using G-**

184   **Power 3.1 software. For the novel tank test, we considered an alpha = 0.05,**

185   **power = 0.85, and effect size = 0.4 (resulting in *n* = 14 per group), while for the light-**

186   **dark test we considered an alpha = 0.05, power = 0.85, and effect size = 0.5 (resulting**

187   **in *n* = 10 per group)**. Normality of data and homogeneity of variances were analyzed

188   using Kolmogorov–Smirnov and Bartlett's tests, respectively. **Due to the parametric**

189   **distribution and data homoscedasticity, results were expressed as means ± standard**

190   **error of means (S.E.M.) and analyzed by two-way analysis of variance (ANOVA),**

191   **followed by Student-Newman-Keuls multiple comparison test whenever necessary.**

192   The inter-rater reliability was estimated using Spearman correlation and all significances

193   were set at $p \leq 0.05$.

194

195   **3. Results**

196   *3.1. Nicotine prevents CAS-induced aversive responses without changing locomotion*

197   **Fig. 2** shows the behavioral effects of nicotine and CAS in the novel tank test.

198   Independently of the treatment, no significant changes were observed in locomotor-

199   related parameters **(Fig. 2A)**. Regarding the vertical activity **(Fig. 2B)**, **nicotine**

200   **increased the time spent in top ($F_{(1,53)} = 25.57$, $p < 0.0001$) and prevented CAS-**

201   **induced effects on this behavior ($F_{(1,53)} = 16.83$, $p = 0.0001$ for the interaction term).**

202   **Both CAS and NIC/CAS groups showed reduced transitions to top area ($F_{(1,53)} =$**

203   **17.01, $p = 0.0001$) and CAS also increased the latency to enter the top ($F_{(1,53)} = 8.172$,**

204   **$p = 0.0061$) when compared to control.** Fig. 3 shows the effects of nicotine and CAS on

205   aversive behaviors. **We observed a significant nicotine x CAS interaction for the**

206   **number ($F_{(1,53)} = 5.189$, $p = 0.0268$) and duration of freezing ($F_{(1,53)} = 13.05$, $p =$**

0.0007), as well as for the number ($F_{(1,53)}$ = 9.792, p = 0.0028), and duration of erratic movements ($F_{(1,53)}$ = 8.394, p = 0.0055). In general, CAS increased defensive behaviors and nicotine prevented these effects.

3.2. Nicotine prevents CAS-induced exacerbation on scototaxis

Fig. 4 shows the behavioral effects of nicotine and CAS in the light-dark apparatus. **CAS decreased the time spent in lit area, while nicotine showed a preventive effect on this behavioral endpoint ($F_{(1,36)}$ = 5.099, p = 0.0301 for the interaction term).** Nicotine-treated fish showed reduced shuttling ($F_{(1,36)}$ = 12.04, p = 0.0014) and increased average duration of entry in the lit area in the absence and presence of CAS ($F_{(1,36)}$ = 15.37, p = 0.0004). **Nicotine also abolished the effects of CAS on the number of risk assessment episodes ($F_{(1,36)}$ = 8.655, p = 0.0056, for the interaction term).**

4. Discussion

The current study examined the behavioral effects of nicotine pretreatment on CAS-induced aversive responses in zebrafish. Evidence shows that acute nicotine treatment in this aquatic species positively modulates cognitive responses and exerts anxiolytic-like responses in various behavioral tasks (Eddins et al., 2009; Levin et al., 2007; Singer et al., 2016; Ziani et al., 2018). Since CAS exacerbates defensive behaviors, it has been considered a chemical cue that elicits behavioral phenotypes that closely parallel those observed in patients with anxiety-, stress- and/or trauma-related disorders (Canzian et al., 2017; Lima et al., 2016; Maximino et al., 2018). To our knowledge, our data represent the first evidence showing that nicotine prevents anxiogenic-like behaviors in zebrafish, possibly due to its anxiolytic actions following an acute exposure.

232   In the last decade, zebrafish has emerged as an attractive model organism in

233   psychiatry and translational neuroscience research to investigate the neural bases

234   involved in anxiety-like responses (Fontana et al., 2018; Stewart et al., 2014). The novel

235   tank and the light-dark tests are suitable protocols to evaluate defensive responses in

236   zebrafish (Maximino et al., 2012). While in the novel tank diving test the motivational

237   aspect is surface escaping (Blaser and Rosemberg, 2012), the main stimulus in the light-

238   dark test is scototaxis (Maximino et al., 2012). Similar to previous findings, we observed

239   that CAS-exposed fish exhibit typical behavioral patterns during habituation to novelty

240   stress, such as increased erratic movements, freezing, and geotaxis (Quadros et al., 2016;

241   Speedie and Gerlai, 2008). Zebrafish also showed robust scototaxis, as well as more risk

242   assessment episodes in the light-dark test. These set of data reflect typical behavioral

243   patterns associated with anxiety-like responses following acute CAS exposure.

244   Importantly, nicotine-treated fish showed increased the average duration of entry in the

245   lit area in the light-dark test and spent more time in top area in the novel tank diving test,

246   suggesting anxiolysis. Moreover, nicotine pretreatment abolished CAS-mediated

247   responses, revealing a preventive effect on aversive behaviors. Because CAS and nicotine

248   did not affect locomotion, the responses measured here reflect changes on anxiety-like

249   behaviors instead of a simple modulation of motor patterns.

250   **Evidence shows that CAS is naturalistic alarm cue responsible for**

251   **triggering alarm reactions in ostariophysian (Maximino et al., 2018; Quadros et al.,**

252   **2016). Although its exact chemical composition has not been fully elucidated,**

253   **aversive responses are attributed to the presence of hypoxanthine 3-N-oxide and**

254   **chondroitin sulphate, which show similar effects when tested alone (Parra et al.,**

255   **2009). Importantly, the occurrence of aversive behaviors (*e.g.*, freezing, erratic**

256   **movements, and bottom-dwelling) is highly reproducible in the literature (Canzian**

et al., 2017; Egan et al., 2009; Maximino et al., 2018). A single CAS exposure elicits prolonged avoidance to the conditioning side in the conditioned place aversion paradigm, possibly due to a time-dependent sensitization of stress response (Lima et al., 2016; Maximino et al., 2018) . Thus, different from other anxiogenic agents, a brief CAS exposure normally induces prominent behavioral phenotypes paralleling those observed in post-traumatic stress disorder (Lima et al., 2016). Although the mechanisms underlying the behavioral effects of CAS are still poorly explored, an involvement of the CNS in CAS-mediated behaviors has been postulated. For example, CAS activates *c-fos* expression in habenula, a brain region responsible for fear responses in the teleost fish (Ogawa et al., 2014). Various signaling molecules, such as biogenic amines, amino acids, peptides, and steroids play a role in anxiety, thereby modulating defensive behaviors (Benson et al., 2015; Strohle and Holsboer, 2003). **In zebrafish, CAS acutely activates sympathetic nervous system and increases whole-body cortisol, blood glucose, norepinephrine, and epinephrine, as well as serotonin and 5-hydroxyindoleacetic acid levels in the CNS, culminating in 'fight or flight' responses (Maximino et al., 2014; Mezzomo et al., 2019; Quadros et al., 2018). Furthermore, an influence of cholinergic system in aversive responses of zebrafish is predicted, since CAS increases acetylcholinesterase activity in the brain (Canzian et al., 2017) and nicotine facilitates contextual fear conditioning following a single exposure to alarm cues (Ziani et al., 2018). These set of data reinforce the growing utility of CAS as a naturalistic stimulus to model alarm reactions and aversive responses in translational neurobehavioral research.**

Anxiolytic compounds are commonly used to reduce the frequency of aversive behaviors. In humans, smoking is considered a key factor to minimize anxiety due to the relieving properties of nicotine (Fidler and West, 2009). Nicotine may promote anxiolysis

282   or anxiogenesis depending on the species, concentration, administration route, and

283   exposure period (Sackerman et al., 2010; Zarrindast and Khakpai, 2018). Acutely, the

284   activation of nAChRs also facilitates the release of GABA (Maggi et al., 2001), which

285   could play a role in anxiolytic effects of nicotine (Sullivan and Covey, 2002). Similar to

286   mammals, zebrafish show high sensitivity to nicotine, which elicits consistent and robust

287   anxiolytic-like responses following acute exposure (Levin et al., 2007; Sackerman et al.,

288   2010; Singer et al., 2016). Importantly, the administration of methyllycaconitine and

289   dihydro-β-erythroidine increases bottom dwelling in the presence of nicotine, implying a

290   key role of both α7 and α4β2 nicotinic receptors in nicotine-mediated anxiolysis (Bencan

291   and Levin, 2008). **Since most neuronal nAChRs in the brain modulate the release of**

292   **various neurotransmitters that influence mood and anxiety (*e.g.*, aetylcholine,**

293   **serotonin, glutamate, and dopamine) (Dani and Bertrand, 2007), the underlying**

294   **mechanisms involved in the preventive role of nicotine against CAS-mediated**

295   **responses still require further scrutiny.**

296

297   **5. Conclusion**

298   To our knowledge, this is the first study showing a positive effect of nicotine on

299   anxiogenic responses in zebrafish, supporting the growing utility of this aquatic species

300   to investigate the neurobehavioral effects of nicotine in vertebrates. Because distinct

301   neurotransmitter systems regulate anxiety, thereby modulating defensive behaviors, the

302   use of zebrafish is a promising tool to assess the molecular mechanisms underlying

303   nicotine-mediated anxiolysis. **Our results also further support a deeper**

304   **pharmacological investigation of the cholinergic signaling as a potential mechanism**

305   **associated to fear responses in translational neuropsychiatric research.** Overall,

306   paralleling clinical and rodent studies, our data strengthen the beneficial properties of

307 nicotine in zebrafish, reinforcing practical advantages to explore the neurobiological

308 bases involved in emotional impairments and correlated behaviors.

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321

322 **Conflict of Interest**

323 The authors declare that no competing interests exist.

324

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471 **Figures**
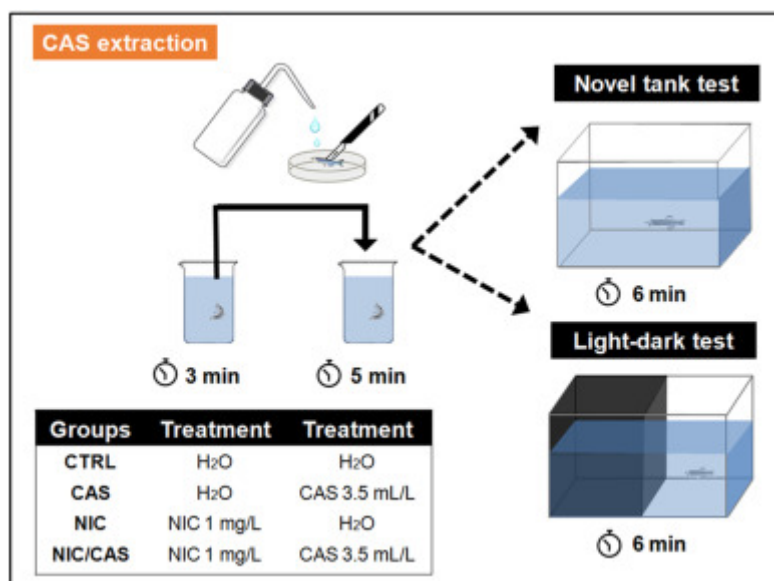

473 **Fig. 1.** Schematic representation of the experimental design and behavioral tests used for

474 assessing the effects of nicotine on CAS-induced anxiogenic-like responses.
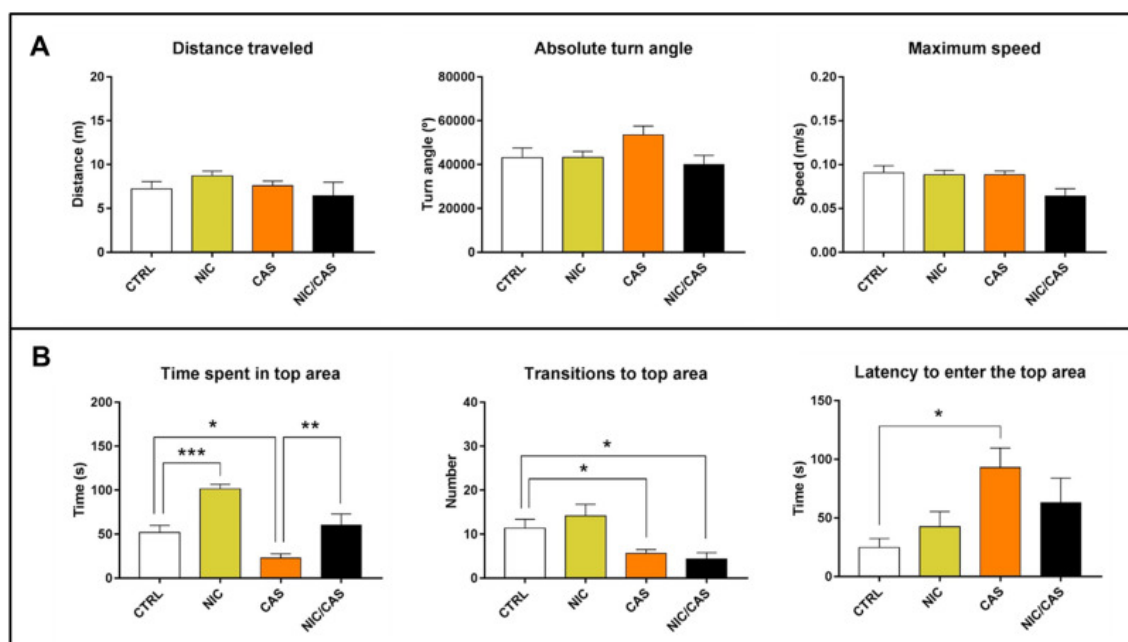
476 **Fig. 2.** Locomotor and exploratory activities of zebrafish in the novel tank test. **(A)**

477 Locomotion-related behavioral endpoints. **(B)** Vertical exploration. Data were expressed

478 as means ± S.E.M. and analyzed by two-way ANOVA, followed by Student-Newman-

479 Keuls multiple comparison test whenever necessary (* $p < 0.05$; ** $p < 0.01$; *** $p <$

480 0.005, *n* = **14 per group**).
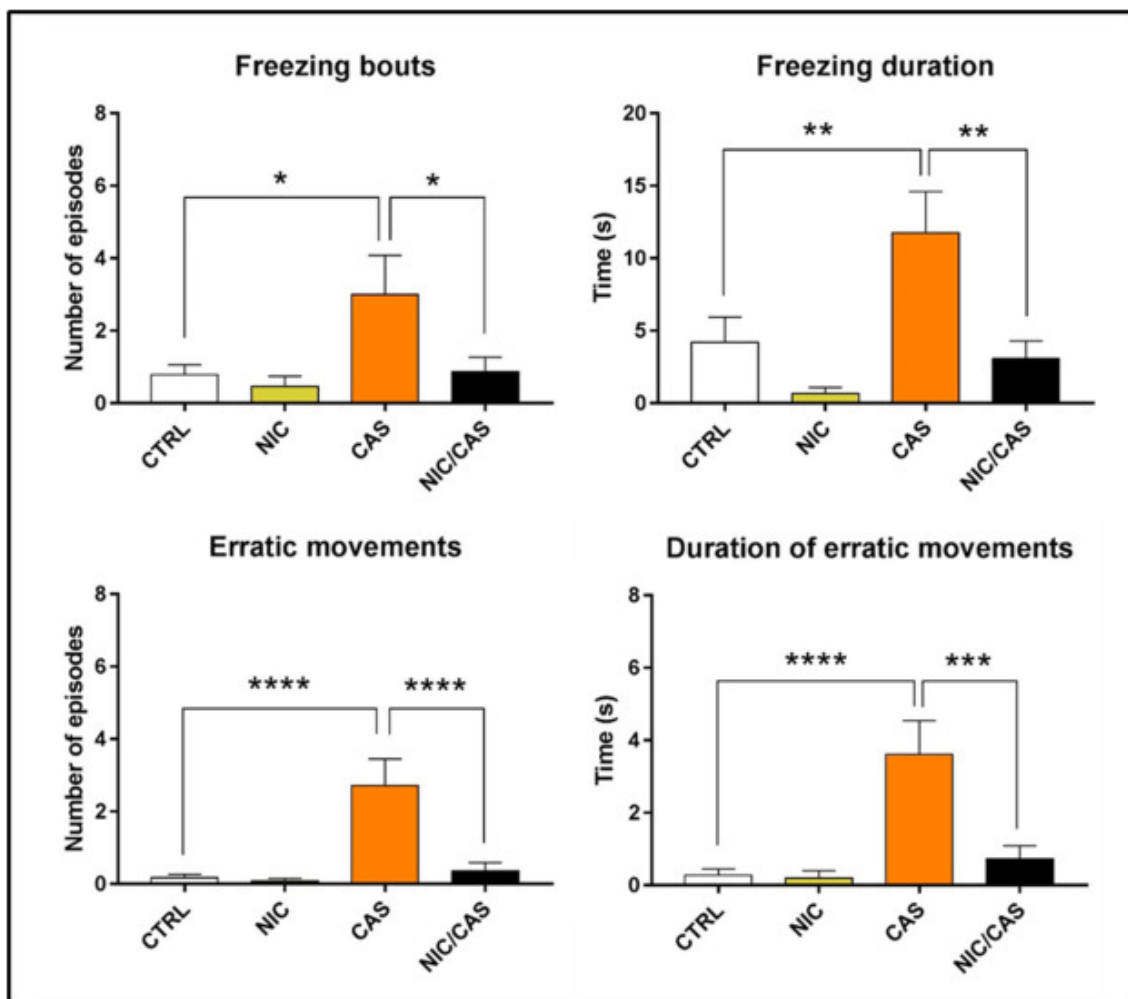
Fig. 3. Preventive effects of nicotine on CAS-induced aversive behaviors. Data were expressed as means ± S.E.M. and analyzed by two-way ANOVA, followed by Student-Newman-Keuls multiple comparison test whenever necessary (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$; **** $p < 0.001$, *n* = **14 per group**).
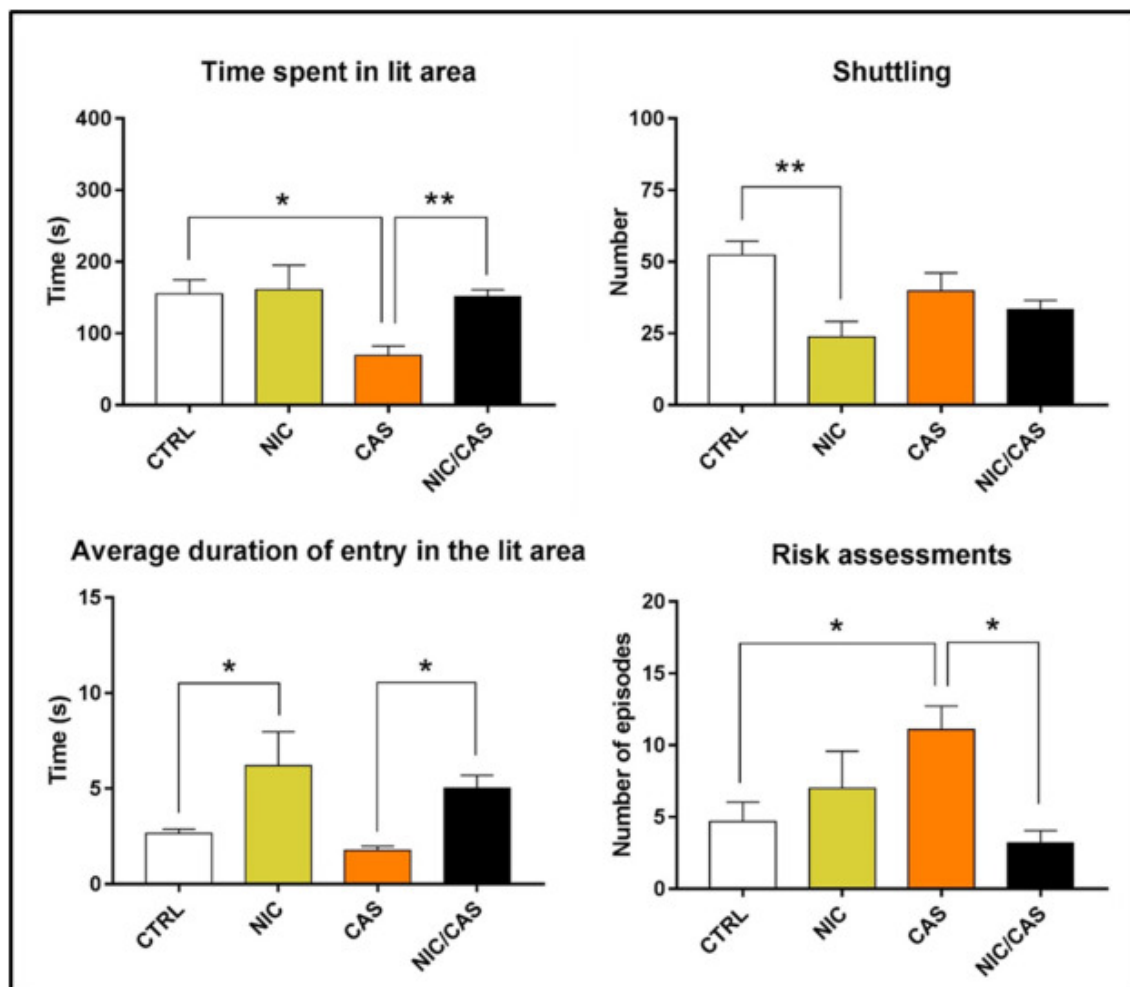
Fig. 4. Effects of nicotine and CAS on scototaxis. Data were expressed as means ± S.E.M. and analyzed by two-way ANOVA, followed by Student-Newman-Keuls multiple comparison test whenever necessary (* $p < 0.05$; ** $p < 0.01$, **$n = 10$ per group**).