

1 Understanding the neurobiological effects of drug abuse: lessons from zebrafish  
2 models

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**32 Abstract**

33 Drug abuse and brain disorders related to drug consumption are public health problems  
34 with harmful individual and social consequences. The identification of therapeutic targets and  
35 precise pharmacological treatments to these neuropsychiatric conditions associated with drug  
36 abuse are urgently needed. Understanding the link between neurobiological mechanisms and  
37 behavior is a key aspect of elucidating drug abuse-related targets. Due to various molecular,  
38 biochemical, pharmacological, and physiological features, the zebrafish (*Danio rerio*) has been  
39 considered a suitable vertebrate for modeling complex processes involved in drug abuse  
40 responses. In this review, we discuss how the zebrafish has been successfully used for modeling  
41 neurobehavioral phenotypes related to drug abuse and review the effects of opioids, cannabinoids,  
42 alcohol, nicotine, and psychedelic drugs on the central nervous system (CNS). Moreover, we  
43 summarize recent advances in zebrafish-based studies and outline potential advantages and  
44 limitations of the existing zebrafish models to explore the neurochemical bases of drug abuse and  
45 addiction. Finally, we discuss how the use of zebrafish models may present fruitful approaches to  
46 provide valuable clinically translatable data.

47  
48 **Keywords:** zebrafish-based models; abuse drugs; neurobehavioral assays; addictive behaviors;  
49 brain disorders.

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## 59 1. Introduction

60 Drug abuse is a public health problem with severe negative consequences for the  
61 individual and the society leading to increased risk for disability and premature death (WHO,  
62 2014). In 2015, 450,000 people died as a result of drug use (WDR, 2017), mortality that is mostly  
63 attributed to the use of legal drugs, such as tobacco and alcohol, and less so to illicit drugs  
64 (Peacock et al., 2018).

65 The etiology of brain disorders caused by drug abuse is complex, involving a combination  
66 of factors that include individual genotype, social environment, and age or developmental stage  
67 (Samochowiec et al., 2014; Wall et al., 2016). In general, drugs act by changing neuronal function  
68 at molecular, cellular, circuitry, macro-structural, and numerous other functional levels leading to  
69 physiological and behavioral alterations (Berridge, 2017; Volkow et al., 2016). Understanding the  
70 link among genes, brain structure, neuronal function, and behavior is a key aspect of identifying  
71 new therapeutic targets aimed at pharmacological treatment of addiction (NIDA, 2016).

72 Non-traditional model organisms, including the zebrafish (*Danio rerio*), have been  
73 successfully used for modeling complex processes involved in drug abuse responses (Mathur and  
74 Guo, 2010; Stewart et al., 2011). Neurobehavioral assays specifically developed or adopted for the  
75 zebrafish provide useful methods to explore reinforcing effects of drugs, and to study drug use  
76 associated phenomena including sensitization, tolerance, withdrawal, drug seeking, extinction, and  
77 relapse (Braida et al., 2007; Cachat et al., 2010; Darland and Dowling, 2001; Kily et al., 2008;  
78 Ninkovic and Bally-Cuif, 2006; Petzold et al., 2009). The zebrafish may serve as a valuable tool  
79 for high-throughput large scale behavioral screening for compounds (small molecules) affecting  
80 drug use and abuse-related responses as well as for mutations modifying drug effects. Such  
81 screens therefore may identify efficacious drugs as well as molecular targets for which such drugs  
82 may be developed in an efficient manner.

83 Here, we discuss the growing utility of zebrafish models to investigate the neural  
84 mechanisms related to drug abuse, focusing on how opioids, cannabinoids, alcohol, nicotine, and  
85 psychedelic drugs influence neurochemical and behavioral functions in zebrafish. These drugs of

86 abuse were selected based on results from official world reports (WDR, 2017; WHO, 2014, 2015,  
87 2018) that demonstrate the high human consumption, prevalence and harmful health effects of  
88 these substances.

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## 90 **2. Why zebrafish has been considered a suitable model organism for studying drug abuse?**

91 The zebrafish is a promising non-traditional organism for assessing the effects of drug  
92 abuse on the brain. Drug abuse-related research with both adult and larval zebrafish has been  
93 found to be translationally relevant. For example, due to the evolutionary conservation of  
94 biological mechanisms associated with drug abuse related processes between fish and mammals,  
95 the zebrafish have been found to respond to human (mammalian) drugs in a predictable manner  
96 (Klee et al., 2012; Stewart et al., 2011). Moreover, similar to humans, the zebrafish show drug  
97 abuse-related phenomena, including development of tolerance and withdrawal syndrome (Klee et  
98 al., 2012; Mathur and Guo, 2010; Meshalkina et al., 2017; Stewart et al., 2011). The advantages of  
99 zebrafish include low cost, prolific breeding, and ease of genetic manipulation (Parker et al., 2012;  
100 Rico et al., 2011; Stewart et al., 2014; Stewart et al., 2015a). Additionally, both larvae and adult  
101 zebrafish are easy to maintain and have small body size, other important characteristics that  
102 facilitate medium/high throughput screens (Bilotta et al., 1999; Kalueff et al., 2013). Furthermore,  
103 delivery of drugs or suspected efficacious compounds can be quite simple, as zebrafish may be  
104 immersed into drug solution from which the fish can rapidly absorb the drug through the gills and  
105 body surface (Rosemberg et al., 2012; Tran et al., 2015a). This method of drug delivery is  
106 effective for water soluble compounds but may also be employed with more lipophilic substances  
107 with the help of dimethyl sulfoxide (DMSO), a solvent to which zebrafish is relatively insensitive  
108 at lower concentrations (Altenhofen et al., 2017; Ibrahim et al., 2014).

109 The zebrafish genome has been fully sequenced (Collier and Echevarria, 2013; Howe et  
110 al., 2013) and a number of genetic tools (*e.g.*, efficient reverse and forward genetic methods) are  
111 available to investigate how various drugs modulate molecular functions in the vertebrate central  
112 nervous system (CNS) (Dooley and Zon, 2000; Rinkwitz et al., 2011). The zebrafish's haploid

113 genome size is 1.7 gigabases, with 25 chromosomes, 12,062 known protein-coding genes and  
114 additional 7,465 predicted protein-coding genes (Klee et al., 2012). The nucleotide sequence of  
115 zebrafish genes shows approximately 70% homology with the human counterparts (MacRae and  
116 Peterson, 2015; Howe et al. 2013), and various orthologs involved with drug abuse-related  
117 processes have been described in the zebrafish (Table 1). Despite the apparent anatomical  
118 differences found between the teleost and the mammalian brain, the zebrafish brain has numerous  
119 areas that have been identified as homologous to mammalian brain regions serving similar  
120 functions to those of mammalian areas (Randlett et al., 2015; Ullmann et al., 2010). For example,  
121 the lateral pallium of the telencephalic area of the zebrafish is responsible for memory processing,  
122 a region homologous to the mammalian hippocampus, which is known to be the center of memory  
123 in mammals, similarly the zebrafish habenula is associated with fear responses, a brain area that  
124 corresponds to the mammalian amygdala (Agetsuma et al., 2010; Perathoner et al., 2016).  
125 Furthermore, dopaminergic projections of the zebrafish forebrain parallel the mesolimbic system  
126 involved in drug addiction in mammals (Rink and Wullimann, 2002). Although the genome  
127 duplication event in teleost fish modified the number of genes encoding proteins related to  
128 synthesis, transport and signaling within the neurotransmitter systems, the expression pattern of  
129 these proteins in zebrafish is similar to those of other vertebrates (Horzmann and Freeman, 2016).  
130 These features make the zebrafish a translationally relevant model organism, and considering that  
131 the zebrafish possesses relatively complex cognitive processing and decision-making abilities, one  
132 can argue that investigation of evolutionarily conserved phenotypes underlying drug abuse,  
133 reward and addiction should be possible using this species.

134 Behavioral mechanisms underlying drug abuse can include sensitization, increased drug  
135 tolerance, withdrawal syndrome, drug seeking, extinction, and relapse. Behavioral phenomena  
136 related to the above together with underlying genetic mechanisms have been investigated using  
137 zebrafish (Klee et al., 2012; Stewart et al., 2011). Zebrafish neurobehavioral assays provide a  
138 suitable platform to explore drug response and their associated addiction related effects, such as  
139 withdrawal, locomotor activation, and drug seeking behavior (Cachat et al., 2010; Darland and

140 Dowling, 2001; Kily et al., 2008; Lau et al., 2006; Ninkovic and Bally-Cuif, 2006). The  
141 conditioned place preference (CPP) task is an often employed method to assess the rewarding  
142 properties of drugs, including morphine and ethanol, in zebrafish (Mathur et al., 2011b; Stewart et  
143 al., 2011; Webb et al., 2009). Behavioral responses following drug exposure in zebrafish have  
144 been found generally similar to those observed in humans or rodents (Meshalkina et al., 2017).  
145 Zebrafish larvae also display robust drug-evoked neurobehavioral phenotypes and offer one the  
146 ability to assess multiple animals simultaneously in a high-throughput manner (Stewart et al.,  
147 2011). Moreover, genetic factors contribute to zebrafish behavioral responses, demonstrating a  
148 clear relationship among genes, reward phenotypes and withdrawal syndrome (Egan et al., 2009;  
149 Klee et al., 2012; Ninkovic and Bally-Cuif, 2006; Webb et al., 2009). Behaviors following  
150 withdrawal are frequently measured using the novel tank test (NTT), in which increased geotaxis  
151 (bottom dwelling), freezing, and erratic movements reflect anxiety-like responses that correlate  
152 with higher whole-body cortisol levels (Cachat et al., 2010). Importantly, adult zebrafish exhibit a  
153 wide spectrum of behavioral phenotypes, which can be analyzed spatio-temporally using three-  
154 dimensional automated video tracking tools (Cachat et al., 2011; Rosa et al., 2018; Rosemberg et  
155 al., 2011). Similarly to humans, withdrawal effects in zebrafish include sedation, altered  
156 sociability, and epileptic seizures (da Silva Chaves et al., 2018; Mathur and Guo, 2010; Muller et  
157 al., 2017). The above suggests that discovery of novel therapeutic approaches for treating drug  
158 abuse related disorders may be facilitated by research using zebrafish. **Fig. 1** summarizes the most  
159 frequently utilized behavioral tests employed to assess the effects of drugs of abuse in zebrafish,  
160 and **Table 2** lists the quantified phenotypes or endpoints measured in these tests.

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### 162 **3. Limitations of zebrafish models to assess the neurobehavioral mechanisms of drug abuse**

163 The first limitation we want to emphasize is a fundamental question related to using  
164 animal models, and it may also be viewed as an advantage of the zebrafish. Fish are evolutionarily  
165 more primitive than mammals and thus one may not expect a fish model of, say drug addiction, to  
166 recapitulate all aspects of the mammalian, and the human, condition. For this reason, a fish model

167 may only be employed as a simplified, “first-pass” approach, one with which the investigator may  
168 gain information about biological processes in a cost-effective manner. However, the discoveries  
169 obtained using fish models must be subsequently verified using mammalian model organisms. In  
170 other words, the zebrafish offers a reductionist approach that may speed up the discovery process.

171 Other limitations are more practical in nature. Although various experimental protocols  
172 have been successfully employed for modeling drug abuse and addiction in zebrafish (**Table 3**),  
173 such models present limitations. For example, usual indices of addiction, such as escalating self-  
174 administration, CPP, relapse or reinstatement after extinction, and incubation, have either not been  
175 demonstrated in zebrafish, or have been published on only sporadically with some controversial  
176 data presented. For example, in zebrafish, some studies show anxiety-like behaviors after ethanol  
177 withdrawal (da Silva Chaves et al., 2018; Muller et al., 2017; Tran et al., 2015a), while other  
178 reports (Cachat et al., 2010) do not observe significant behavioral responses. Zebrafish have been  
179 found not to show reinforcement responses to all categories of drugs (*e.g.*, cannabinoids), because  
180 some drugs or xenobiotics can trigger aversive responses rather than exhibiting rewarding  
181 properties required in CPP tests (Brock et al., 2017; da Rosa et al., 2016). Another limitation is  
182 that drug delivery via immersion of the fish in the drug solution is not feasible with lipophilic  
183 drugs, and this method of drug delivery is also complicated from the perspective of precise control  
184 of the amount of drug to be delivered. In addition, the pH of the drug solution also can influence  
185 the toxicity of the drug as well as its bioavailability (Rubinstein, 2006). Perhaps the biggest  
186 disadvantage of the zebrafish in psychopharmacology is that knowledge of pharmacokinetics is  
187 still limited for this species, and the drug concentration that reaches different tissues is poorly  
188 explored (Chatterjee and Gerlai, 2009; Rosemberg et al., 2012). A seemingly trivial, but often  
189 encountered problem is that sexing zebrafish is not easy at the young age of these animals.  
190 Furthermore, due to the small size of this species certain procedures (*e.g.*, injections) can  
191 constitute a methodological challenge. Another important limitation arises from the known  
192 genome duplication in teleosts (Glasauer and Neuhauss, 2014). Thus, investigation of certain  
193 molecular or genetic mechanisms may be complicated by multiplicity of functionally similar

194 molecular components not found in mammals. Although, some regards this as an advantage as  
195 knocking out particular gene targets may not lead to embryonic lethality and may only have a  
196 graded effect, allowing the analysis of the effects of reduced gene product. Furthermore, although  
197 the existence of distinct zebrafish strains (*e.g.*, AB, TU, WIK, TL, WT, leopard) can serve as an  
198 interesting tool to assess how genes modulate drug response (Kenney et al., 2017; Quadros et al.,  
199 2016; Rosa et al., 2018), differences in the genetic background and other experimental variables  
200 (*e.g.*, housing, feeding, environmental factors) may influence behavioral data among laboratories  
201 (Gerlai, 2019; Parker et al., 2012). It is also notable that most “standard” strains of the zebrafish,  
202 including the above listed ones, are not fully inbred, and thus, unlike with mice, genetic stability is  
203 not warranted from generation to generation, and across laboratories (Gerlai et al., 2019).

204         Despite these limitations, however, the zebrafish has been found to be a valuable tool  
205 complementing the existing rodent literature in the analysis of numerous CNS disorders including  
206 those associated with drug abuse. This has been possible because most neurobiological  
207 phenomena share fundamental, evolutionarily ancient, conserved mechanisms that may be found  
208 both in fish and mammals.

209

## 210 **4. Ethanol**

### 211 *4.1 General information*

212         Socio-cultural traditions and behavioral stereotypes facilitate alcohol (ethanol, ethyl  
213 alcohol) consumption, and early experience with its use, easy access, relatively low cost and  
214 association with leisure activities may all contribute to the development of alcohol use disorders  
215 (AUD) (Sudhinaraset et al., 2016). Social drinking elicits positive mood states and anxiety/stress-  
216 relieving effects (Harrison et al., 2017; Hendler et al., 2013). However, long-term consumption  
217 may lead to AUD, which are classified as neuropsychiatric conditions, and the patients exhibit  
218 alcohol craving and alcohol seeking behavior (Reus et al., 2018). Alcohol abuse and alcoholism  
219 represent major public health issues due to their high prevalence and multidimensional health  
220 consequences, including complex brain alterations (Preuss et al., 2018; WHO, 2014). Alcohol is



221 the most harmful drug of abuse both in terms of its effects on the individual and on the society  
222 (Nutt et al., 2010; van Amsterdam et al., 2015; WHO, 2014). Indeed, alcohol consumption is  
223 responsible for approximately 5.9% of deaths worldwide and 5.1% of the global burden of  
224 diseases (WHO, 2014).

225 Compulsive alcohol consumers exhibit loss of behavioral control, as well as tolerance and  
226 withdrawal symptoms, which may include anxiety, depressive episodes, reduced sociability,  
227 insomnia, nausea, and epileptic seizures (Becker and Mulholland, 2014; Enoch et al., 2003).  
228 Importantly, both genetic and environmental factors influence the predisposition to alcoholism  
229 (Wall et al., 2016). Despite the remarkable impacts of AUD on public health, pharmacological  
230 treatments for alcohol dependence are substantially underutilized. The reason for lack of  
231 efficacious drugs one could employ as pharmacotherapy for AUD is the lack of understanding of  
232 the effects of this pharmacologically and functionally complex drug (Antonelli et al., 2018; Goh  
233 and Morgan, 2017).

234

#### 235 *4.2. Putative mechanisms of ethanol in the CNS*

236 The effects of ethanol on brain functions occur from the low range to 100 millimolar, but  
237 the exact mechanisms involved in ethanol-mediated responses are not fully elucidated. Different  
238 from other psychotropic drugs, ethanol has a rather complex pharmacological effect profile in the  
239 CNS, influencing several molecular targets and biochemical reactions directly and an even larger  
240 number of these mechanisms indirectly. Ethanol easily crosses the blood–brain barrier and  
241 modulates brain activity, thereby affecting behavior in a dose-dependent manner (Esel and Dinc,  
242 2017; Spanagel, 2009). Acute exposure to low to moderate doses of ethanol promote disinhibition  
243 of punished behavior, euphoria, and anxiolysis, while higher doses induce sedation, lack of  
244 coordination, somnolence, and memory impairments (Hendler et al., 2013). Chronically, ethanol  
245 induces neuroadaptive processes and triggers tolerance, dependence, and withdrawal syndrome,  
246 which include psychiatric symptoms, overt neurotoxicity, and severe cognitive disruptions  
247 (Abraham et al., 2017; Banerjee, 2014; Hammoud and Jimenez-Shahed, 2019).

248 Ethanol modulates both excitatory and inhibitory signal transduction pathways in the  
249 CNS (Chastain, 2006; Esel and Dinc, 2017). Ethanol decreases metabolic activity (Wang et al.,  
250 2000), inhibits glutamatergic neurotransmission (Hwa et al., 2017; Roberto and Varodayan, 2017),  
251 and potentiates gamma-aminobutyric acid- (GABA) and glycine-mediated synapses (Breese et al.,  
252 2006; Soderpalm et al., 2017; Zhu and Lovinger, 2006). Additionally, ethanol reduces the  
253 transmembrane flow of calcium ( $\text{Ca}^{2+}$ ) by inhibiting the functioning of L-type  $\text{Ca}^{2+}$  channels  
254 (Hendricson et al., 2003), which play a key role in ethanol-induced depressive behaviors (Dopico  
255 et al., 2014), CPP (Hill et al., 2003; Newton et al., 2008), and neuroadaptation (Mulholland et al.,  
256 2011; Nimitvilai et al., 2016). Impaired CNS homeostasis is associated with ethanol-induced  
257 sedative and anxiolytic effects (Lovinger and Roberto, 2013), as well as memory deficits affecting  
258 cognitive performance (Costardi et al., 2015; Lovinger and Roberto, 2013; Weiss et al., 2014).

259 Ethanol indirectly activates dopaminergic and serotonergic neurons, which innervate the  
260 reward system (Erdozain and Callado, 2014). Low ethanol doses increase dopamine and serotonin  
261 release, thereby facilitating reinforcement (Morel et al., 2018; Nutt et al., 2015), and emotion-  
262 related behaviors (Marcinkiewicz, 2015), respectively. Conversely, chronic ethanol consumption  
263 reduces both dopamine and serotonin levels, and negative emotional states are observed during  
264 ethanol withdrawal. Ethanol also affects the homeostasis of cholinergic (Davis and de Fiebre,  
265 2006; Hendrickson et al., 2013), noradrenergic (Haass-Koffler et al., 2018; Rossetti et al., 1992),  
266 opioid (Costardi et al., 2015; Gianoulakis, 2009), and endocannabinoid (Lavanco et al., 2018;  
267 Sloan et al., 2017) systems (**see details in Fig. 2A**). These sets of mechanisms help explain how  
268 ethanol modulates learning, memory, as well as the rewarding properties of ethanol (White, 2003;  
269 Zorumski et al., 2014).

270 A number of proteins with ethanol-binding sites have been identified. These include  
271 enzymes involved in ethanol pharmacokinetics, such as alcohol and acetaldehyde dehydrogenases  
272 (*ADH/ALDH*), cytochrome P450 2E1 (*CYP2E1*), and adenylyl cyclase – an enzyme that produces  
273 the second messenger adenosine 3',5'-monophosphate (cAMP) from adenosine triphosphate  
274 (ATP) (Pereira et al., 2015; Yoshimura et al., 2006). Corticotropin releasing factor (*CRF*) plays an

275 important role on stress-induced relapses to ethanol drinking because its positive regulation is  
276 directly involved in negative emotional states (Heilig and Koob, 2007). Acute and chronic ethanol  
277 consumption also modulates cAMP-responsive element binding (*CREB*) protein in the brain  
278 (Morrow et al., 2004), and downstream effects on several important *CREB*-related genes, such as  
279 neuropeptide Y (*NPY*), brain derived neurotrophic factor (*BDNF*), activity-regulated cytoskeleton-  
280 associated protein (*ARC*), and corticotrophin-releasing hormone (*CRH*). These *CREB*-related  
281 genes play a crucial roles in the genetic predisposition to alcoholism contributing to the behavioral  
282 effects of ethanol (Moonat et al., 2010).

283

#### 284 *4.3. Ethanol and zebrafish: neurobehavioral studies*

285         The first study to analyze the behavioral effects of acute ethanol exposure in zebrafish  
286 date from 2000, a study in which ethanol was found to elicit a range of behavioral changes  
287 analogous to what was found in mammals (Gerlai et al., 2000). For example, low-to-moderate  
288 concentrations (0.25 and 0.5% v/v) of ethanol increased locomotor activity while higher  
289 concentrations (1.0% v/v) inhibited locomotion, causing sedation in the zebrafish (Gerlai et al.,  
290 2000). Importantly, ethanol-mediated effects on behavior have been found concentration- and  
291 time-dependent, effects that correlated with changes in brain ethanol levels (Rosemberg et al.,  
292 2012). After acute exposure to low-to-moderate ethanol concentrations, a substantial decrease in  
293 anxiety-like behaviors, such as erratic movement (Egan et al., 2009), freezing (Blaser and  
294 Penalosa, 2011), predator induced avoidance (Pannia et al., 2014), and bottom dwelling (Wong et  
295 al., 2010) have been reported in zebrafish. At lower concentrations, ethanol also triggers  
296 aggressive behavior, affects shoaling, and modulates fear-like responses (Fontana et al., 2016;  
297 Fontana et al., 2018b; Gerlai, 2003; Gerlai et al., 2000). Brief ethanol exposure (1.5% v/v for 20  
298 min) have also been found to enhance ethanol preference in a CPP paradigm, confirming  
299 positively-reinforcing properties of ethanol in zebrafish (Mathur et al., 2011a; Mathur et al.,  
300 2011b).

301 Acute ethanol exposure increases dopaminergic and serotonergic activity in the brain of  
302 AB zebrafish (Chatterjee and Gerlai, 2009; Chatterjee et al., 2014; Tran et al., 2016; Tran et al.,  
303 2015c). However, ethanol decreases the levels of aspartate, taurine, GABA, glutamate and glycine  
304 in a concentration-dependent manner (Chatterjee et al., 2014). Furthermore, ethanol acutely  
305 enhances acetylcholinesterase activity and alters antioxidant defenses by decreasing superoxide  
306 dismutase (SOD) and increasing catalase (CAT) activities, which result in lipid peroxidation  
307 (Rosemberg et al., 2010). Ethanol acutely stimulates mitochondrial respiration and consequently  
308 the bioenergetics efficiency, reinforcing the ethanol stimulatory effect on mitochondrial O<sub>2</sub>  
309 consumption (Müller et al., 2019). Mounting data also support the involvement of purinergic  
310 signaling in ethanol-mediated responses, since ethanol and its metabolite, acetaldehyde, modulate  
311 the hydrolysis of extracellular nucleotides in zebrafish (Rico et al., 2007; Rico et al., 2008).  
312 Because zebrafish neurotransmitter systems show a robust functional correspondence and  
313 homology compared to those of mammals, investigators have concluded that the main functions,  
314 enzymes, receptors, transporters affected by ethanol are evolutionarily conserved (Cox et al.,  
315 2005; Kaslin et al., 2004; Kim et al., 2004; Maximino et al., 2013; Moly et al., 2014; Panula et al.,  
316 2006; Rico et al., 2003; Senger et al., 2004).

317 Protocols for studying ethanol tolerance and withdrawal (Dlugos and Rabin, 2003; Gerlai  
318 et al., 2009; Mathur and Guo, 2011; Tran et al., 2015b) have been validated for zebrafish.  
319 Administration can be performed by using an intermittent (Mathur and Guo, 2011; Muller et al.,  
320 2017) or a continuous (Damodaran et al., 2006; Dlugos and Rabin, 2003; Egan et al., 2009)  
321 ethanol exposure. Both protocols induce tolerance to the locomotor stimulant, anxiolytic, and  
322 anxiogenic effects of ethanol. The intermittent protocol provides translational relevance because it  
323 mimics the ethanol consumption observed in humans. This repeated ethanol exposure elicits  
324 defensive responses by stimulating social behavior, geotaxis, and scototaxis, which reflect  
325 anxiogenesis (Muller et al., 2017). These behavioral responses can be associated with changes in  
326 oxidant processes in the brain since ethanol decreases antioxidant defenses like SOD and CAT  
327 activities and non-protein thiol (NPSH) levels, and increases lipid peroxidation (Muller et al.,

328 2017). Intermittent ethanol exposure also impairs the electrons flow between I- and II- complexes  
329 of mitochondrial electron transport chain, which is associated with ROS formation (Müller et al.,  
330 2019).

331 Continuous ethanol exposure appears to lead to a more robust level of tolerance and  
332 removes the potential effects of repeated withdrawal from the drug as it happens in the repeated  
333 intermittent protocol. After continuous ethanol exposure, AB zebrafish show increased levels of  
334 dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin, 5-hydroxyindoleacetic acid  
335 (5HIAA), glutamate, aspartate, and glycine in the CNS following withdrawal (Chatterjee et al.,  
336 2014). Zebrafish continually exposed to ethanol also exhibit decreased choline acetyltransferase  
337 activity, changes in antioxidant enzymatic defenses, and increased reactive oxygen species  
338 production (Agostini et al., 2018). Continuous chronic exposure followed by ethanol withdrawal  
339 also impairs aversive learning in the inhibitory avoidance learning task (Amorim et al., 2017;  
340 Luchiari et al., 2015). Withdrawal protocols using zebrafish have also produced some conflicting  
341 results. Some studies report robust effects of withdrawal on anxiety-like behavior (da Silva  
342 Chaves et al., 2018; Muller et al., 2017; Tran et al., 2015a), while other reports (Cachat et al.,  
343 2010) do not observe significant behavioral responses. A possible explanation for data  
344 heterogeneity may be related to differences in ethanol concentration (e.g., 0.25, 0.5, or 1.0 % (v/v)  
345 concentration range) and exposure period (e.g., acute vs. chronic, intermittent or continuous  
346 exposure) employed in a wide range of protocols (Cachat et al., 2010; da Silva Chaves et al.,  
347 2018; Chatterjee et al., 2014; Müller et al., 2017).

348 A model of voluntary ethanol intake, using ethanol mixed with gelatin, has been  
349 characterized in zebrafish. Stimulatory effects on locomotion, reduced anxiety, potentiation of  
350 aggressive behaviors, and increased expression of galanin (*gal*) and orexin (*ox*) in specific  
351 hypothalamic areas were observed (Sterling et al., 2015). Importantly, the effects of ethanol on  
352 brain circuitry and behavior of zebrafish may occur in a strain-dependent manner (de Esch et al.,  
353 2012; Dlugos and Rabin, 2003; Gerlai et al., 2009), as well as dependent on the time of the day in  
354 which ethanol is administered (Tsang et al., 2018; Vera et al., 2018).

355 In addition to the analysis of the effects of acute and chronic ethanol administration,  
356 investigation of the consequences of embryonic ethanol exposure has also been started using the  
357 zebrafish. The goal of this research has been to model behavioral and physiological phenotypes  
358 associated with human fetal alcohol spectrum disorders (FASD). Some features as high fecundity,  
359 external fertilization, embryo transparency and rapid development, make the zebrafish especially  
360 well-suited for modeling and studying the pathology of FASD (Lovely et al., 2016). Early  
361 zebrafish FASD models recapitulated some features of gross morphological abnormalities seen in  
362 the most severe forms of fetal alcohol syndrome (Carvan et al., 2004). More recently, however,  
363 attempts have been made to model the milder and more prevalent forms of FASD. These studies  
364 found lasting behavioral defects without apparent morphological abnormalities, particularly in the  
365 domains of social behavior (shoaling) and anxiety-like responses (Fernandes et al., 2019; Parker et  
366 al., 2014), cognition, learning and memory (Amorim et al., 2017; Cleal and Parker, 2018; Lutte et  
367 al., 2018), with higher concentrations of ethanol also leading to structural defects in various  
368 tissues (brain, sensory organs, heart and craniofacial phenotypes) (Baggio et al., 2018; Fernandes  
369 et al., 2018; Ramlan et al., 2017). Embryonic ethanol exposure, changes the expression of genes  
370 involved in developmental processes, such as development of the neural tube, forebrain, and eye  
371 formation (*six3b* and *gli1*), resulting in altered somite development, decreased body length, and  
372 intraocular distance (Loucks and Ahlgren, 2012). Moreover, early embryonic ethanol exposure  
373 has been found to impair neurotransmitter systems, including the serotonergic, dopaminergic  
374 (Baggio et al., 2018; Buske and Gerlai, 2011; Mahabir et al., 2018) and purinergic systems in  
375 zebrafish (Lutte et al., 2018). In summary, both larval and adult zebrafish have been successfully  
376 used to explore the neurobehavioral mechanisms involved in ethanol abuse and addiction.

377

## 378 **5. Opioids**

### 379 *5.1. General information*

380 Opioids are a class of drugs that include opiates (*e.g.*, opium, diamorphine, morphine,  
381 codeine, thebaine, and oripavine) and semi- and synthetic drugs (*e.g.*, meperidine, pethidine,  
382 hydromorphone, methadone, hydrocodone, oxycodone, and fentanyl) that have similar actions to  
383 those of opiates (Volkow et al., 2011). Non-medical use of opioids is considered a major public  
384 health challenge in the last two decades (CBHSQ, 2015). The repeated exposure to escalating  
385 dosage of opioids can result in development of tolerance, addiction (intense drug craving and  
386 compulsive use), and dependence (to avoid a withdrawal syndrome) (Kosten and George, 2002).

387 Opioid dependence and addiction are serious, chronic, and often relapsing disorders of the  
388 CNS associated with altered brain homeostasis (Kolodny et al., 2015). The brain abnormalities  
389 that produce addiction are complex and long-lasting, and are influenced by environmental (*e.g.*,  
390 stress, social context, and psychological conditioning) as well as genetic factors (*e.g.* brain  
391 pathways that were abnormal even before the first dose of opioid was taken) (Kosten and George,  
392 2002). Altered brain function can produce craving that leads to relapse months or even years after  
393 the last use of the drug (Koob and Volkow, 2010). Opioid dependence and addiction are  
394 considered chronic diseases, like hypertension, schizophrenia, and diabetes (Dennis and Scott,  
395 2007). Thus, novel therapeutical strategies for treating drug addiction are necessary, and novel  
396 approaches to circumvent opioid addiction are imperative.

397

## 398 *5.2. Putative mechanisms of opioids in the CNS*

399 Opioid molecules travel in the bloodstream to the brain and attach to specific proteins at  
400 the surfaces of opiate-sensitive neurons, known as opioid receptors (Kosten and George, 2002). In  
401 humans, there are three classical opioid receptors: the mu-opioid receptor (MOP), the kappa-  
402 opioid receptor (KOP), and the delta-opioid receptor (DOP), encoded by the genes *OPRM1*,  
403 *OPRK1*, and *OPRD1*, respectively (Al-Hasani and Bruchas, 2011). These genes encode G-  
404 protein-coupled receptors that interact with heterotrimeric (Gi/Go) G proteins (Dhawan et al.,  
405 1996). Opioids activate the mesolimbic (mindbrain) reward system, which plays a role in  
406 motivating the user to repeatedly abuse the drug (Ikemoto, 2010). This pathway is mainly

407 responsible for generating signals in the ventral tegmental area (VTA), culminating in the release  
408 of dopamine in the nucleus accumbens (NAc) that causes feeling of pleasure (Adinoff, 2004).  
409 Opioids also affect other brain areas involved in associative learning and memory, which associate  
410 good feelings with the environment in which they occurred (Volkow et al., 2002). These  
411 memories often result in drug craving when abusers reencounter this environment (*e.g.*, person,  
412 places, or objects), facilitating drug use relapse and abuse (Kosten and George, 2002). In early  
413 stages, the opioid's stimulation of reward systems is the primary reason for drug-seeking  
414 behavior. However, the compulsion to use opioids is constantly increased and after repeated  
415 exposure to the drug is also driven by developed tolerance leading to drug dependence in the  
416 patient (Koob and Le Moal, 2001).

417         Mounting evidence suggests that tolerance and dependence-like phenomena can occur at  
418 cellular levels and include changes in cyclic adenosine 3',5'-monophosphate (cAMP) dependent  
419 biochemical pathways (Anand et al., 2010; Dhawan et al., 1996; Gupta and Kulhara, 2007;  
420 McFadzean, 1988; Sharma et al., 1977). Furthermore, the chronic use of morphine results in a  
421 feedback inhibition of endogenous opioid synthesis as it decreases/increases the expression of  
422 proenkephalin mRNA and enkephalin peptides depending on the cell type affected (Gonzalez-  
423 Nunez et al., 2013; Le Merrer et al., 2009). Although opioid-induced changes in the above  
424 mentioned genes are a key factor related to opioid addiction and withdrawal, the mechanisms  
425 underlying addiction involve a potentially large number of other factors, leading to alterations in  
426 the dopaminergic system (Volkow, 2010), ion conductance (Grudt and Williams, 1995), cAMP  
427 (Chan and Lutfy, 2016; Dziedzicka-Wasylewska and Przewlocki, 1995), endogenous ligands  
428 (Weber, 1983), and glutamate (Peters, 2012) all contributing drug-seeking behavior in a complex  
429 manner (**see details in Fig. 2B**). Clearly, further studies are necessary to elucidate the mechanistic  
430 complexity underlying chronic effects of opioids.

431

432 *5.3. Opioids and zebrafish: neurobehavioral studies*



433 Zebrafish have an evolutionarily conserved opioid system, which makes this fish a  
434 valuable tool for studying the molecular bases of human opioid addiction. The initial studies  
435 related to opioid system in zebrafish analyzed ZFOR<sub>1</sub>, a delta-opioid receptor isolated using a  
436 probe from rat MOP (Barrallo et al., 1998). Phylogenetic analyses showed a high degree of  
437 nucleotide sequence conservation for all opioid receptor genes of zebrafish when compared to  
438 their respective human counterparts (Dreborg et al., 2008). Orthlogs of three classical human  
439 opioid receptors were characterized in zebrafish (Stevens, 2009). While the *oprml*, *oprkl*, *oprll*  
440 genes have single homologs in zebrafish, a pair of *oprdl* can be found in this species (Herrero-  
441 Turrión et al., 2014).

442 As it occurs in rodents, opioid drug exposure results in behavioral changes, affecting  
443 locomotion and anxiety-like response in the zebrafish too. For example, diacetylmorphine (heroin)  
444 evokes hyperlocomotion without changing anxiety-like behaviors in zebrafish (Stewart and  
445 Kalueff, 2014). Although morphine elicits analgesia in zebrafish (Costa et al., 2019; Magalhaes et  
446 al., 2017; Taylor et al., 2017), exposure to this drug can also have anxiolytic effects in zebrafish  
447 and decrease erratic movements and increase the time spent near the water surface in the NTT  
448 (Wong et al., 2010). The larval zebrafish exhibits preference for morphine in the choice chamber  
449 test, and such response is attenuated by opioid and dopamine antagonists (Bretaud et al., 2007).

450 Dopaminergic and serotonergic neurons have been shown to play essential roles in drug-  
451 seeking behavior in humans (Rogers, 2011). Confirming such findings, adult zebrafish exhibit  
452 increased conditioned preference for both food and morphine, a response that is blocked by  
453 naloxone (an opioid antagonist) as well as by dopamine receptor antagonists (Lau et al., 2006).  
454 Other opioid drugs such as fentanyl, oxycodone, tetracaine, phencyclidine, and chlorpheniramine  
455 also exhibit rewarding properties in zebrafish CPP test (Brock et al., 2017). Importantly, a  
456 method of self-administration of opioids has been recently developed for the zebrafish (Bosse and  
457 Peterson, 2017), providing a novel tool for assessing drug addiction in this species. Briefly,  
458 zebrafish shoals trained for five days were found to self-administer hydrocodone hydrochloride, a  
459 MOP-dependent behavior blocked by dopamine and glutamate receptor antagonists (Bosse and

460 Peterson, 2017). Finally, drug withdrawal induced responses have also been observed in zebrafish:  
461 increased anxiety-like behaviors and whole-body cortisol levels have been found (Cachat et al.,  
462 2010). The above results demonstrating robust drug seeking-behavior and withdrawal responses  
463 reinforce the utility of zebrafish as a vertebrate model organism appropriate for studying the  
464 molecular bases underlying opioid addiction and dependence processes.

465

## 466 **6. Nicotine**

### 467 *6.1 General informations*

468 Nicotine is an alkaloid found in tobacco plants that affects the CNS in a varied manner  
469 (Jaffe and Kanzler, 1979; Levin and Chen, 2004; Levin and Rezvani, 2000). This alkaloid the  
470 major active constituent of tobacco responsible for the development of smoking induced drug  
471 dependence (Stolerman and Jarvis, 1995). Nicotine addiction is considered a chronic brain  
472 disorder, and tobacco smoking represents a risk factor for developing several chronic diseases,  
473 including cancers and cardiovascular disorders (Berrendero et al., 2010). Smoking-related  
474 diseases are serious world health problems since tobacco use alone causes approximately six  
475 million deaths per year worldwide (WHO, 2015). According to recent estimates, there are nearly  
476 1.3 billion of smokers in the world, making tobacco smoking one the most prevalent addiction  
477 over the world (D'Souza, 2016). Various factors contribute to nicotine addiction, including  
478 chronic stress, low socioeconomic status, individual susceptibility, etc. (Jarvis, 2004; Morris et al.,  
479 2016). Moreover, psychiatric disorders, including schizophrenia, bipolar disorder and depression  
480 also increase susceptibility to smoking (Lucatch et al., 2018; Suemaru et al., 2006). Smoking  
481 induces changes in the brain that can lead to nicotine craving, relapse, and withdrawal induced  
482 symptoms (Bruijnzeel, 2017). Despite pharmacological treatments available to combat nicotine  
483 addiction, the number of smokers remains high and smoking related health issues continue to  
484 represent a major medical problem (Herman et al., 2014).

485

### 486 *6.2 Putative mechanisms of nicotine in the CNS*

487           After smoking, nicotine is absorbed into the blood through the lungs and reaches the  
488 brain in ten seconds (Benowitz, 2009). Nicotine absorption is pH-dependent, where nicotine is  
489 non-ionized and quickly crosses cellular membranes at physiological pH (Le Houezec, 2003).  
490 Nicotine interacts with a number of nicotinic acetylcholine receptors (nAChRs) that are formed as  
491 homopentamers and heteropentamers from subunits in the CNS (Gotti et al., 2009). In the  
492 mammalian brain, there are eight  $\alpha$  subunits ( $\alpha_2$ – $\alpha_{10}$ ) and three  $\beta$  subunits ( $\beta_2$ – $\beta_4$ ) (Karlin, 2002;  
493 Subramaniyan and Dani, 2015), and the most commonly expresses are  $\alpha_4\beta_2$  or  $\alpha_7$  nAChR (Dani  
494 and Bertrand, 2007).

495           Nicotine activates nAChRs and promotes the release of various neurotransmitters  
496 including acetylcholine, dopamine, serotonin, GABA, glutamate, and norepinephrine in the brain  
497 (Alkondon et al., 2000; Dani and Bertrand, 2007; Dehkordi et al., 2018; Gao et al., 2010;  
498 Mansvelder et al., 2002; Mao et al., 2011; Wonnacott, 1997). Nanomolar concentrations of  
499 nicotine enhance both glutamatergic and cholinergic neurotransmission (McGehee et al., 1995).  
500 Nicotine also stimulates the release of GABA in the rodent brain (Zhu and Chiappinelli, 1999),  
501 and increases the firing rate of dopaminergic and non-dopaminergic neurons (Yin and French,  
502 2000). The activation of nAChRs increases intracellular levels of  $\text{Ca}^{2+}$  (De Biasi and Dani, 2011;  
503 Nakayama and Nakashima, 2004), and modulates both the opioid and endocannabinoid systems  
504 (Maldonado and Berrendero, 2010; Maldonado et al., 2006; Zarrindast and Khakpai, 2018).  
505 Importantly, the broad action of nicotine via nAChRs in the brain modulates anxiety, learning,  
506 memory, pain, body weight and temperature (Zarrindast and Khakpai, 2018).

507           Nicotine dependence has different phases (D'Souza and Markou, 2013). The acquisition  
508 and maintenance of nicotine seeking occurs by the positive reinforcing effects of nicotine (*e.g.*,  
509 mild euphoria, relaxation, increased arousal, and decreased fatigue) (Henningfield et al., 1985).  
510 Chronically, nicotine induces neuroadaptation in the brain reward systems, leading to the  
511 development of dependence (D'Souza and Markou, 2013). The rewarding properties of nicotine  
512 are mediated by dopaminergic neurons in the mesocorticolimbic system (Zarrindast and Khakpai,  
513 2018). Stimulation of the  $\alpha_4\beta_2$  nAChR of the dopamine cell bodies in the VTA results in

514 dopamine release in the NAc and prefrontal cortex (PFC) (Herman et al., 2014). Similarly to the  
515 effects of some other drugs of abuse, nicotine induced increase of dopamine in the NAc mediates  
516 the rewarding and pleasurable effects of this drug, which is considered the mechanism responsible  
517 for initiation and maintenance of nicotine addiction (Herman et al., 2014). When nicotine  
518 administration is stopped, withdrawal symptoms appear (D'Souza and Markou, 2013). In humans,  
519 withdrawal produces negative affective effects, such as depressed mood, dysphoria, irritability,  
520 anxiety, and craving (Shiffman and Jarvik, 1976). The “physical” or somatic symptoms include  
521 insomnia, gastrointestinal discomfort, bradycardia, and increased appetite (Hughes et al., 1991).  
522 Besides of nAChR, opioid and endocannabinoid systems, neuropeptides, and changes in signal  
523 transduction pathways are also implicated in nicotine withdrawal syndrome (Jackson et al., 2015).  
524 After the development of nicotine dependence, the endogenous release of CRF and the resulting  
525 negative mood state can drive nicotine intake (negative reinforcement) (Bruijnzeel, 2017). These  
526 pathways have been considered key targets for the development of new treatments to reduce  
527 withdrawal symptoms (Bruijnzeel, 2017; D'Souza, 2016; Jackson et al., 2015).

528         Nicotine target proteins and enzymes related to nicotine pharmacokinetics include the  
529 liver enzymes cytochrome P450 2A6 (*CYP2A6*), UDP glucuronosyltransferase (*UGT*), flavin-  
530 containing monooxygenase (*FMO*) (Benowitz, 2009). Nicotine also promotes neuroadaptation by  
531 increasing cAMP-responsive element-binding protein (*pCREB*) levels in dopaminergic neurons  
532 after chronic administration of this drug. Nicotine exposure in adolescent rats also suppresses  
533 basal adenylyl cyclase activity and eventually compromises the response to beta-adrenergic  
534 receptor stimulation, culminating in several alterations of neural circuits in adulthood (Slotkin et  
535 al., 2008). Although BDNF levels do not change during chronic exposure to nicotine, a prominent  
536 increase of the level of this neurotrophic factor is observed in the NAc, VTA, and substantia nigra  
537 during abstinence from nicotine (Kivinummi et al., 2011). Overall, experimental findings support  
538 the involvement of *BDNF* and *pCREB* in nicotine-induced neurochemical changes observed in  
539 dopaminergic neurons. Specific mechanisms involved in the effects of nicotine on the CNS are  
540 summarized in **Fig. 2C**.

541

542 *6.3 Nicotine and zebrafish: neurobehavioral studies*

543           The zebrafish has been argued to be a suitable tool to study the effects of nicotine on the  
544 CNS (Braidà et al., 2014). Eight zebrafish neuronal cDNA of nAChRs subunits have been cloned  
545 ( $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 6$ ,  $\alpha 7$ ,  $\beta 2$ ,  $\beta 3$ , and  $\beta 4$ ) (Ackerman et al., 2009; Papke et al., 2012; Welsh et al., 2009;  
546 Zirger et al., 2003), showing high degree of similarities (nucleotide sequence homologies across  
547 genes) when compared to their respective human and rat orthologs (Papke et al., 2012).  
548 Importantly, the well-known nAChR antagonists (*e.g.*, mecamylamine and dihydro- $\beta$ -  
549 erythroidine) reverse the effects of nicotine in zebrafish just like in mammals, supporting the notion  
550 that there is strong conservation between zebrafish and humans nAChR (Bencan and Levin, 2008;  
551 Eddins et al., 2009; Levin et al., 2006).

552           During the last decade, several studies aiming to understand how nicotine influences  
553 zebrafish behavior and memory have been performed (Cousin et al., 2014; Faillace et al., 2017;  
554 Klee et al., 2012). Acute nicotine exposure alters the shoal polarization (coordinated directional  
555 movement of shoal members) and reduces social cohesion (increases inter-individual distance) in  
556 zebrafish (Miller et al., 2013). Moreover, nicotine attenuates contextual fear-like responses in  
557 zebrafish exposed to conspecific alarm substance, and a putative role of cholinergic signaling in  
558 aversive memory has also been predicted in this species (Ziani et al., 2018). Nicotine also exerts a  
559 dual role in anxiety-like behavior in zebrafish similarly to mammals (Levin et al., 2007;  
560 Sackerman et al., 2010; Singer et al., 2016), whereby acute exposure has anxiolytic (Sackerman et  
561 al., 2010), and chronic exposure anxiogenic effects (File et al., 2000; Stewart et al., 2015b).  
562 Nicotine administered acutely may reduce swimming speed, increase vertical activity in an open  
563 tank task in both male and female zebrafish (Singer et al., 2016). Furthermore, both  $\alpha 7$  and  $\alpha 4\beta 2$   
564 nicotinic receptors play a role in nicotine-mediated reduction of anxiety-like responses in  
565 zebrafish (Bencan and Levin, 2008).

566           When administered chronically, nicotine induces conditioned place preference (CPP) in  
567 zebrafish (Faillace et al., 2017), and this effect may persist even after 3 weeks of abstinence from

568 the drug (Kily et al., 2008). Moreover, changes in the expression of 868 genes from adult  
569 zebrafish brain (50:50 male:female ratio) were detected following 30  $\mu\text{mol/L}$  nicotine exposure 20  
570 min each day over a 4-week period (Kily et al., 2008). Conversely, CPP-mediated reinforcing  
571 effects were blocked when nicotine was co-administered with cystine derivatives, showing that this  
572 response was due to binding to high-affinity heteromeric receptors (except  $\alpha_7$  receptors) and that  
573 these molecules may contribute to the induction of smoke cessation (Ponzoni et al., 2014).

574 Evidence shows that nicotine, similarly to its effects in mammals, improves cognitive  
575 function in zebrafish (Levin and Chen, 2004; Levin et al., 2006; May et al., 2016). For example,  
576 nicotine increases the discrimination index in a virtual object recognition test (VORT) (Braida et  
577 al., 2014), enhances familiar object preference (May et al., 2016), and at lower doses improves  
578 memory in a delayed spatial alternation test (Levin and Chen, 2004). In zebrafish, the VORT  
579 evaluates the selective attention behavior using 2D geometrical shapes, located on two opposite  
580 walls of the tank. Each fish is subjected to a familiarization trial, and the discrimination index is  
581 calculated at different time intervals (5 min to 96 h) following the novel shape recognition (Braida  
582 et al., 2014). Nicotine significantly improves the percent of correct accuracy in the spatial position  
583 discrimination test (Levin et al., 2006). This latter positive effect was observed 20–40 min post  
584 administration, and declined after 80 and 160 min (Levin et al., 2006). Interestingly, the neural  
585 bases associated with the positive effects of nicotine on learning in zebrafish involve the  
586 dopaminergic system, because nicotine exposure increases both acquisition rates and DOPAC  
587 (metabolite of dopamine) levels in the brain (Eddins et al., 2009). Furthermore, nAChRs play a  
588 modulatory role in zebrafish cognition, and learning performance changes in an inverted U-shaped  
589 nicotine dose-dependent manner with moderate nicotine doses improving while high doses  
590 impairing learning performance (Braida et al., 2014).

591 Nicotine exposure in *Danio rerio* gill (DrG) cell lines and gill tissue causes depletion of  
592 antioxidant enzymes, leading to reduced glutathione (GSH), SOD, CAT, glutathione S-transferase  
593 (GST) and glutathione peroxidase (GPx1a) (Nambi, 2017). Nicotine also increases lipid  
594 peroxidation and the expression of apoptosis-related genes, *p53* and *cas3* (Nambi, 2017).

595 Importantly, nicotine has embryotoxic effects and its administration during embryonic  
596 development leads to abnormal morphology and impaired heart rate (Yoo et al., 2018). Acute  
597 exposure increases embryonic motor output (Thomas et al., 2009), and evokes locomotor  
598 responses in zebrafish embryos 36 hours post-fertilization (hpf) (Mora-Zamorano et al., 2016),  
599 whereas chronic larval exposure results in changes in notochord length, reduced eye diameter,  
600 altered behavior and decreased survival (Parker and Connaughton, 2007). Moreover, adult female  
601 zebrafish exposed to nicotine exhibit downregulation of expression of myelin-related transcription  
602 regulators. Because myelin plays a key role in the nervous system, deficits in myelin are often  
603 related to various neuropsychiatric conditions and drug addiction (Zhao et al., 2014).

604

## 605 **7. Cannabinoids**

### 606 *7.1 General information*

607 For many centuries, *Cannabis sativa* has been used due to its psychoactive and medicinal  
608 properties (Ameri, 1999). About 147 million people consume cannabis worldwide (WHO, 2018)  
609 and approximately 13.1 million people are cannabis-dependent (Degenhardt et al., 2013). The  
610 extract of cannabis contains over 100 compounds called cannabinoids (CB), classified as a  
611 collective group of molecules that act on cannabinoid receptors. The most abundant CB is  $\Delta^9$ -  
612 tetrahydrocannabinol ( $\Delta^9$ -THC), its precursor cannabidiol (CBD), and cannabinol (CBN) (Panlilio et  
613 al., 2015). CBD and CBN do not exhibit the psychoactive properties of  $\Delta^9$ -THC, but have clinical  
614 interest because their antipsychotic, anticonvulsive, neuroprotective, and anxiolytic effects (Bonini  
615 et al., 2018; Izzo et al., 2009; Niesink and van Laar, 2013).

616 Interaction between genetic and environmental factors explain psychological, social, and  
617 physiological effects of cannabis following acute as well as long-term consumption (Danielsson et  
618 al., 2016). Inhalation of *C. sativa* smoke is the generally preferred route of administration because it  
619 produces rapid effects. Acute effects last for approximately 2–3 h and are often described as  
620 pleasant and relaxing (Panlilio et al., 2015).  $\Delta^9$ -THC mainly acts on the CNS, producing a mixture  
621 of psychotomimetic and depressant effects, along with several centrally mediated autonomic effects

622 (Curran et al., 2016). The mixture of depressant and stimulant effects is characterized by euphoria,  
623 talkativeness, sedation, easy laughter, distortion of time perception, increased perception of external  
624 stimuli, and memory lapses (Fratta and Fattore, 2013). Users typically experience peripheral effects,  
625 such as increased appetite, dry mouth, tachycardia, increased blood pressure, and bronchodilation  
626 (Panlilio et al., 2015). Overall, chronic CB use is associated with addiction, which results in  
627 cognitive impairment, poor educational outcome, diminished life satisfaction and achievement, and  
628 increased risk of psychotic disorders (Fratta and Fattore, 2013; Volkow et al., 2014).

629

## 630 *7.2 Putative mechanisms of cannabinoids in the CNS*

631 The first studies investigating the biology of cannabinoid signaling were conducted shortly  
632 after the two primary receptors, cannabinoid receptor 1 (CB<sub>1</sub>) and cannabinoid receptor 2 (CB<sub>2</sub>),  
633 were cloned in the early 1990s (Matsuda et al., 1990; Munro et al., 1993). CB<sub>1</sub> and CB<sub>2</sub> are  
634 transmembrane G-protein coupled receptors (GPCR) well-conserved in vertebrates. CB<sub>1</sub> is abundant  
635 in the brain and is widely distributed throughout the CNS, as well as in various peripheral tissues  
636 (Pertwee et al., 2010). CB<sub>2</sub> was identified within peripheral tissues and in glial cells of the brain  
637 (Cabral et al., 2008; Onaivi, 2006).

638 CB receptors are found in presynaptic terminals and modulate the release of  
639 neurotransmitters, including glutamate, GABA, glycine, acetylcholine, norepinephrine, dopamine,  
640 serotonin, and cholecystokinin (CCK) (Klee et al., 2012; Lupica et al., 2004). The activation of CB<sub>1</sub>  
641 and CB<sub>2</sub> receptors triggers the inhibition of adenylyl cyclase, voltage-gated Ca<sup>2+</sup> channels, and  
642 activation of potassium channels (Onaivi, 2006). These mechanisms are implicated in cannabinoid-  
643 evoked inhibition of neurotransmitter release (Fratta and Fattore, 2013). CB receptors also influence  
644 gene expression either directly by activating mitogen-activated protein (MAP) kinase or indirectly  
645 by reducing protein kinase A activity as a consequence of reduced adenylyl cyclase activity (Onaivi  
646 et al., 2002). These mechanisms may explain the different effects of cannabis on working memory,  
647 planning and decision-making, response speed, motor coordination, mood and cognition, as well as  
648 the role of endocannabinoid signaling in various disorders (*e.g.*, anxiety, depression, and addiction)



649 (Madras, 2015).

650 The processes involved in the release of endocannabinoids, activation of CB receptors, and  
651 their resulting behavioral and psychological effects, are not completely understood. The increased  
652 concentration of intracellular  $\text{Ca}^{2+}$  is probably an important cellular trigger, since  $\text{Ca}^{2+}$  activates  
653 enzymes involved in endocannabinoid biosynthesis (Robbe et al., 2002). Endocannabinoids  
654 suppress presynaptic glutamate release, leading to a depolarization-induced suppression of  
655 excitation (DSE), and inhibit presynaptic GABA release, leading to depolarization-induced  
656 suppression of inhibition (DSI) (Fratta and Fattore, 2013). Importantly, cannabinoid agonists not  
657 only act via presynaptic  $\text{CB}_1$  to inhibit the release of glutamate and GABA in the striatum, but also  
658 cause long-term effects on synaptic plasticity (long-term potentiation and depression), that can last  
659 hours or weeks (Hoffman and Lupica, 2001). **Fig. 2D** depicts some mechanisms associated with  
660 cannabinoid-mediated effects on the CNS.

661

### 662 *7.3. Cannabinoids and zebrafish: neurobehavioral studies*

663 Although zebrafish has emerged as an efficient model organism in addiction studies  
664 (Stewart et al., 2011), addictive properties of exogenous CB are poorly explored in this species.  
665 Nevertheless, the zebrafish has been claimed to be an appropriate tool to the study CB signaling  
666 because the endocannabinoid system (eCB) has been found extensively conserved between  
667 zebrafish and mammals (Klee et al., 2012).  $\text{CB}_1$  (Lam et al., 2006; Migliarini and Carnevali,  
668 2009) and  $\text{CB}_2$  (Elphick, 2012; Rodriguez-Martin et al., 2007) receptors, as well as some  
669 endocannabinoid key enzymes, such as fatty acid amide hydrolase, prostaglandin-endoperoxide  
670 synthase 2, transient receptor potential cation channel 1A, and peroxisome proliferator activated  
671 receptor alpha have homologous functions and high nucleotide sequence homology when  
672 compared to corresponding human genes (Klee et al., 2012). Although  $\Delta^9$ -THC exposure does not  
673 change the zebrafish behavior in the CPP task (Brock et al., 2017), fish acutely exposed to  $\Delta^9$ -  
674 THC exhibit activation of extracellular signal-regulated kinases (ERK) signaling in the lateral

675 pallium associated with deficits in spatial memory performance (Ruhl et al., 2014). The zebrafish  
676 eCB system seems modulate associative learning and memory, in which the stimulation of the  
677 CB<sub>1</sub> receptor may play a specific role in acquisition and storage of aversive learning and memory,  
678 while CB<sub>1</sub> blockade enhances cognitive functions (Ruhl et al., 2015). Moreover, CB<sub>1</sub> activation by  
679  $\Delta^9$ -THC has been found to inhibit acquisition of fear learning, possibly by impairing stimulus  
680 encoding processes in the pallial area of the zebrafish brain (Ruhl et al., 2017).

681         Similar to rodents, acute  $\Delta^9$ -THC exposure causes hypolocomotion in zebrafish  
682 (Smirnov and Kiyatkin, 2008; Stewart and Kalueff, 2014), while high  $\Delta^9$ -THC concentrations  
683 impair locomotor activity of zebrafish larvae (Akhtar et al., 2013). THC and CBD treatments alter  
684 synaptic activity at neuromuscular junctions, and fluorescent labeling of primary and secondary  
685 motor neurons reveals a change in branching patterns and a reduction in the number of axonal  
686 branches in the trunk musculature, culminating in reduced heart rates, axial malformations, and  
687 shorter trunks in zebrafish embryos (Ahmed et al., 2018). CBD exposure at blastula increases  
688 developmental dysmorphologies, especially jaw malformation (Carty et al., 2018). CB<sub>1</sub> and CB<sub>2</sub>  
689 double mutant zebrafish have impaired liver development and function (Liu et al., 2016).  
690 Specifically, inhibition of CB receptor activity has been found to disrupt liver development and  
691 metabolic function in zebrafish, affecting hepatic differentiation and liver size due to fewer  
692 hepatocytes and reduced liver-specific gene expression and cell proliferation (Liu et al., 2016).  
693 Moreover, both endocannabinoid system and retinoic acid signaling pathway influence lipid  
694 deposition during zebrafish embryogenesis, with additive function in lipid abundance during  
695 development (Fraher et al., 2015). In general, these data support zebrafish as a useful model to  
696 evaluate the neurobehavioral mechanisms of cannabinoids, as well as the potential involvement of  
697 endocannabinoid system in regulating different biochemical pathways. However, more studies  
698 about the circuit mechanisms underlying eCBs's role in reward, addiction, and anxiety are needed.

699

## 700 **8. Psychedelics**

### 701 *8.1. General information*

702            Psychedelic drugs are psychoactive substances that affect behavior, consciousness or  
703 thinking, perception, emotion, and other cognitive processes (Belouin and Henningfield, 2018;  
704 Nichols, 2016). Classical tryptamine chemicals-derived, such as lysergic acid (LSD), a well-  
705 known hallucinogenic agent, and Ayahuasca, a natural compound used in sacramental beverage  
706 (Johnson et al., 2018; McKenna and Riba, 2015) are examples of psychedelic compounds. In  
707 2013, the National Survey on Drug Use and Health published that more than 24.8 million people  
708 have used LSD at least once in their life.

709            Psychedelic reactions are subjective, variable, and unpredictable, and the abuse of these  
710 drugs lead to risks to individuals and society (Das et al., 2016). Some users feel positive euphoric  
711 feelings and hallucinogenic sights and sensations, while other report bad feelings including  
712 anxiety, panic, fear, mental confusion, and prolonged psychosis (Carbonaro and Gatch, 2016; Das  
713 et al., 2016; Johnson et al., 2018). Psychedelic drugs can also produce dependence when users  
714 increase drug amount and frequency of consumption, and withdrawal symptoms that include  
715 psychological and physical effects (NIH, 2016). Psychedelic compounds have received interest in  
716 clinical research since classic psychedelics have shown promising effects for treating cancer-  
717 related psychological distress, addictions, and depression (Johnson et al., 2018; Tupper et al.,  
718 2015).

719

### 720 *8.2. Putative mechanisms of psychedelics in the CNS*

721            The effects of psychedelic drugs on brain functioning are complex, but LSD and  
722 Ayahuasca, at least in part, share the same mechanism of action. LSD triggers pleiotropic  
723 mechanisms, and its psychosensory effects are mediated by agonism or partial agonism of  
724 serotonergic receptors (5-HT<sub>2A</sub> mainly, 5-HT<sub>1A</sub>, 5-HT<sub>2C</sub>, and 5-HT<sub>6</sub>) (Halberstadt and Geyer,  
725 2011; Nichols, 2016), as well as via dopaminergic, glutamatergic, and adrenergic modulation (De  
726 Gregorio et al., 2016; Kyzar et al., 2017; Nichols, 2004; Passie et al., 2008). Chronically, LSD

727 causes a persistent increase in 5-HT synthesis and turnover, and a downregulation of cortical  
728 5HT<sub>2A</sub> receptors (Diaz and Huttunen, 1971; Gresch et al., 2005; Lee and Geyer, 1980).

729 Ayahuasca preparation is basically composed of N,N-dimethyltryptamine (DMT) plus  $\beta$ -  
730 carboline alkaloids (McKenna and Riba, 2018). DMT acts on 5-HT<sub>1A</sub>, 5-HT<sub>2A</sub>, and 5-HT<sub>2C</sub>  
731 serotonin receptors (Dos Santos et al., 2012; Smith et al., 1998), and the agonism of 5-HT<sub>2A</sub>  
732 receptors also seems to depend on metabotropic glutamate receptors (mGluR) (Gonzalez-Maeso et  
733 al., 2008; Moreno et al., 2011).  $\beta$ -carboline alkaloids have monoamine oxidase-inhibiting  
734 properties, which results in increased brain monoamine levels (*e.g.*, serotonin, dopamine,  
735 norepinephrine) (Dominguez-Clave et al., 2016; Grella et al., 2003).  $\beta$ -carbolines protect DMT  
736 from degradation in the liver and gut, thus enabling it to cross the blood–brain barrier to exert its  
737 effects on the CNS (McKenna and Towers, 1984). Chronically, Ayahuasca increases the number  
738 of 5-HT receptors localized on platelets (Callaway et al., 1994), expanding the effects triggered by  
739 serotonergic modulation on peripheral tissues. Evolutionarily conserved mechanisms involved in  
740 the effects of psychedelics in the vertebrate brain are summarized in **Fig. 2E**.

741

### 742 *8.3. Psychedelics and zebrafish: neurobehavioral studies*

743 The use of zebrafish models to explore hallucinogen pharmacology and psychedelic  
744 mechanisms is fairly recent (Kyzar and Kalueff, 2016). Neurochemical and molecular studies  
745 emphasize the growing utility of zebrafish to investigate the role of psychedelics on the  
746 serotonergic neurotransmitter system (Volgin et al. 2019). Chemical manipulations of the  
747 serotonergic neurotransmitter system produce similar behavioral and neuroendocrinological  
748 effects in zebrafish and mammals (Maximino and Herculano, 2010; Maximino et al., 2013).  
749 Serotonergic enzymes, transporters, and receptors, as well as drug effects on 5-HT receptors have  
750 been found evolutionarily conserved in zebrafish (Aldeco et al., 2011; Herculano and Maximino,  
751 2014; Maximino et al., 2015; Norton et al., 2008; Schneider et al., 2012).

752 The first study characterizing the effects of LSD in zebrafish aimed to explore how LSD  
753 affects stress and behavior (Grossman et al., 2010). LSD (250  $\mu$ g/L, for 20 min) found increased

754 whole-body cortisol levels in zebrafish and also a wide range of behavioral changes induced by  
755 the drug, including increased the time spent in top and lit areas of the test tank, thigmotaxis,  
756 decreased freezing, and impaired shoaling responses (Green et al., 2012; Grossman et al., 2010).  
757 Although lower Ayahuasca concentrations (0.1 and 0.5 ml/L) were found not to affect locomotion  
758 or to reduce anxiety-like behaviors in zebrafish, higher concentrations (1.0 and 3.0 ml/L) reduced  
759 locomotion and triggered anxiety-like behaviors in a concentration-dependent manner (Savoldi et  
760 al., 2017). Importantly, high Ayahuasca concentrations have been found responsible for inducing  
761 locomotor deficits and developmental abnormalities in zebrafish embryos, including hatching delay,  
762 loss of equilibrium, edema, and accumulation of red blood cells (Andrade et al., 2018).  
763 Chronically, Ayahuasca has been found to impair discriminative performance and to trigger  
764 locomotor alterations in adult zebrafish (Lobao-Soares et al., 2018). In sum, the zebrafish has  
765 turned out to be a sensitive tool for the analysis of psychedelic drug-induced functional changes in  
766 the vertebrate brain. Although psychedelic drug induced behavioral responses are beginning to be  
767 well characterized in zebrafish, there is a growing need for elucidation of molecular biomarkers of  
768 hallucinogenic action on various neurotransmitter pathways, including serotonin, dopamine,  
769 glutamate, cannabinoid, opioid, and acetylcholine receptors (Neelkantan, 2013), a research area  
770 where zebrafish may be particularly useful in the future.

771

## 772 **9. Concluding remarks**

773 This review showcased the zebrafish from the perspectives of how this species may be  
774 utilized in studying complex neurobehavioral phenotypes related to drug abuse. The conserved  
775 physiological and molecular mechanisms, as well as the well-characterized behaviors and external  
776 development of zebrafish, make this species a valuable tool with which one can explore drug  
777 abuse related mechanisms (Fontana et al., 2018a; MacRae and Peterson, 2015). Multiple drug  
778 abuse-related phenomena have been explored in zebrafish, which shows a wide range of behaviors  
779 with clinically translatable data. Although addiction research in zebrafish are still on its infancy,  
780 future directions and questions that remain open with regard to the use of the zebrafish in

781 modeling drug abuse and addiction-related phenotypes are summarized in **Table 4**. In sum, a  
782 growing number of studies conducted with zebrafish shows how this species may be utilized in  
783 the analysis of molecular and cellular mechanisms underlying behavioral changes induced by  
784 drugs of abuse. These studies suggest that the zebrafish will have utility in the development of  
785 potential therapeutic strategies for drug abuse related disorders. Furthermore, the often complex  
786 mechanistic nature of these disorders necessitates comprehensive and high throughput essays,  
787 including pharmaceutical compound or mutation screens, approaches that are particularly efficient  
788 with this small and simple vertebrate. Validation of drug abuse-related zebrafish protocols and  
789 characterization of novel zebrafish behaviors will further enhance the utility of this species in  
790 addiction research.

791

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803

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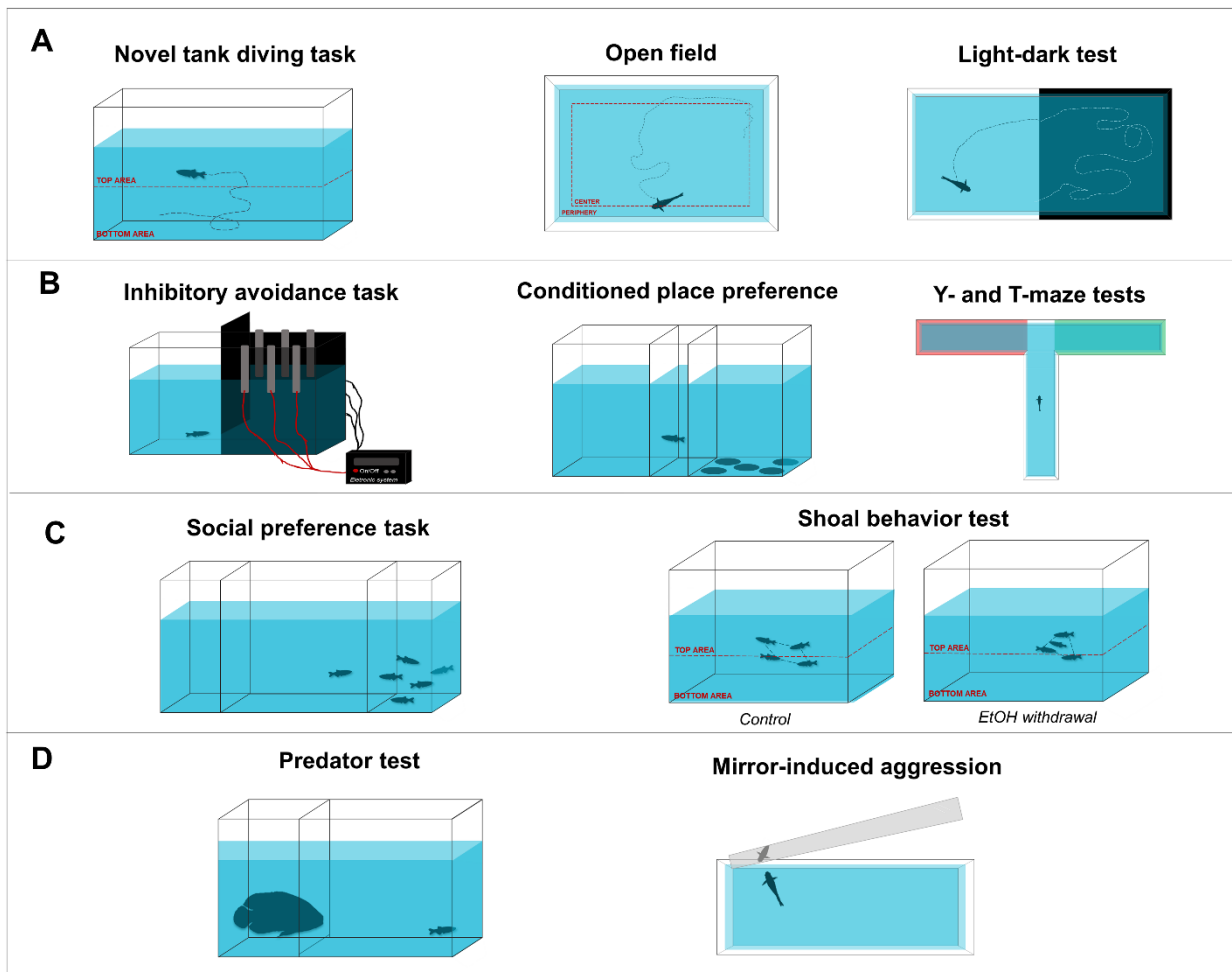
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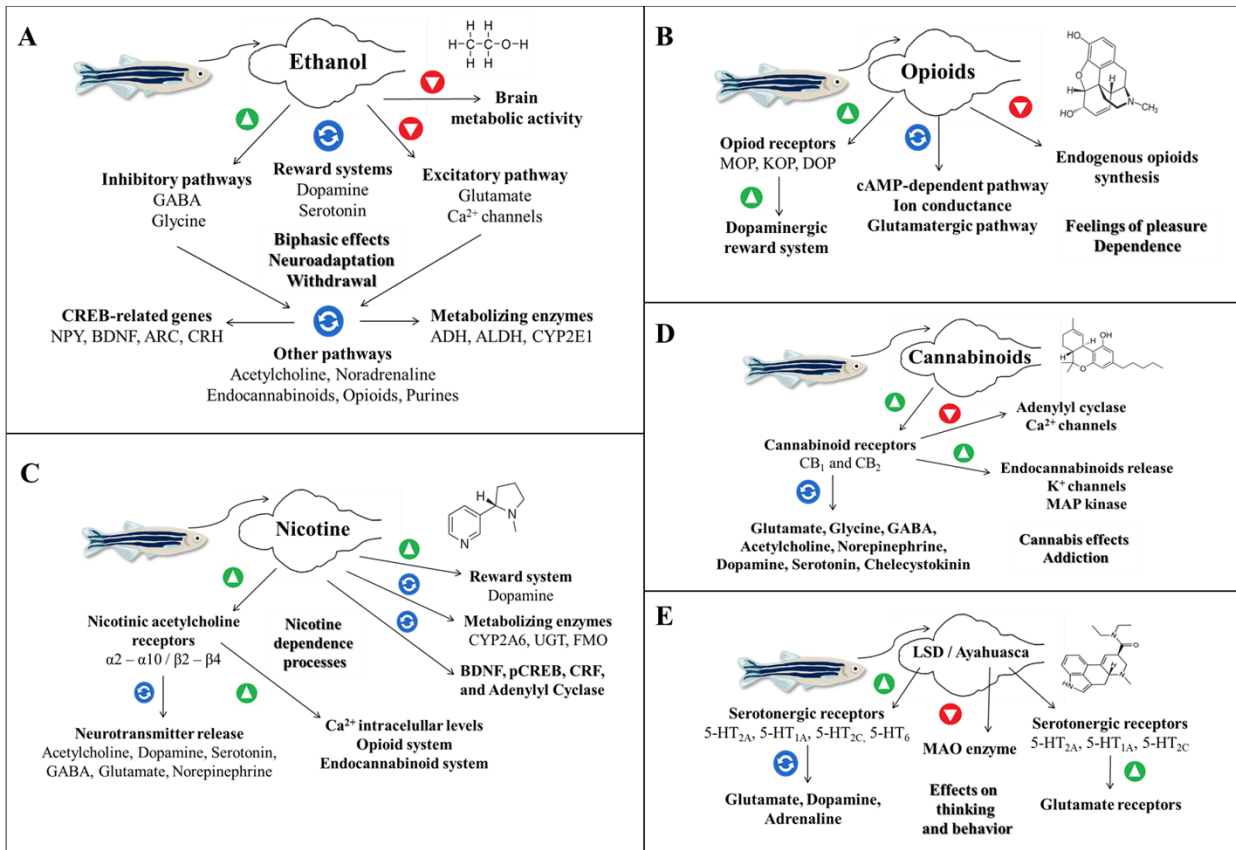
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1648 **Figures**

1649  
 1650 **Fig. 1.** Main behavioral tests used to assess drug abuse- and addiction-related phenotypes in  
 1651 zebrafish. **(A)** Novel tank diving task, open field, light-dark test (locomotion, exploration, and  
 1652 anxiety-like behaviors); **(B)** inhibitory avoidance task, conditioned place preference, Y- and T-  
 1653 maze tests (aversive and spatial memory tasks); **(C)** social preference task, shoal behavior test  
 1654 (seeking for conspecifics and social interaction, respectively); **(D)** predator test, mirror induced-  
 1655 aggression test (fear/avoidance responses and aggression behavior, respectively).



1656

1657 **Fig. 2.** Overview of the main mechanisms triggered by ethanol, opioids, nicotine, cannabinoids,

1658 LSD, and Ayahuasca on zebrafish CNS. **(A)** Ethanol directly decreases the brain metabolic

1659 activity, inhibits glutamatergic neurotransmission, transmembrane flow of  $\text{Ca}^{2+}$ , and potentiates

1660 GABA- and glycine-mediated synapses. Indirectly, ethanol activates dopaminergic and

1661 serotonergic neurons, which innervate the reward system. Ethanol also affects the homeostasis of

1662 cholinergic, noradrenergic, opioid, endocannabinoid, and purinergic systems. Ethanol

1663 metabolizing enzymes such as ADH, ALDH, and CYP2E1, as well as CREB-related genes, such

1664 as NPY, BDNF, ARC, and CRH are modulated by ethanol consumption. These changes are

1665 involved, at least in part, in biphasic effects, neuroadaptation, and withdrawal symptoms. **(B)**

1666 Opioids positively modulate MOP, KOP, DOP opioids receptors, and activate the reward system,

1667 culminating in the release of dopamine. cAMP dependent biochemical pathways, ion conductance,

1668 and glutamatergic pathway are also affected by opioids. The endogenous opioid synthesis is

1669 chronically inhibited by opioid consumption. These actions are involved in the feeling of pleasure

1670 effects, and dependence. **(C)** Nicotine activates nAChRs ( $\alpha 2$ – $\alpha 10$ ,  $\beta 2$ – $\beta 4$ ), and promotes the

1671 release of neurotransmitters, including acetylcholine, dopamine (reward system), serotonin,  
1672 GABA, glutamate, and norepinephrine in the brain. The activation of nAChRs also increases  
1673 intracellular levels of  $\text{Ca}^{2+}$ , and modulates both the opioid and endocannabinoid systems. Nicotine  
1674 metabolizing enzymes such as CYP2A, UGT, and FMO, as well as the levels of pCREB, CRF,  
1675 BDNF, and the adenylyl cyclase activity are modulated by nicotine. These changes are involved in  
1676 the nicotine dependence processes. **(D)** Cannabinoids act on  $\text{CB}_1$  and  $\text{CB}_2$  cannabinoids receptors  
1677 and modulate the release of neurotransmitters, including glutamate, GABA, glycine,  
1678 acetylcholine, norepinephrine, dopamine, serotonin, and cholecystokinin. The activation of CB  
1679 receptors triggers the inhibition of adenylyl cyclase, voltage-gated  $\text{Ca}^{2+}$  channels, and activation  
1680 of potassium channels, and MAP kinase activity, and endocannabinoids release. These alterations  
1681 are involved in the cannabis effects and addiction. **(E)** LSD effects are mediated by agonism or  
1682 partial agonism of serotonergic receptors ( $5\text{-HT}_{2A}$  mainly,  $5\text{-HT}_{1A}$ ,  $5\text{-HT}_{2C}$ , and  $5\text{-HT}_6$ ), as well as  
1683 via dopaminergic, glutamatergic, and adrenergic modulation. Ayahuasca preparation acts on  $5\text{-}$   
1684  $\text{HT}_{1A}$ ,  $5\text{-HT}_{2A}$ , and  $5\text{-HT}_{2C}$  serotonin receptors, and the agonism of  $5\text{-HT}_{2A}$  receptors activates  
1685 metabotropic glutamate receptors. Ayahuasca compounds have monoamine oxidase-inhibiting  
1686 properties, which results in increased brain monoamine levels. Basically, these alterations are  
1687 involved in the effects of thinking and behavior trigger by LSD, and Ayahuasca consumption.

1688 *Abbreviations:* Gamma-aminobutyric acid (GABA), neuropeptide Y (NPY), brain derived  
1689 neurotrophic factor (BDNF), activity-regulated cytoskeleton-associated protein (ARC),  
1690 corticotrophin-releasing hormone (CRH), alcohol dehydrogenase (ADH), acetaldehyde  
1691 dehydrogenase (ALDH), cytochrome P450 2E1 (CYP2E1), cyclic adenosine 3',5'-monophosphate  
1692 (cAMP), cAMP-responsive element-binding protein (CREB), cytochrome P450 2A6 (CYP2A6),  
1693 UDP glucuronosyltransfease (UGT), flavin-containing monooxygenase (FMO), corticotropin  
1694 releasing factor (CRF), mitogen-activated protein (MAP), monoamine oxidase (MAO). Green, red  
1695 and blue symbols indicate activation, inhibition, and concentration-dependent modulatory effects,  
1696 respectively.

**Table 1.** Main molecular targets of drugs of abuse and homology of corresponding genes between zebrafish and mammalian orthologs.

Drug	Gene	Encoded protein	Main role in drug abuse/addiction	Zebrafish orthologs	Nucleotide sequence similarity (%)		
					Human vs Zebrafish	Human vs Mice	Mice vs Zebrafish
Ethanol	<i>aldh2</i>	Aldehyde dehydrogenase 2	Oxidative pathway of alcohol metabolism	<i>aldh2a, aldh2b</i>	76.67 & 77.35	87.23	72.79 & 73.67
	<i>bdnf</i>	Brain derived neurotrophic factor	Ethanol self-administration processes	<i>bdnf</i>	78.81	91.77	79.10
	<i>creb1</i>	cAMP responsive element binding protein 1	Genetic predisposition and behavioral effects of ethanol	<i>creb1a, creb1b</i>	77.45 & 74.21	95.05	78.51 & 77.09
	<i>crh</i>	Adenylyl cyclase	Stress-induced alcohol consumption	<i>crh</i>	66.02	67.97	70.64
	<i>drd1</i>	Dopamine receptor D1	Reward and reinforcement properties of ethanol	<i>drd1</i>	70.99	74.56	69.38
	<i>drd2</i>	Dopamine receptor D2	Alcohol intake, reward and reinforcement effects	<i>drd2b, drd2c</i>	76.29 & 78.43	87.92	77.99 & 72.01
	<i>gabral</i>	Gamma-aminobutyric acid type A1 receptor	Consumption and ethanol inhibitory behaviors	<i>gabral</i>	78.08	84.14	77.98
	<i>gabra6</i>	Gamma-aminobutyric acid type A6 receptor	Intoxication and withdrawal alcohol symptoms	<i>gabra6a</i>	71.45	77.15	68.58
	<i>grin1</i>	Glutamate ionotropic receptor NMDA	Excitatory and withdrawal effects of ethanol	<i>grin1a, grin1b</i>	78.81 & 92.86	87.19	78.63 & 86.36
	<i>glral</i>	Glycine receptor alpha1	Ethanol-mediated inhibitory actions	<i>glral</i>	77.29	86.80	77.98
<i>npy</i>	Neuropeptide Y	Modulation of ethanol intake and dependence-related processes	<i>npy</i>	75.00	80.89	74.86	
<i>th</i>	Tyrosine hydroxylase	Dopamine-mediated behavior and locomotor effects of ethanol	<i>th</i>	100	83.04	67.25	
Opioids	<i>oprm1</i>	Opioid receptor mu1	Opioid dependence, target of endogenous opioids agents	<i>oprm1</i>	77.68	84.70	78.98
	<i>oprk1</i>	Opioid receptor kappa1	Addictive properties, endogenous and synthetic opioids ligands	<i>oprk1</i>	76.42	73.73	74.36
	<i>oprd1</i>	Opioid receptor delta1	Modulation of opioid reward and addiction	<i>oprd1a, oprd1b</i>	73.79 & 76.99	86.70	73.36 & 75.76
	<i>opr11</i>	Opioid related nociceptin receptor 1	Neurobehavioral responses to opioids	<i>opr11</i>	70.21	84.00	68.39
Cannabinoids	<i>cnr1</i>	Cannabinoid receptor 1	Withdrawal and effects of cannabinoids on mood and cognition	<i>cnr1</i>	75.53	82.22	75.68
	<i>cnr2</i>	Cannabinoid receptor 2	Reward and psychoactive effects of cannabinoids	<i>cnr2</i>	100	79.21	66.06
	<i>faah2</i>	Fatty acid amide hydrolase 2	Endocannabinoid catabolism, cannabis dependence	<i>faah2a, faah2b</i>	67.25 & 65.70	70.67	77.05 & 86.36
	<i>ptgs2</i>	Prostaglandin-endoperoxide synthase 2	Involved in endocannabinoid metabolism	<i>ptgs2a, ptgs2b</i>	69.47 & 71.60	72.70	70.74 & 72.27
	<i>trpa1</i>	Transient receptor potential cation channel 1A	Ionotropic cannabinoid receptor activated by THC	<i>trpa1a, trpa1b</i>	67.88 & 71.20	80.99	63.97 & 77.57
	<i>ppara</i>	Peroxisome proliferator activated receptor alpha	Activation of intracellular cannabinoids signalling cascades	<i>pparaa, pparab</i>	71.96 & 73.30	79.30	71.52 & 73.45

<b>Drug</b>	<b>Gene</b>	<b>Encoded protein</b>	<b>Main role in drug abuse/addiction</b>	<b>Zebrafish orthologs</b>	<b>Nucleotide sequence similarity (%)</b>			
					<b>Human Zebrafish</b>	<b>vs Human Mice</b>	<b>vs Mice</b>	<b>1705 vs Zebrafish</b>
	<i>chrna2</i>	Cholinergic receptor nicotinic alpha 2	Increases the risk of nicotine dependence, self-administration	<i>chrna2a, chrna2b</i>				1703
	<i>chrna3</i>	Cholinergic receptor nicotinic alpha 3	Nicotine dependence and abstinence, anxiety-like behaviors	<i>chrna3</i>	79.08	88.52	76.83	1704
	<i>chrna5</i>	Cholinergic receptor nicotinic alpha 5	Increases nicotine intake, anxiety, and withdrawal	<i>chrna5</i>	71.59	84.16	70.97	
<b>Nicotine</b>	<i>chrna6</i>	Cholinergic receptor nicotinic alpha 6	Increases risk of nicotine dependence, withdrawal symptoms	<i>chrna6</i>	73.76	81.01	74.70	1706
	<i>chrna7</i>	Cholinergic receptor nicotinic alpha 7	Nicotine-stimulated dopamine release, abstinence	<i>chrna7</i>	72.11	87.58	71.92	
	<i>chrnb2</i>	Cholinergic receptor nicotinic beta 2	Nicotine self-administration and conditioning reinforcement	<i>chrnb2</i>	80.32	81.90	79.98	1707
	<i>chrnb4</i>	Cholinergic receptor nicotinic beta 4	Nicotine dose-dependent tolerance development	<i>chrnb4</i>	79.57	83.05	76.53	1708
	<i>htr1a</i>	5-hydroxytryptamine receptor 1A	Stimulant effects, self-boundaries and cognitive control	<i>htr1aa, htr1ab</i>	70.88 & 67.60	81.58	77.35 & 67.57	1709
	<i>htr2a</i>	5-hydroxytryptamine receptor 2A	Development of tolerance, facilitates dopamine neurotransmission	<i>htr2a</i>	67.31	87.12	71.48	
<b>Psychedelics</b>	<i>htr2c</i>	5-hydroxytryptamine receptor 2C	Self-administration, addictive effects of hallucinogens	<i>htr2cl1</i>	69.67	83.25	69.23	1710
	<i>mao a</i>	Monoamine oxidase A	Psychedelic monoamine-oxidase-inhibiting properties	<i>mao</i>	69.95	81.10	69.87	
	<i>tph1</i>	Tryptophan hydroxylase 1	Biosynthesis of serotonin, hyper serotonergic activity	<i>tph1a, tph1b</i>	71.43 & 71.69	84.12	73.31 & 71.55	1711
				<b>Mean</b>	<b>77.02</b>	<b>82.63</b>	<b>76.00</b>	1712
								1713

**Note:** NCBI database was used to assess the nucleotide sequence and to obtain the nucleotide identity rate (%) through blast analyses.

1716 **Table 2.** Overview of main behavioral tests and endpoints used to evaluate the effects of drugs of abuse and addiction behavior in adult zebrafish.

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Behavioral test	Major endpoints	References
<b>Novel tank diving task</b>	<b>Locomotor parameters:</b> Distance traveled, Absolute turn angle, Maximum speed, Immobility. <b>Anxiolytic-like behavior:</b> ↑ Time spent in the top area, ↑Transitions to the top area, ↓Latency to enter the top area. <b>Anxiogenic-like behavior:</b> ↑Time spent in bottom area, ↑Freezing/Erratic movements.	Egan et al., 2009; Gerlai et al., 2000; Rosemberg et al., 2011; Stewart et al., 2014; Tran et al., 2016
<b>Open field</b>	<b>Thigmotaxis behavior:</b> Entries to the center area, Time in the center area, Entries to periphery area, Time in the periphery area. <b>Exploratory behavior:</b> Distance traveled, Maximum speed, Time immobile.	Baiamonte et al., 2016; Champagne et al., 2010; Grossman et al., 2010; Nema et al., 2018
<b>Light-dark test</b>	<b>Anxiolytic-like behavior:</b> ↑Time spent in the lit area. <b>Anxiogenic-like behavior:</b> ↑Time spent in the dark area, ↑Freezing, ↑Risk assessment episodes.	Chaves et al., 2018; Faccioli et al., 2019; Holcombe et al., 2014; Maximino et al., 2010
<b>Inhibitory avoidance task</b>	<b>Aversive memory:</b> Latency to enter in the aversive stimulus area. Time in the aversive stimulus area.	Blank et al., 2009; Manuel et al., 2014; Nazario et al., 2015; Cleal and Parker, 2018.
<b>Conditioned place preference</b>	<b>Drug-seeking behavior:</b> Preference for the drug-associated compartment.	Brock et al., 2017; Collier et al., 2014; Mathur et al., 2011
<b>Y- and T-maze tests</b>	<b>Spatial memory and visual discrimination learning:</b> Total arm entries, Time spent in each arm, Center entries, Freezing, Directional preference, Frequency of choice, Repetitive alternation.	Aoki et al., 2015; Cognato et al., 2012; Grossman et al., 2010
<b>Social preference task</b>	<b>Seeking for conspecifics:</b> Time spent near the conspecific area, Transitions to the conspecific area..	Baggio et al., 2018; Fontana et al., 2018; Grossman et al., 2010
<b>Shoal behavior test</b>	<b>Shoaling behavior:</b> Inter-fish distance, Nearest neighbor distance, Farthest neighbor distance, Shoal area. <b>Anxiolytic-like behavior:</b> Shoaling disruption. <b>Anxiogenic-like behavior:</b> ↑Shoal cohesion.	Fontana et al., 2018; Müller et al., 2017; Canzian et al. 2017; Fernandes et al., 2015
<b>Predator test</b>	<b>Avoidance and fear-like responses:</b> Time in the predator area, Transitions to the predator area, Freezing episodes, Risk assessment episodes.	Gerlai et al., 2000; Fontana et al., 2018; Ladu et al., 2015
<b>Mirror-induced aggression test</b>	<b>Aggressive-like behavior:</b> Time in the mirror area, Transitions to the mirror area, Aggressive episodes, Duration of aggressive episodes.	Echevarria et al., 2011; Fontana et al., 2016; Gerlai et al., 2000

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1720 **Table 3.** Overview of the main experimental protocols for modeling drug abuse and addiction-related behaviors in zebrafish.

<b>Drug</b>	<b>Experimental protocol</b>	<b>Biological response</b>	<b>References</b>
<b>Ethanol</b>	<b>Acute ethanol exposure</b> (0.25, 0.5, 1.0% v/v for 1 h)	U-shaped response. Ethanol modulates locomotor-, aggression-, anxiety-, and fear-like behaviors. Increase brain alcohol levels, trigger oxidative stress and neurochemical changes.	Gerlai et al., 2000; Rosemberg et al., 2012; Fontana et al., 2016; Chatterjee and Gerlai, 2009; Chatterjee, Shams and Gerlai, 2014
	<b>Ethanol conditioned place preference</b> (1.5% v/v for 20 min)	Increases alcohol preference, measures positive-reinforcing qualities of alcohol.	Mathur and Guo 2011; Mathur, Lau, and Guo, 2011
	<b>Intermittent ethanol exposure</b> (1.0 % v/v, 20 min per day, for 8 consecutive days)	Elicits defensive responses by stimulating social behavior, geotaxis, and scototaxis, which reflect angiogenesis. Induces tolerance and withdrawal effects and trigger oxidative stress.	Mathur and Guo, 2011; Müller et al., 2017
	<b>Continuous ethanol exposure</b> (0.5% v/v applied continuously for 10 weeks)	Tolerance and withdrawal effects. Ethanol triggers anxiety-like responses, and impairs aversive learning. Changes in neurochemicals levels and oxidative stress are observed.	Damodaran et al., 2006; Dlugos and Rabin, 2003; Egan et al., 2009; Gerlai et al., 2009; Agostini et al., 2018; Chaves et al., 2018; Cachat et al., 2010
	<b>Voluntary ethanol intake</b> (10% or 20% ethanol (v/v) mixed with gelatin)	Stimulatory effects on locomotion, reduces anxiety, potentiates aggressive behaviors, and increases the expression of galanin and orexin in specific hypothalamic areas.	Sterling et al., 2015
	<b>Fetal alcohol spectrum disorder</b> (0.12% ethanol from 2 to 9 dpf)	Behavioral deficits in social conduct and anxiety-like responses. Cognition, learning, and memory are altered. Neurochemical changes and teratogenic ethanol effects are observed.	Parker et al., 2014; Fernandes et al., 2019; Lutte et al., 2018; Amorim et al., 2017; Cleal and Parker, 2018; Baggio et al., 2018; Fernandes et al., 2018; Ramlan et al., 2017
<b>Opioids</b>	<b>Acute diacetylmorphine exposure</b> (15 and 25 mg/L, for 20 min)	Evokes hyperlocomotion without changing anxiety-like behaviors.	Stewart and Kalueff, 2014
	<b>Morphine acute exposure</b> (2.0 mg/L, for 15 min)	Elicits anxiolysis in zebrafish by decreasing erratic movements and increasing the time in the top area of the tank.	Taylor et al., 2017
	<b>Morphine conditioned preference paradigm</b> (1 mg/mL, for 30 min)	Conditioned morphine preference is increased, which is blocked by naloxone (an opioid antagonist) and dopaminergic antagonists.	Lau et al., 2006; Wong et al., 2010
	<b>Morphine larval exposure</b> (0.4, 0.8, and 1.5 $\mu$ M, for 1 h)	Exposure modulates the choice preference and such response is attenuated by opioid and dopamine antagonists	Bretaud et al., 2007
	<b>Self-administration of hydrocodone hydrochloride</b> (1.5 $\mu$ g of hydrocodone from a solution of 6mg/L)	Robust self-administration of hydrocodone hydrochloride, which is mu-opioid receptor-dependent and is blocked by dopaminergic and glutamatergic antagonists	Bossé and Peterson, 2017
	<b>Morphine withdrawal</b> (1.5 mg/L, for 2 weeks)	Evokes angiogenesis and changes in whole-body cortisol levels	Cachat et al., 2010



Drug	Experimental protocol	Biological response	References
Nicotine	<b>Acute nicotine exposure</b> (4 or 8 mg/L, for 3 min)	Anxiolysis, reduces shoal cohesion and swimming speed of zebrafish. Improves aversive and spatial memories, and is related to changes in cholinergic and dopaminergic signaling.	Ziani et al., 2018; Miller et al., 2013; Braida et al., 2014; May et al., 2016; Levin and Chen et al., 2004; Eddins et al., 2009
	<b>Chronic nicotine exposure</b> (1-2mg/L, for 4 days)	Increases shoaling and facilitates anxiogenic responses.	Stewart et al., 2015; File et al., 2000
	<b>Nicotine conditioned place paradigm</b> (15 mg/L, 20 min per day, for 14 days)	Dose-dependent acute nicotine reinforcement response that persists following 3 weeks of abstinence.	Failacce et al., 2018
	<b>Nicotine embryo exposure</b> (5, 10, or 20 mg/L, for 10 days)	Changes the notochord length, reduces eye diameter, alters behavior and decreases survival	Parker and Conhaughton, 2007
Cannabinoids	<b>Acute exposure to <math>\Delta^9</math>-tetrahydrocannabinol</b> (100 nM, for 1h)	Triggers hypolocomotion. <a href="#">Extracellular signal-regulated kinases</a> signaling activation and deficits in spatial memory performance and associative learning are observed.	Ruhl et al., 2014; Ruhl et al., 2015; Ruhl et al., 2017; Stewart and Kalueff, 2014; Smirnov and Kiyatkin, 2008
	<b><math>\Delta^9</math>-tetrahydrocannabinol and canabidiol embryo exposure</b> (2, 4, 6, 8 and 10 mg/L of $\Delta^9$ -THC, and 1, 2, 3 and 4 mg/L of CB, from 5.45-48 hpf)	$\Delta^9$ -THC impairs locomotor activity of larvae. $\Delta^9$ -THC and CB treatments reduce the number of axonal branches in the trunk musculature, culminating in reduced heart rates, axial malformations, and shorter trunks in embryos.	Akhtar et al., 2013; Ahmed et al., 2018; Carty et al., 2018
Psychedelics	<b>Acute lysergic acid exposure</b> (250 $\mu$ g/L, for 20 min)	Increases whole-body cortisol levels and induces behavioral changes, such as increases the time in top and lit areas, thigmotaxis, decreases freezing, and impairs shoaling responses.	Green et al., 2012; Grossman et al., 2010
	<b>Acute Ayahuasca exposure</b> (0.1, 0.5, 1.0, and 3.0 ml/L, 1 h)	Higher concentrations (1.0 and 3.0 ml/L) reduce locomotion and trigger anxiogenic-like behaviors in a concentration-dependent manner.	Savoldi et al., 2017
	<b>Ayahuasca embryo exposure</b> (0.064; 0.3; 1.6; 8; 40; 106 200 and 1000 mg/L, for 96 h)	High Ayahuasca concentrations causes locomotor deficits and developmental abnormalities, including hatching delay, loss of equilibrium, edema, and accumulation of red blood cells.	Andrade et al., 2018
	<b>Chronic Ayahuasca exposure</b> 0.1 and 0.5 ml/L, for 13 days	Impairs discriminative learning performance and causes locomotor changes in adults.	Lobao-Soares et al., 2018

\* The experimental exposure protocols may vary according to the study. *Abbreviations:*  $\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -THC), canabidiol (CB).

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1725 **Table 4.** Selected open questions in the field of zebrafish modeling drug abuse and addiction.

Questions
• Can zebrafish help elucidate unknown molecular pathways evolutionarily conserved involved in addiction?
• Can zebrafish serve as a suitable tool to identify novel therapeutic targets and pharmacological treatments against drug addiction?
• Can novel drug abuse- and addiction-related behavioral phenotypes still be validated in zebrafish models?
• Can comorbidities associated with drug abuse and addiction be studied in zebrafish?
• Can zebrafish help investigate the role of external factors ( <i>e.g.</i> , depression, social deprivation, and individual susceptibility) in drug addiction models?
• Can anti-addiction drugs treat addiction-related symptoms in zebrafish?
• Are epigenetic processes involved in drug abuse and addiction in zebrafish?
• Do sex differences play a key role in drug abuse and addiction in zebrafish models?
• <b>Are there any genetic, biochemical, and behavioral differences among zebrafish strains that contribute to drug addiction response?</b>
• Can duplicated genes <b>be differently modulated after drug exposure, playing a role in the effects of drug abuse and addiction?</b>

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