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

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Abstract

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

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

1. Introduction

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

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

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

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

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

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

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

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

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

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

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

3. Limitations of zebrafish models to asesss the neurobehavioral mechanisms of drug abuse

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

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

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

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

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

4. Ethanol

4.1 General information

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

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

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

4.2. Putative mechanisms of ethanol in the CNS

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

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

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

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

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

4.3. Ethanol and zebrafish: neurobehavioral studies

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

Acute ethanol exposure increases dopaminergic and serotonergic activity in the brain of AB zebrafish (Chatterjee and Gerlai, 2009; Chatterjee et al., 2014; Tran et al., 2016; Tran et al., 2015c). However, ethanol decreases the levels of aspartate, taurine, GABA, glutamate and glycine in a concentration-dependent manner (Chatterjee et al., 2014). Furthermore, ethanol acutely enhances acetylcholinesterase activity and alters antioxidant defenses by decreasing superoxide dismutase (SOD) and increasing catalase (CAT) activities, which result in lipid peroxidation (Rosemberg et al., 2010). Ethanol acutely stimulates mitochondrial respiration and consequently the bioenergetics eficience, reinforcing the ethanol stimulatory effect on mitochondrial O_2 consumption (Müller et al., 2019). Mounting data also support the involvement of purinergic signaling in ethanol-mediated responses, since ethanol and its metabolite, acetaldehyde, modulate the hydrolysis of extracellular nuceleotides in zebrafish (Rico et al., 2007; Rico et al., 2008). Because zebrafish neurotransmitter systems show a robust functional correspondence and homology compared to those of mammals, investigators have concluded that the main functions, enzymes, receptors, transporters affected by ethanol are evolutionarily conserved (Cox et al., 2005; Kaslin et al., 2004; Kim et al., 2004; Maximino et al., 2013; Moly et al., 2014; Panula et al., 2006; Rico et al., 2003; Senger et al., 2004).

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

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

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

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

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

5. Opioids

5.1. General information

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

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

5.2. Putative mechanisms of opioids in the CNS

Opioid molecules travel in the bloodstream to the brain and attach to specific proteins at the surfaces of opiate-sensitive neurons, known as opioid receptors (Kosten and George, 2002). In humans, there are three classical opioid receptors: the mu-opioid receptor (MOP), the kappa-opioid receptor (KOP), and the delta-opioid receptor (DOP), encoded by the genes *OPRM1*, *OPRK1*, and *OPRD1*, respectively (Al-Hasani and Bruchas, 2011). These genes encode G-protein-coupled receptors that interact with heterotrimeric (Gi/Go) G proteins (Dhawan et al., 1996). Opioids activate the mesolimbic (mindbrain) reward system, which plays a role in 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 chonic effects of opioids.

431

432 *5.3. Opioids and zebrafish: neurobehavioral studies*

Zebrafish have an evolutionarily conserved opioid system, which makes this fish a valuable tool for studying the molecular bases of human opioid addiction. The initial studies related to opioid system in zebrafish analyzed $ZFOR_1$, a delta-opioid receptor isolated using a probe from rat MOP (Barrallo et al., 1998). Phylogenetic analyses showed a high degree of nucleotide sequence conservation for all opioid receptor genes of zebrafish when compared to their respective human counterparts (Dreborg et al., 2008). Orthlogs of three classical human opioid receptors were characterized in zebrafish (Stevens, 2009). While the *oprm1*, *oprk1*, *oprl1* genes have single homologs in zebrafish, a pair of *oprd1* can be found in this species (Herrero-Turrion et al., 2014).

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

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

After smoking, nicotine is absorbed into the blood through the lungs and reaches the brain in ten seconds (Benowitz, 2009). Nicotine absorption is pH-dependent, where nicotine is non-ionized and quickly crosses cellular membranes at physiological pH (Le Houezec, 2003). Nicotine interacts with a number of nicotinic acetylcholine receptors (nAChRs) that are formed as homopentamers and heteropentamers from subunits in the CNS (Gotti et al., 2009). In the mammalian brain, there are eight α subunits (α_2–α_{10}) and three β subunits (β_2–β_4) (Karlin, 2002; Subramaniyan and Dani, 2015), and the most commonly expresses are $\alpha_4\beta_2$ or α_7 nAChR (Dani and Bertrand, 2007).

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

Nicotine dependence has different phases (D'Souza and Markou, 2013). The acquisition and maintenance of nicotine seeking occurs by the positive reinforcing effects of nicotine (*e.g.*, mild euphoria, relaxation, increased arousal, and decreased fatigue) (Henningfield et al., 1985). Chronically, nicotine induces neuroadaptation in the brain reward systems, leading to the development of dependence (D'Souza and Markou, 2013). The rewarding properties of nicotine are mediated by dopaminergic neurons in the mesocorticolimbic system (Zarrindast and Khakpai, 2018). Stimulation of the α4β2 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 glucuronosyltransfease (*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 neurotorphic 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**.

6.3 Nicotine and zebrafish: neurobehavioral studies

The zebrafish has been argued to be a suitable tool to study the effects of nicotine on the CNS (Braida et al., 2014). Eight zebrafish neuronal cDNA of nAChRs subunits have been cloned (α2, α3, α4, α6, α7, β2, β3, and β4) (Ackerman et al., 2009; Papke et al., 2012; Welsh et al., 2009; Zirger et al., 2003), showing high degree of similarities (nucleotide sequence homologies across genes) when compared to their respective human and rat orthologs (Papke et al., 2012). Importantly, the well-known nAChR antagonists (*e.g.*, mecamylamine and dihydro-β-erythroidine) reverse the effects of nicotine in zebrafish just like in mamals, supporting the notion that there is strong conservation between zebrafish and humans nAChR (Bencan and Levin, 2008; Eddins et al., 2009; Levin et al., 2006).

During the last decade, several studies aiming to understand how nicotine influences zebrafish behavior and memory have been performed (Cousin et al., 2014; Faillace et al., 2017; Klee et al., 2012). Acute nicotine exposure alters the shoal polarization (coordinated directional movement of shoal members) and reduces social cohesion (increases inter-individual distance) in zebrafish (Miller et al., 2013). Moreover, nicotine attenuates contextual fear-like responses in zebrafish exposed to conspecific alarm substance, and a putative role of cholinergic signaling in aversive memory has also been predicted in this species (Ziani et al., 2018). Nicotine also exerts a dual role in anxiety-like behavior in zebrafish similarly to mammals (Levin et al., 2007; Sackerman et al., 2010; Singer et al., 2016), whereby acute exposure has anxiolytic (Sackerman et al., 2010), and chronic exposure anxiogenic effects (File et al., 2000; Stewart et al., 2015b). Nicotine administered acutely may reduce swimming speed, increase vertical activity in an open tank task in both male and female zebrafish (Singer et al., 2016). Furthermore, both α_7 and $\alpha_{4\beta2}$ nicotinic receptors play a role in nicotine-mediated reduction of anxiety-like responses in zebrafish (Bencan and Levin, 2008).

When administered chronically, nicotine induces conditioned place preference (CPP) in 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 μmol/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 derivates, showing that this
572 response was due to binding to high-affinity heteromeric receptors (except α_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). Furthremore, 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 Δ^9-
612 tetrahydrocannabinol (Δ^9-THC), its precursor cannabidiol (CBD), and cannabinol (CBN) (Panlilio et
613 al., 2015). CBD and CBN do not exhibit the psychoactive properties of Δ^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). Δ^9-THC mainly acts on the CNS, producing a mixture
621 of psychotomimetic and depressant effects, along with several centrally mediated autonomic effects

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

7.2 Putative mechanisms of cannabinoids in the CNS

The first studies investigating the biology of cannabinoid signaling were conducted shortly after the two primary receptors, cannabinoid receptor 1 (CB_1) and cannabinoid receptor 2 (CB_2), were cloned in the early 1990s (Matsuda et al., 1990; Munro et al., 1993). CB_1 and CB_2 are transmembrane G-protein coupled receptors (GPCR) well-conserved in vertebrates. CB_1 is abundant in the brain and is widely distributed throughout the CNS, as well as in various peripheral tissues (Pertwee et al., 2010). CB_2 was identified within peripheral tissues and in glial cells of the brain (Cabral et al., 2008; Onaivi, 2006).

CB receptors are found in presynaptic terminals and modulate the release of neurotransmitters, including glutamate, GABA, glycine, acetylcholine, norepinephrine, dopamine, serotonin, and cholecystokinin (CCK) (Klee et al., 2012; Lupica et al., 2004). The activation of CB_1 and CB_2 receptors triggers the inhibition of adenylyl cyclase, voltage-gated Ca^{2+} channels, and activation of potassium channels (Onaivi, 2006). These mechanisms are implicated in cannabinoid-evoked inhibition of neurotransmitter release (Fratta and Fattore, 2013). CB receptors also influence gene expression either directly by activating mitogen-activated protein (MAP) kinase or indirectly by reducing protein kinase A activity as a consequence of reduced adenylyl cyclase activity (Onaivi et al., 2002). These mechanisms may explain the different effects of cannabis on working memory, planning and decision-making, response speed, motor coordination, mood and cognition, as well as 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 Ca^{2+} is probably an important cellular trigger, since 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 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-mediates 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). CB_1 (Lam et al., 2006; Migliarini and Carnevali,
668 2009) and 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 funtions and high nucleotide sequence homology when
672 compared to corresponding human genes (Klee et al., 2012). Although Δ^9-THC exposure does not
673 change the zebrafish behavior in the CPP task (Brock et al., 2017), fish acutely exposed to Δ^9-
674 THC exhibit activation of extracellular signal-regulated kinases (ERK) signaling in the lateral

pallium associated with deficits in spatial memory performance (Ruhl et al., 2014). The zebrafish eCB system seems modulate associative learning and memory, in which the stimulation of the CB_1 receptor may play a specific role in acquisition and storage of aversive learning and memory, while CB_1 blockade enhances cognitive functions (Ruhl et al., 2015). Moreover, CB_1 activation by Δ^9-THC has been found to inhibit acquisition of fear learning, possibly by impairing stimulus encoding processes in the pallial area of the zebrafish brain (Ruhl et al., 2017).

Similar to rodents, acute Δ^9-THC exposure causes hypolocomotion in zebrafish (Smirnov and Kiyatkin, 2008; Stewart and Kalueff, 2014), while high Δ^9-THC concentrations impair locomotor activity of zebrafish larvae (Akhtar et al., 2013). THC and CBD treatments alter synaptic activity at neuromuscular junctions, and fluorescent labeling of primary and secondary motor neurons reveals a change in branching patterns and a reduction in the number of axonal branches in the trunk musculature, culminating in reduced heart rates, axial malformations, and shorter trunks in zebrafish embryos (Ahmed et al., 2018). CBD exposure at blastula increases developmental dysmorphologies, especially jaw malformation (Carty et al., 2018). CB_1 and CB_2 double mutant zebrafish have impaired liver development and function (Liu et al., 2016). Specifically, inhibition of CB receptor activity has been found to disrupt liver development and metabolic function in zebrafish, affecting hepatic differentiation and liver size due to fewer hepatocytes and reduced liver-specific gene expression and cell proliferation (Liu et al., 2016). Moreover, both endocannabinoid system and retinoic acid signaling pathway influence lipid deposition during zebrafish embryogenesis, with additive function in lipid abundance during development (Fraher et al., 2015). In general, these data support zebrafish as a useful model to evaluate the neurobehavioral mechanisms of cannabinoids, as well as the potential involvement of endocannabinoid system in regulating different biochemical pathways. However, more studies about the circuit mechanisms underlying eCBs's role in reward, addiction, and anxiety are needed.

8. Psychedelics

8.1. General information

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

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

8.2. Putative mechanisms of psychedelics in the CNS

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

causes a persistent increase in 5-HT synthesis and turnover, and a downregulation of cortical $5HT_{2A}$ receptors (Diaz and Huttunen, 1971; Gresch et al., 2005; Lee and Geyer, 1980).

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

8.3. Psychedelics and zebrafish: neurobehavioral studies

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

The first study characterizing the effects of LSD in zebrafish aimed to explore how LSD affects stress and behavior (Grossman et al., 2010). LSD (250 µg/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 locomor 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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802

803

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Figures

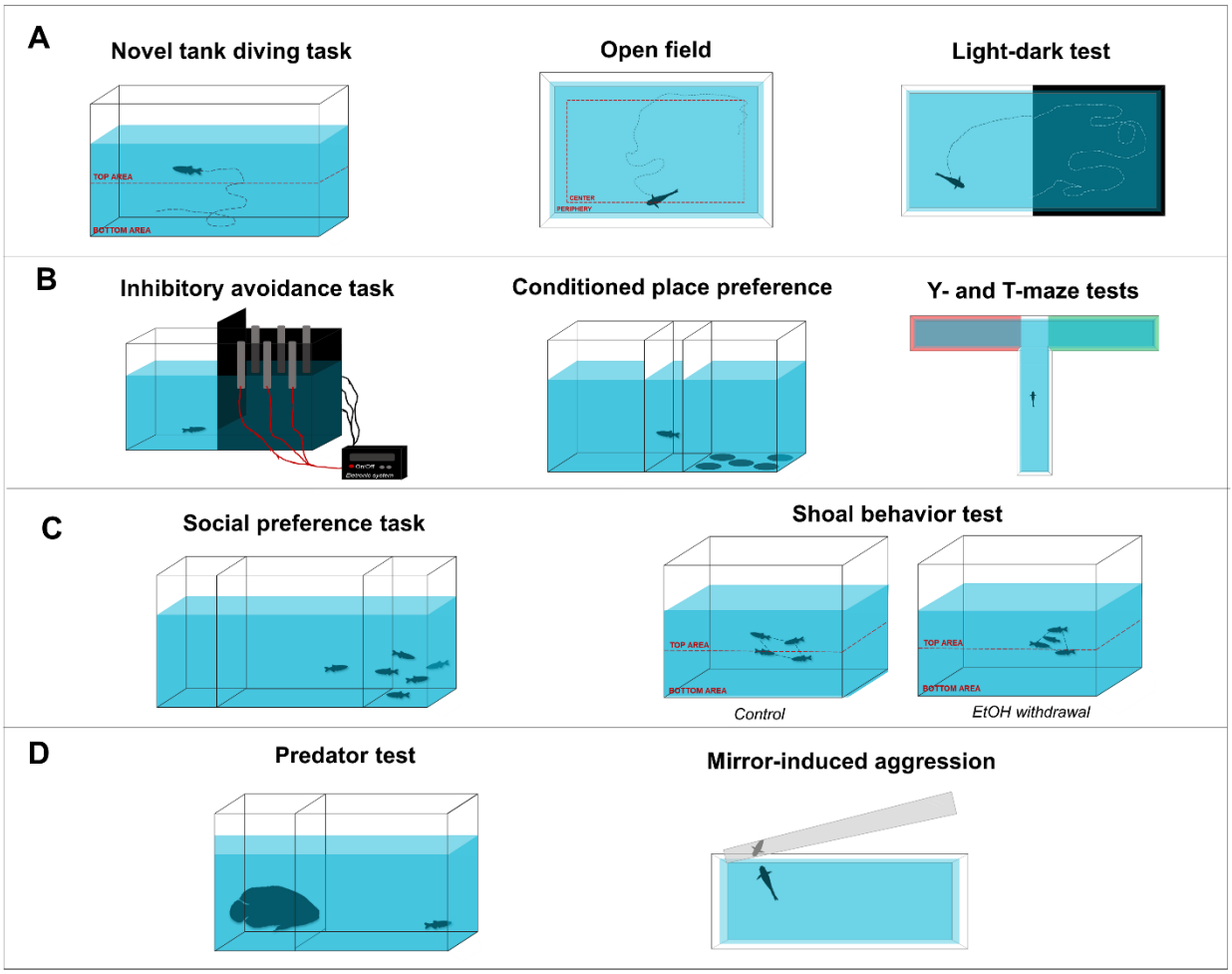

Fig. 1. Main behavioral tests used to assess drug abuse- and addiction-related phenotypes in zebrafish. **(A)** Novel tank diving task, open field, light-dark test (locomotion, exploration, and anxiety-like behaviors); **(B)** inhibitory avoidance task, conditioned place preference, Y- and T-maze tests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

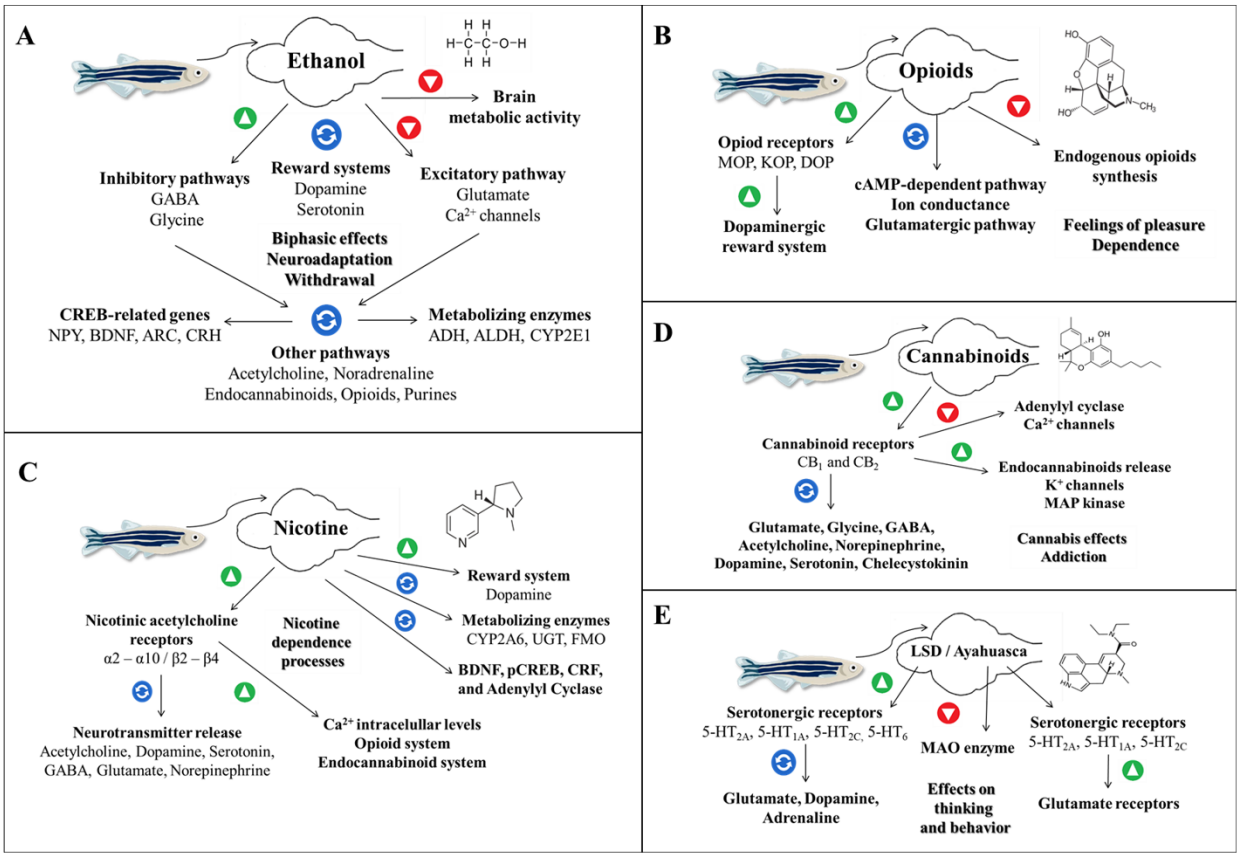

Fig. 2. Overview of the main mechanisms triggered by ethanol, opiods, nicotine, cannabinoids, LSD, and Ayahuasca on zebrafish CNS. **(A)** Ethanol directly decreases the brain metabolic activity, inhibits glutamatergic neurotransmission, transmembrane flow of Ca^{2+}, and potentiates GABA- and glycine-mediated synapses. Indirectly, ethanol activates dopaminergic and serotonergic neurons, which innervate the reward system. Ethanol also affects the homeostasis of cholinergic, noradrenergic, opioid, endocannabinoid, and purinergic systems. Ethanol metabolizing enzymes such as ADH, ALDH, and CYP2E1, as well as CREB-related genes, such as NPY, BDNF, ARC, and CRH are modulated by ethanol consumption. These changes are involved, at least in part, in biphasic effects, neuroadaptation, and withdrawal symptoms. **(B)** Opioids positively modulate MOP, KOP, DOP opioids receptors, and activate the reward system, culminating in the release of dopamine. cAMP dependent biochemical pathways, ion conductance, and glutamatergic pathway are also affected by opioids. The endogenous opioid synthesis is chronically inhibited by opioid consumption. These actions are involved in the feeling of pleasure effects, and dependence. **(C)** Nicotine activates nAChRs (α2–α10, β2–β4), and promotes the

release of neurotransmitters, including acetylcholine, dopamine (reward system), serotonin, GABA, glutamate, and norepinephrine in the brain. The activation of nAChRs also increases intracellular levels of Ca^{2+}, and modulates both the opioid and endocannabinoid systems. Nicotine metabolizing enzymes such as CYP2A, UGT, and FMO, as well as the levels of pCREB, CRF, BDNF, and the adenylyl cyclase activity are modulated by nicotine. These changes are involved in the nicotine dependence processes. **(D)** Cannabinoids act on CB_1 and CB_2 cannabinoids receptors and modulate the release of neurotransmitters, including glutamate, GABA, glycine, acetylcholine, norepinephrine, dopamine, serotonin, and cholecystokinin. The activation of CB receptors triggers the inhibition of adenylyl cyclase, voltage-gated Ca^{2+} channels, and activation of potassium channels, and MAP kinase activity, and endocannabinoids release. These alterations are involved in the cannabis effects and addiction. (E) LSD effects are mediated by agonism or partial agonism of serotonergic receptors (5-HT_{2A} mainly, 5-HT_{1A}, 5-HT_{2C}, and 5-HT_6), as well as via dopaminergic, glutamatergic, and adrenergic modulation. Ayahuasca preparation acts on 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} serotonin receptors, and the agonism of 5-HT_{2A} receptors activates metabotropic glutamate receptors. Ayahuasca compounds have monoamine oxidase-inhibiting properties, which results in increased brain monoamine levels. Basically, these alterations are involved in the effects of thinking and behavior trigger by LSD, and Ayahuasca consumption. *Abbreviations:* Gamma-aminobutyric acid (GABA), neuropeptide Y (NPY), brain derived neurotrophic factor (BDNF), activity-regulated cytoskeleton-associated protein (ARC), corticotrophin-releasing hormone (CRH), alcohol dehydrogenase (ADH), acetaldehyde dehydrogenase (ALDH), cytochrome P450 2E1 (CYP2E1), cyclic adenosine 3′,5′-monophosphate (cAMP), cAMP-responsive element-binding protein (CREB), cytochrome P450 2A6 (CYP2A6), UDP glucuronosyltransfease (UGT), flavin-containing monooxygenase (FMO), corticotropin releasing factor (CRF), mitogen-activated protein (MAP), monoamine oxidase (MAO). Green, red and blue symbols indicate activation, inhibition, and concentration-dependent modulatory effects, respectively.

Tables

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	*aldh2*	Aldehyde dehydrogenase 2	Oxidative pathway of alcohol metabolism	*aldh2a, aldh2b*	76.67 & 77.35	87.23	72.79 & 73.67
	bdnf	Brain derived neurotrophic factor	Ethanol self-administration processes	*bdnf*	78.81	91.77	79.10
	creb1	cAMP responsive element binding protein 1	Genetic predisposition and behavioral effects of ethanol	*creb1a, creb1b*	77.45 & 74.21	95.05	78.51 & 77.09
	crh	Adenylyl cyclase	Stress-induced alcohol consumption	*crh*	66.02	67.97	70.64
	drd1	Dopamine receptor D1	Reward and reinforcement properties of ethanol	*drd1*	70.99	74.56	69.38
	drd2	Dopamine receptor D2	Alcohol intake, reward and reinforcement effects	*drd2b, drd2c*	76.29 & 78.43	87.92	77.99 & 72.01
	gabra1	Gamma-aminobutiric acid type A1 receptor	Consumption and ethanol inhibitory behaviors	*gabra1*	78.08	84.14	77.98
	gabra6	Gamma-aminobutiric acid type A6 receptor	Intoxication and withdrawal alcohol symptoms	*gabra6a*	71.45	77.15	68.58
	grin1	Glutamate ionotropic receptor NMDA	Excitatory and withdrawal effects of ethanol	*grin1a, grin1b*	78.81 & 92.86	87.19	78.63 & 86.36
	glra1	Glycine receptor alpha1	Ethanol-mediated inhibitory actions	*glra1*	77.29	86.80	77.98
	npy	Neuropeptide Y	Modulation of ethanol intake and dependence-related processes	*npy*	75.00	80.89	74.86
	th	Tyrosine hydroxylase	Dopamine-mediated behavior and locomotor effects of ethanol	*th*	100	83.04	67.25
Opiods	*oprm1*	Opioid receptor mu1	Opioid dependence, target of endogenous opioids agents	*oprm1*	77.68	84.70	78.98
	oprk1	Opioid receptor kappa1	Addictive proprieties, endogenous and synthetic opioids ligands	*oprk1*	76.42	73.73	74.36
	oprd1	Opioid receptor delta1	Modulation of opioid reward and addiction	*oprd1a, oprd1b*	73.79 & 76.99	86.70	73.36 & 75.76
	oprl1	Opioid related nociceptin receptor 1	Neurobehavioral responses to opioids	*oprl1*	70.21	84.00	68.39
Cannabinoids	*cnr1*	Cannabinoid receptor 1	Withdrawal and effects of cannabinoids on mood and cognition	*cnr1*	75.53	82.22	75.68
	cnr2	Cannabinoid receptor 2	Reward and psychoactive effects of cannabinoids	*cnr2*	100	79.21	66.06
	faah2	Fatty acid amide hydrolase 2	Endocannabinoid catabolism, cannabis dependence	*faah2a, faah2b*	67.25 & 65.70	70.67	77.05 & 86.36
	ptgs2	Prostaglandin-endoperoxide synthase 2	Involved in endocannabinoid metabolism	*ptgs2a, ptgs2b*	69.47 & 71.60	72.70	70.74 & 72.27
	trpa1	Transient receptor potential cation channel 1A	Ionotropic cannabinoid receptor activated by THC	*trp1a, trpa1b*	67.88 & 71.20	80.99	63.97 & 77.57
	ppara	Peroxisome proliferator activated receptor alpha	Activation of intracellular cannabinoids signalling cascades	*pparaa, pparab*	71.96 & 73.30	79.30	71.52 & 73.45

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

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
Novel tank diving task	**Locomotor parameters:** Distance traveled, Absolute turn angle, Maximum speed, Immobility. **Anxiolytic-like behavior:** ↑ Time spent in the top area, ↑Transitions to the top area, ↓Latency to enter the top area. **Anxiogenic-like behavior:** ↑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
Open field	**Thigmotaxis behavior:** Entries to the center area, Time in the center area, Entries to periphery area, Time in the periphery area. **Exploratory behavior:** Distance traveled, Maximum speed, Time immobile.	Baiamonte et al., 2016; Champagne et al., 2010; Grossman et al., 2010; Nema et al., 2018
Light-dark test	**Anxiolytic-like behavior:** ↑Time spent in the lit area. **Anxiogenic-like behavior:** ↑Time spent in the dark area, ↑Freezing, ↑Risk assessment episodes.	Chaves et al., 2018; Facciol et al., 2019; Holcombe et al., 2014; Maximino et al., 2010
Inhibitory avoidance task	**Aversive memory**: 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.
Conditioned place preference	**Drug-seeking behavior:** Preference for the drug-associated compartment.	Brock et al., 2017; Collier et al., 2014; Mathur et al., 2011
Y- and T-maze tests	**Spatial memory and visual discrimination learning:** 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
Social preference task	**Seeking for conspecifics:** Time spent near the conspecific area, Transitions to the conspecific area..	Baggio et al., 2018; Fontana et al., 2018; Grossman et al., 2010
Shoal behavior test	**Shoaling behavior:** Inter-fish distance, Nearest neighbor distance, Farthest neighbor distance, Shoal area. **Anxiolytic-like behavior:** Shoaling disruption. **Anxiogenic-like behavior:** ↑Shoal cohesion.	Fontana et al., 2018; Müller et al., 2017; Canzian et al. 2017; Fernandes et al., 2015
Predator test	**Avoidance and fear-like responses:** 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
Mirror-induced aggression test	**Aggressive-like behavior:** 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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Table 3. Overview of the main experimental protocols for modeling drug abuse and addiction-related behaviors in zebrafish.

Drug	Experimental protocol	Biological response	References
Ethanol	**Acute etanol exposure** (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
	Ethanol conditioned place preference (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
	Intermittent ethanol exposure (1.0 % v/v, 20 min per day, for 8 consecutive days)	Elicits defensive responses by stimulating social behavior, geotaxis, and scototaxis, which reflect anxiogenesis. Induces tolerance and withdrawal effects and trigger oxidative stress.	Mathur and Guo, 2011; Müller et al., 2017
	Continuous ethanol exposure (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
	Voluntary ethanol intake (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
	Fetal alcohol spectrum disorder (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
Opioids	**Acute diacetylmorphine exposure** (15 and 25 mg/L, for 20 min)	Evokes hyperlocomotion without changing anxiety-like behaviors.	Stewart and Kalueff, 2014
	Morphine acute exposure (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
	Morphine conditioned preference paradigm (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
	Morphine larval exposure (0.4, 0.8, and 1.5 µM, for 1 h)	Exposure modulates the choice preference and such response is attenuated by opioid and dopamine antagonists	Bretaud et al., 2007
	Self-administration of hydrocodone hydrochloride (1.5 µg 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
	Morphine withdrawal (1.5 mg/L, for 2 weeks)	Evokes anxiogenesis and changes in whole-body cortisol levels	Cachat et al., 2010

Drug	Experimental protocol	Biological response	References
Nicotine	**Acute nicotine exposure** (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
	Chronic nicotine exposure (1-2mg/L, for 4 days)	Increases shoaling and facilitates anxiogenic responses.	Stewart et al., 2015; File et al., 2000
	Nicotine conditioned place paradigm (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
	Nicotine embryo exposure **(** 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	**Acute exposure to Δ^9-tetrahydrocannabinol** (100 nM, for 1h)	Triggers hypolocomotion. Extracellular signal-regulated kinases 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
	Δ^9-tetrahydrocannabinol and canabidiol embryo exposure (2, 4, 6, 8 and 10 mg/L of Δ^9-THC, and 1, 2, 3 and 4 mg/L of CB, from 5.45-48 hpf)	Δ^9-THC impairs locomotor activity of larvae. Δ^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	**Acute lysergic acid exposure** (250 µg/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
	Acute Ayahuasca exposure (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
	Ayahuasca embryo exposure (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
	Chronic Ayahuasca exposure 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:* Δ^9-tetrahydrocannabinol (Δ^9-THC), canabidiol (CB).

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 (*e.g.*, 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?
• Are there any genetic, biochemical, and behavioral differences among zebrafish strains that contribute to drug addiction response?
• Can duplicated genes **be differently modulated after drug exposure, playing a role in the effects of** drug abuse and **addiction**?

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