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Fishing for a deeper understanding of nicotine effects using zebrafish behavioural models

Olga Wronikowska[a], Agnieszka Michalak[b], Krystyna Skalicka-Woźniak[c], Alexander D. Crawford[d], Barbara Budzyńska[a,*]

[a] Independent Laboratory of Behavioral Studies, Medical University of Lublin, Jaczewskiego 4, 20-090 Lublin, Poland
[b] Department of Pharmacology and Pharmacodynamics, Medical University of Lublin, 4a Chodźki Str., 20-093 Lublin, Poland
[c] Independent Laboratory of Natural Products Chemistry, Department of Pharmacognosy, Medical University of Lublin, 1 Chodźki Str., 20-093 Lublin, Poland
[d] Faculty of Veterinary Medicine, Norwegian University of Life Sciences (NMBU), Ullevålsveien 72, 0454 Oslo, Norway

A B S T R A C T

Nicotine, the primary psychoactive component of tobacco, is the most widely used drug of abuse. Although the substance is well-known, there is still a lack of information concerning its long-term neurological and physiological effects and its mechanisms of action. In order to search for new, effective drugs in the therapy of nicotinism, as well as to design new drugs that exert positive nicotine-like effects, further experiments are needed, ideally also using new behavioural models and paradigms. A wide range of complex behaviours – including aggression, anxiety, long- and short-term memory, object discrimination and colour preference – have recently been comprehensively classified and characterized in the zebrafish model. Zebrafish offer an attractive experimental platform, based on a microscale in vivo bioassays, which can be used to investigate psychoactive drugs, their effects on the central nervous system and potential treatments of drug addictions. In this review, we present recent data revealing the potential of the zebrafish model to evaluate the effects and molecular mechanisms of nicotine by taking into consideration its impact on anxiety, learning and memory, addiction and social behaviours.

1. Introduction

Nicotine is a psychoactive component of tobacco and one of the most widely used drugs.

According to the latest report of the World Health Organisation (WHO), tobacco use kills over 7 million people each year (WHO, 2017). In humans, nicotine exerts its effects by activation of nicotinic as well as muscarinic cholinergic receptors (nAChR and mAChRs, respectively) (Benowitz, 2009). The activation of nAChRs leads to the release of several neurotransmitters, including dopamine, noradrenaline, acetylcholine, glutamate and gamma-aminobutyric acid (Papke, 2014). The pentameric neuronal nAChRs consist of different nicotinic receptor subunits: α (210) and β (Avdesh et al., 2012; Bencan and Levin, 2008; Benowitz, 2009), composing heteromeric or homomeric subtypes (Wu et al., 2016). The $\alpha_4\beta_2$ receptors dominate in the mesocorticolimbic system and their activation results in an increase of dopamine release, especially in the nucleus accumbens (NAC), which is linked with the rewarding effects of nicotine. nAChRs containing β_2 subunit are responsible for these rewarding effects and the affective symptoms of nicotine withdrawal syndrome, while α_5 and α_7 subtypes control the physical (somatic) withdrawal symptoms (Jackson et al., 2008; Picciotto et al., 1998). The homomeric α_7 receptors, meanwhile, are responsible for synaptic transmission and play a role in learning processes (Rezvani and Levin, 2001). Moreover, glutamatergic neurons with a high expression of the α_7 nAChRs reveal high permeability for calcium ions (Ca^{2+}) and take part in inducing long-term potentiation (LTP), a form of synaptic plasticity underlying memory formation (Jain et al., 2008).

The zebrafish (*Danio rerio*), a small freshwater fish from South-East Asia, is an increasingly popular model organism in neuropharmacology and pharmacogenetics. Advantages of this model include its genetic tractability, small size, easy maintenance and breeding, low cost, and the presence of translucent embryos. Moreover, similarities in physiology and morphology of the central nervous system (CNS) make the zebrafish suitable for modelling human neurological disease (Fontana et al., 2018; Kalueff et al., 2013). Rewarding properties of nicotine, as well as its influence on learning and memory processes and emotional states, result from the activation of the AChRs, expressed in the cortex, ventral tegmental area (VTA), NAC, amygdala, hippocampus, striatum and cerebellum in mammals. Similar receptors and neuronal circuits are observed in zebrafish. Importantly, eight nAChRs subunits have been identified in the zebrafish model to date (α2, α3, α4, α6, α7, β2, β3 and β4) (Ackerman et al., 2009; Zirger et al., 2003), emphasizing a high resemblance with nAChRs in mammals, and their high expression levels in brain regions that are homologous to mammalian brain structures (Papke et al., 2001).

* Corresponding author.
E-mail address: basia.budzynska@umlub.pl (B. Budzyńska).

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Although very well-established in the field of psychopharmacology, rodent models of CNS disorders do have important limitations – e.g. time-consuming experimental protocols, high cost, and ethical concerns – which make carrying out larger numbers of pharmacological experiments (such as drug screening) challenging or infeasible. A wide range of complex behaviours observed in mammalian models – including, among others, aggression, anxiety, long- and short-term memory, object discrimination, colour and place preference, among others – have been comprehensively classified and characterized in zebrafish (Fontana et al., 2018). Thus, zebrafish can successfully fill the gap between cell culture and rodents, allowing an early in vivo assessment of potentially effective drugs that could be used to treat different neuropsychiatric diseases, including nicotine addiction.

The abovementioned findings indicate that zebrafish is a promising model to study the.

behavioural effects of nicotine including its additive potential using e.g. conditioned place preference (CPP) paradigm as well as nicotine withdrawal by evaluation of nicotine cessation on anxiety level and memory disturbances. The evaluation of nicotine impact on CNS functions using zebrafish model should not only lead to an improvement in pharmacotherapies of nicotine addiction but also may contribute to a better understanding of the molecular basis of these effects (Klee et al., 2011). Furthermore, zebrafish models can be used as a quick and effective screening tool to search for new drug candidates, which would ideally be similar to nicotine in their mechanism of action, but free from unwanted effects such as addictive potential. The purpose of this review is to summarise the knowledge about zebrafish as a new and complementary animal model for evaluation of nicotine effects, including the most recent reports in this field.

2. Nicotine impact on anxiety

The anxiolytic effects of nicotine have been already well-documented in rodents, as well as in humans (Irvine et al., 1999; Kassel and Unrod, 2000; Levin et al., 2007). However, the growing popularity of zebrafish as a behavioural model, resulting in novel anxiety assays, has given researchers the opportunity to use zebrafish behaviours as useful measures of anxiety-related effects of nicotine. The anxiolytic effects in zebrafish can be investigated using the novel tank diving test (Fig. 1A), which exploits the stress-related response to a new environment in adult zebrafish. Placed in a novel tank, zebrafish express a natural tendency to spend the majority of time at the bottom of a tank since, in the wild, diving can help fish to avoid predators. Interestingly, the choice between the bottom and the top of a tank is perceived as analogous to the choice between the open and closed arms in the elevated plus maze test in rodents (Levin et al., 2007). In the novel tank diving test, anxiolytic effects of drugs are expressed as an increased amount of time spent at the top of a tank, an increased number of entries and a decreased latency to reach the top of a tank (Bencan and Levin, 2008; Levin et al., 2007; Stewart et al., 2015b).

The anxiolytic effects of nicotine in adult zebrafish after acute exposure to the drug were first investigated using the novel tank diving test (Levin et al., 2007; Sackerman et al., 2010). Nicotine ditartrate (50 and 100 mg/L) decreased the time spent in the bottom of a tank, which is linked to the anxiolytic properties of nicotine. Nicotine-induced anxiolytic effects were reversed by the nicotinic antagonist, mecamylamine, but only when given together with nicotine, and not when administered after nicotine exposure but prior to the testing session (Levin et al., 2007). Altogether it is suggested that nicotine effects on bottom-dwelling preference were caused by activation of the primary nicotinic receptors, independently of their continuous stimulation, and that nicotine-induced anxiolytic effects are linked to the initial activation of nicotinic receptors but not to subsequent adaptive changes of these receptors. The anxiolytic effects of nicotine in the novel tank diving test can be reversed by the selective nicotinic α7 receptor antagonist methyllycaconitine (MLA) and the selective nicotinic α4β2 receptors antagonist dihydro-β-erythroidine (DhβE), which implies that both α7 and α4β2 nicotinic receptors play roles in nicotine-induced anxiolytic effects in zebrafish (Bencan and Levin, 2008). Moreover, zebrafish diving in the novel tank test is characterized by within-trial habituation, expressed as gradually decreased bottom-dwelling over experimental time. Nicotine detartrate (100 mg/L) significantly decreased bottom-dwelling in the first minute with a further linear trend over the 5-min session (Levin et al., 2007), thus reducing the characteristic novel tank diving response in zebrafish.

It is well known that changes in locomotor activity may affect results obtained from behavioural studies. Some reports revealed that nicotine increases swim velocity in zebrafish (Bencan and Levin, 2008; Stewart et al., 2015a), which is consistent with its psychostimulant profile. However, Levin et al. (2007) showed that nicotine-treated fish attenuate swimming speed from the third and fourth minute of the 5-min session (100 mg/L and 50 mg/L, respectively), which could be a result of sedative properties of high concentrations of nicotine (Sackerman et al., 2010). Perhaps 3D video-tracking methods (see below) will decisively clarify the correlation between anxiety-related behaviours and swimming activity in zebrafish. An alternative solution could be to choose a different way of presenting the bottom-dwelling scores. As the total distance travelled might be a treatment-dependent

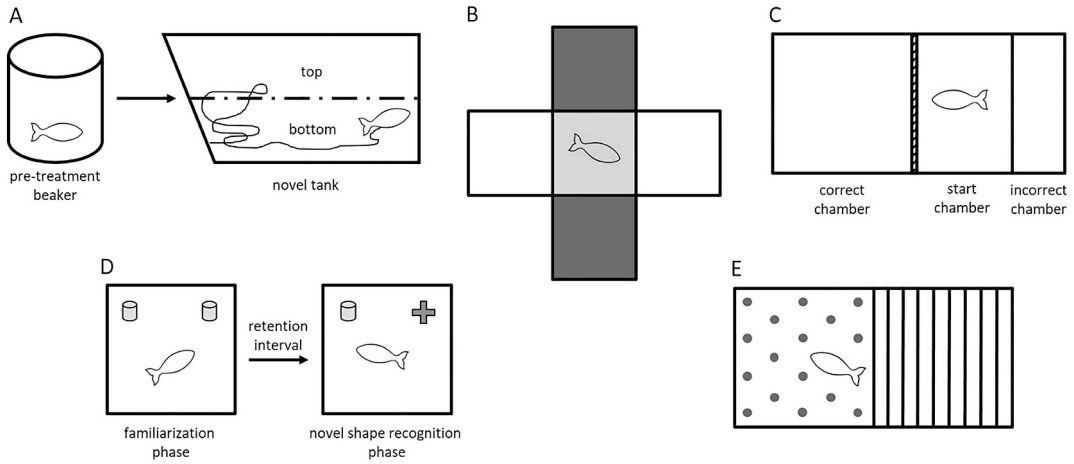

Fig. 1. The different paradigms used for evaluation of nicotine effects in zebrafish: A) the novel tank diving test (according to Cachat et al., 2010); B) the light/dark plus maze test (according to Varga et al., 2018); C) the three-chamber delayed spatial alteration test (according to Levin and Chen, 2004); D) the novel object recognition test (according to Magyary, 2019); E) the CPP apparatus (according to Mathur et al., 2011).

parameter, it could be more appropriate to present bottom-dwelling as the ratio of the distance travelled at the bottom of a tank to the total distance travelled.

Interestingly, anxiolytic effects of nicotine were observed at a much lower concentration when nicotine was administered in a free base form (1 mg/L) (Singer et al., 2016), which is equivalent to an approximately 3-fold higher concentration of nicotine ditartrate. In this report, the researchers observed a reduced swimming speed, an increase in consistency of swimming (reduction of individual variance), reduced latency to enter the top half of the tank and a general tendency to swim in the top half of a tank. When freezing behaviour was measured, no significant effects were recorded. The aim of this study was also to evaluate the role of sex in nicotine-dependent anxiolytic behaviours; however, no sex-specific nicotine effects on anxiety behaviours in zebrafish were found (Singer et al., 2016).

In contrast to acute nicotine administration, chronic nicotine exposure (4 days; 1 mg/L for the first 2 days followed by 2 mg/L for the next 2 days) triggers anxiogenic effects in adult zebrafish without altering the within-trial habituation response (Stewart et al., 2015b). Both the time spent in the top of a tank and the number of transitions to the top was reduced in fish treated with nicotine as compared to the control group. Moreover, the latency to reach the top of a tank was increased, which also suggests an anxiogenic potential of repeated nicotine exposure. In addition, motor patterns (i.e. distance travelled, velocity and mobility) were not impaired by chronic nicotine, which suggests that locomotor activity was not a factor contributing to the elevated level of anxiety in nicotine-treated fish. Furthermore, chronic nicotine did not alter cortisol levels in zebrafish (Stewart et al., 2015b), which could be contributed to its anxiogenic properties. Interestingly, acute nicotine (10 mg/L for 5 min) was found to elevate the whole-body cortisol levels, regardless of observed anxiolytic effects (Cachat et al., 2011). Therefore, it seems that nicotine alters anxiety-related behaviours in zebrafish in a cortisol-independent manner.

Anxiety behaviours in zebrafish can be also investigated using the light/dark plus maze test (Fig. 1B), which is based on the tendency of zebrafish to initially prefer dark over white areas when placed in novel environments. The plus maze includes a neutral centre section and four surrounding arms (two opposite white arms and two opposite black arms). After placing a fish in the centre section, during the 5-min test the amount of time spent motionless in the middle part, the amount of time spent in white arms and the number of crosses into black and white arms can be measured as indicators of anxiety-related responses. Nicotine ditartrate did not decrease anxiety levels in adult AB and WIK zebrafish in the light/dark plus maze test (Sackerman et al., 2010). Nonetheless, the reported concentration of 25 mg/L (3-min immersion time) was lower than concentrations that exerted anxiolytic properties in the novel tank diving test (50 and 100 mg/L) (Bencan and Levin, 2008; Levin et al., 2007).

Another tool for investigating anxiety-related behaviours in zebrafish is the horizontal place preference, also known as thigmotaxis. Thigmotaxis is a behaviour associated with avoiding the centre of the apparatus, tank or arena (depending on the tested species) and moving near its boundaries, which are regarded as a safe zone in a novel environment. Thigmotaxic behaviour is well-known and documented in different species, including rodents, humans and zebrafish (adult and larval) and it is perceived as an index of anxiety, which may be attenuated or enhanced by anxiolytic and anxiogenic drugs, respectively (Schnörr et al., 2012). Therefore, analysing thigmotaxis may be considered as a useful tool for studying nicotine-induced anxiety-related behaviours in zebrafish. While the influence of nicotine on thigmotactic behaviours in larval zebrafish has surprisingly not been published as a full-text paper to date, one conference abstract describes the effects of acute nicotine on complex larval zebrafish behaviours including thigmotaxis, reporting that nicotine (16 μM but not 48 μM) increased thigmotaxis in 5 dpf larvae (Chen and Scalzo, 2015).

The results discussed above were obtained using 2D scoring methods, which for obvious reasons will not fully reflect three-dimensional movement patterns of fish. For this reason, 2D methods of locomotor analysis may lead to incomplete conclusions of the observed behaviours. To address this challenge, 3D tracking analysis was carried out, revealing an interesting swimming pattern in zebrafish treated with nicotine (10 mg/L for 20 min) in the novel tank diving test (Stewart et al., 2015a). Nicotine-treated fish expressed previously observed preference to the top of a tank (Bencan and Levin, 2008; Levin et al., 2007) with stereotypic 'wall hugging' at the water's surface, probably reflecting nicotine psychostimulant profile (Stewart et al., 2015a). As previously discussed, nicotine may affect swim velocity, which would influence the results from the novel tank diving test, given that the bottom/top dwelling is expressed in units of time. As 2D techniques omit dimension, in situations where nicotine-treated fish exert circling behaviour, swimming activity (presented as distance travelled over time) might be underestimated.

Taken together, these findings indicate that zebrafish may serve as a useful model for the evaluation of nicotine-induced anxiety-related behaviours as well as for the examination of mechanisms underlying these effects. Acute nicotine treatment exerts anxiolytic effects, while chronic nicotine treatment exerts anxiogenic effects, in adult zebrafish in the novel tank diving test. Nicotine-induced anxiolytic effects are caused by the initial activation of nicotinic receptors, including α7 and α4β2 receptor subtypes, but not by their continuous stimulation which leads to receptor adaptive changes. Anxiety-like manifestations in zebrafish behaviour have not been correlated with changes in whole-body cortisol levels; however, acute nicotine elevates levels of this stress indicator, despite observed anxiolytic effects. Three-dimensional scoring methods should provide a more insightful and complex analysis of the swimming activity in nicotine-treated fish and its correlation with anxiety-related behaviours. A summary of data on the anxiety-related effects of nicotine in zebrafish is shown in Table 1.

3. Nicotine impact on memory and learning

Procognitive effects of nicotine have been well-documented in a number of different species (e.g. rats. mice, rabbits, monkeys or humans) and for a wide variety of tasks (e.g. the radial arm maze, the water maze, the passive avoidance test and object recognition test) (see Levin et al., 2006b for review).

Zebrafish experimental models for cognition, learning and memory have the potential to bridge the gap between in vitro studies and more advanced animal models, due to several advantages of zebrafish over rodents. In contrast to the phenotypic complexity of mammals, which may lead to difficulties in the prediction of cognitive processes, the simplicity and robustness of zebrafish phenotypes contribute to the facilitation of identifying forms of behaviour (Meshalkina et al., 2017). Stress plays a significant role in memory performance in all species. Similarly to humans, fish release cortisol as a primary stress hormone, and not corticosterone as in the case of rodents (Kalueff et al., 2014). Vision also contributes to learning and memory processes and unlike rodents, zebrafish have a well-developed visual perception, which makes them a potentially better model to study visual memory. However, zebrafish and human vision are not directly comparable, as zebrafish have evolved a tetrachromatic vision which makes them capable of differentiating wider range of colours than humans (Avdesh et al., 2012). These differences between zebrafish, rodents and humans have to be taken into consideration when designing experiments (e.g. colour selection of objects in recognition assays) as well as when interpreting results.

Although there are several key advantages of using zebrafish in learning, memory and cognition studies, certain limitations should also be noted, which mostly are linked to differences in morphology and neurophysiology between zebrafish and mammals. Zebrafish have no cortex, no defined hippocampus, and they lack some midbrain dopaminergic structures such as the substantia nigra or VTA area. Still, there

Table 1
Effects of nicotine on the anxiety-related response in a zebrafish model.

Test	Nicotine ditartrate dose	Immersion time	Dosing-testing interval	Effect	Comments	References
Novel tank diving	50, 100 mg/L	3 min	5, 20, 40 min	+/0	Decreased bottom dwelling in fish treated with nicotine 100 mg/mL under 5- and 20- but not 40-min dosing-testing interval; decreased bottom dwelling in fish treated with nicotine 50 mg/L during the first minute over the 5-min session under 5-min dosing-testing interval	Bencan and Levin, 2008; Levin et al., 2007
	20 mg/L	5, 20 min	none	+	3D tracking analysis revealed stereotypic 'wall hugging' at the water's surface	Cachat et al., 2011; Stewart et al., 2015a
	1 mg/L*	3 min	none	+	Fish were treated with free base nicotine	Singer et al., 2016
	25 mg/L	3 min	none	0	No anxiolytic effects in AB and WIK zebrafish lines	Sackerman et al., 2010
	1–2 mg/L	chronic 4 days	n/a	–	1 mg/L for the first 2 days followed by 2 mg/L for the next 2 days	Stewart et al., 2015b
Light/dark plus maze	25 mg/L	3 min	5 min	0	The test was performed in AB and WIK zebrafish previously introduced to the 5-min session of the novel tank diving test	Sackerman et al., 2010
Horizontal place preference (thigmotaxis)	16 μM	n/k	n/k	–	The results were not published in a full-text paper but in a brief conference abstract; little is known about the materials and methods of this work; experiments were conducted in larvae	Chen and Scalzo, 2015

* Indicates nicotine free base; – indicates anxiogenic effects; + indicates anxiolytic effects; 0 indicates no changes in anxiety-related behaviours; n/a – not applicable; n/k – not known.

are analogous structures in the zebrafish brain that potentially have similar biochemical and functional meaning (Panula et al., 2010; Parker et al., 2013). Moreover, learning is a complex process leading to the development of changes in behaviour after a specific amount of time and experience and many different factors (e.g. social interactions, stress responses, predators avoidance) can contribute to elicited memory effects (Meshalkina et al., 2017). Zebrafish naturally live in shoals and placing them in isolation to perform a test can evoke stress response which may affect cognitive performance (Kalueff et al., 2014). Thus, proper habituation to new experimental conditions is crucial to diminish stress impact on learning performance. Despite some limitations, zebrafish can serve as a valid tool to study memory and cognition in different paradigms, as described further below.

The effects of nicotine on memory performance in zebrafish have been evaluated in several paradigms. For example, nicotine was shown to improve memory using the delayed spatial alteration test (DSA; Fig. 1C), in which after several learning trials allowing zebrafish to learn about correct and incorrect chambers, the choice accuracy and response latency was measured (Eddins et al., 2009; Levin and Chen, 2004; Levin et al., 2006a). A significant improvement in choice accuracy was shown for nicotine (50 and 100 mg/L) when tested immediately after the end of drug administration (Levin and Chen, 2004), as well as for nicotine (100 mg/L), when the 20-min dosing-testing interval was conducted (Eddins et al., 2009). Interestingly, higher doses have shown progressive impairment in choice accuracy, which is referred to as a biphasic dose-dependent nicotine effect on memory function (also known as the inverted U-shaped dose-effect curve) (Levin and Chen, 2004). A procognitive effect of nicotine (0.02 and 0.002 mg/kg, i.p.), with a visible inverted U-shaped dose-dependent effect, was observed using the T-maze task, in which after a training session, the time needed to find a reservoir with grass, stones and marbles in a maze is measured (Braida et al., 2014b). This biphasic effect of nicotine was also seen in rats and monkeys (Levin and Simon, 1998; Levin and Rezvani, 2002).

The dose-dependent effects of nicotine on memory improvement in zebrafish in the DSA test were also connected with the dosing-testing interval. The positive impact on choice accuracy for nicotine (100 mg/L), was observed not earlier than between 20 and 40 min after drug administration and was no longer noticed when the dosing-testing interval was 80 or 160 min (Levin et al., 2006a). Interestingly, in this study, the higher dose of nicotine (200 mg/L) also improved choice accuracy after 40 min a dosing-testing interval. While this contradicts the abovementioned studies and the inverted U-shaped dose-effect hypothesis, a decrease in the concentration of nicotine over 40 min time and its adjustment to an optimal level exerting procognitive effects cannot be excluded (Levin et al., 2006a).

Another test that can be used to evaluate learning and memory in zebrafish is the novel object recognition test (NOR; also known as the novel object preference test, NOP) or its modified version known as the virtual object recognition test (VORT). During the first phase (a familiarization phase), animals were exposed to two identical objects (NOP) or simple 2D geometrical shapes shown to the fish on the iPod (VORT), which were subsequently removed from the tank or were no longer displayed. In the second phase, a fish explored the tank again; however, one of the familiar objects or pictures was replaced by a novel one (Fig. 1D) (Braida et al., 2014a; Faillace et al., 2017; May et al., 2016). The ability to discriminate between two objects and more time spent nearby the novel object is treated as an indicator of positive memory performance and cognitive improvement (Ennaceur and Delacour, 1988).

An increased preference for the novel object indicating memory improvement in the zebrafish NOP test was shown for nicotine (0.02 mg/kg, i.p.) (Braida et al., 2014a). It has been also shown that nicotine (15 mg/L) modifies object preference in the NOP test, but the results differ depending on the look of the objects (Faillace et al., 2017). In comparison to the placebo-treated group, nicotine (15 mg/L)

Table 2
Effects of nicotine on memory and learning processes in a zebrafish model.

Test	Nicotine ditartrate dose	Immersion time	Dosing-testing interval	Effect	Comments	References
Delayed spatial alteration	50, 100, 150, 200, 400, 800 mg/L	3 min	none	+/−	Lower doses of nicotine (50, 100 mg/L) increased choice accuracy and higher doses (150, 200, 400, 800 mg/L) attenuated this effect creating inverted U-shaped response	Levin and Chen, 2004
	100 mg/L	3 min	20 min	+	Increase in choice accuracy	Eddins et al., 2009
	100, 200 mg/L	3 min	20, 40, 80, 160 min	+/−	Increase in choice accuracy seen for 100 mg/L nicotine with dosing-testing interval 20 and 40 min but not 80 and 160 min, and for 200 mg/L with dosing-testing interval 40 min	Levine, 2006
Novel object preference	50 mg/L	3 min	none	−	Familiar object preference	May et al., 2016
	15 mg/L	10 min	1.5 h, 24 h	+	Two innately more preferred objects were replaced with different two innately more preferred objects (novel object preference with dosing-testing interval 1.5 and 24 h)	Faillace et al., 2017
				−	Two innately less preferred objects were replaced with different two innately less preferred objects (familiar object preference with dosing-testing interval 1.5 and 24 h)	
				−/0	Two innately more preferred objects were replaced with one more and one less innately preferred objects (familiar object preference with dosing-testing interval 1.5 h, no effect on preference with dosing-testing interval 24 h)	
				+	One innately more and one innately less preferred objects were replaced with two innately more preferred object (novel object preference with dosing testing interval 1.5 h)	
Virtual object preference	0.02 mg/kg	i.p.	20 min	+	Novel object preference	Braida et al., 2014a
T-maze	0.2, 0.02, 0.002 mg/kg	i.p.	10 min	+/−	Reduced time needed to find reservoir for 0.02 and 0.002 mg/kg but not for 0.2 mg/kg	Braida et al., 2014b
Context-dependent fear response	1 mg/L*	3 min	20 min	+	Procognitive effect of nicotine on associative aversive learning	Ziani et al., 2018

+ indicates positive effects on memory performance; − indicates negative effects on memory performance; 0 indicates no changes in memory performance; i.p. – intraperitoneal injection.
* Indicates nicotine free base.

increased preference for the novel object when one of the innately more preferred objects was replaced with different, but still innately more preferred one. Nicotine-induced enhancement of the preference for the novel object was also seen when one of two innately less preferred objects was replaced with an innately more preferred one. Contrary results were shown for different combinations of innately more and less preferred objects (see details in Table 2), suggesting the importance of carefully selecting the colours and shapes of presented objects in the design of a new experiment, as these can influence the observed effects. Interestingly, the concentration of nicotine (15 mg/L) used in this study (Faillace et al., 2017) was lower than the concentrations used in different memory and learning tasks. Despite this fact, the procognitive effect of nicotine was clearly seen, probably due to the longer time of immersion: 10 min (Faillace et al., 2017) vs 3 min in other tasks assessing memory performance (see Table 2).

Two abovementioned studies (Braida et al., 2014a; Faillace et al., 2017) confirm the procognitive effects of nicotine; however, these results are contradicted by another study in which nicotine (50 mg/L) increased preference for the familiar object (May et al., 2016). This study indicates that zebrafish could be neophobic. However, other studies provided strong evidence of nicotine-induced procognitive effects, thus different experimental conditions must be considered as a reason for the observed discrepancies. In view of the fact that zebrafish have a natural preference for dark environment, and most likely dark objects (Meshalkina et al., 2017), the procognitive effect was observed when simple shapes figures (Faillace et al., 2017) or 2D simple black and white shapes presented on iPod (Braida et al., 2014a), but not when the objects were represented by colourful and big 3D objects (LEGO® figures) (May et al., 2016). Moreover, 3D figures could have been perceived as potential predators and elicited a stress response, in light of the importance of innately preferred colours and shapes (Faillace et al., 2017). Although a lack of interest in the presented objects also cannot be excluded, the main factor that could have contributed to the lack of observed procognitive effects was the timing of nicotine administration. Furthermore, memory improvement was shown when nicotine was administered 20 min (Braida et al., 2014a), 1.5 h or 24 h (Faillace et al., 2017) prior to the novel object recognition phase. However, the effect was not pronounced when the drug was introduced 3 min prior to the habituation and familiarization phase (May et al., 2016). For this reason, nicotine could have enhanced the preference for the familiar object presented as the first one after drug exposure. The discrepant results strongly suggest that colours and shapes of chosen objects, as well as the time of nicotine administration, play a crucial role in the NOP test.

A recent study evaluated the effects of nicotine on fear responses in a context-dependent manner assay, which allows triggering defensive behaviours after a single exposure and may serve as a tool assessing associative learning (Ziani et al., 2018). In this study, a conspecific alarm substance (CAS) was used as an aversive stimulus (triggering defensive behaviours in zebrafish, e.i. freezing, erratic movements, bottom-dwelling or increased social cohesion) to evaluate how specific treatment modulates contextual fear responses. Following a training session allowing zebrafish to learn about certain effects in the specific context, a nicotine impact on associative learning with aversive stimulus was assessed (Fig. 2). The results revealed that CAS itself elicited fear responses, whereas nicotine-treated fish previously exposed to CAS showed a significant increase in freezing behaviours when tested in tanks with similar context during post-training session without CAS. However, no changes were observed when nicotine/CAS fish were tested in an altered context during training and post-training session, which may suggest a positive effect of nicotine on aversive memory in zebrafish. The nicotine concentration used in this study (1 mg/L of pure nicotine) seems to be appreciably lower in comparison to doses or concentrations used in other studies assessing procognitive properties of nicotine. However, this situation was also seen previously in anxiety protocol, in which nicotine at the concentration of 1 mg/L elicited

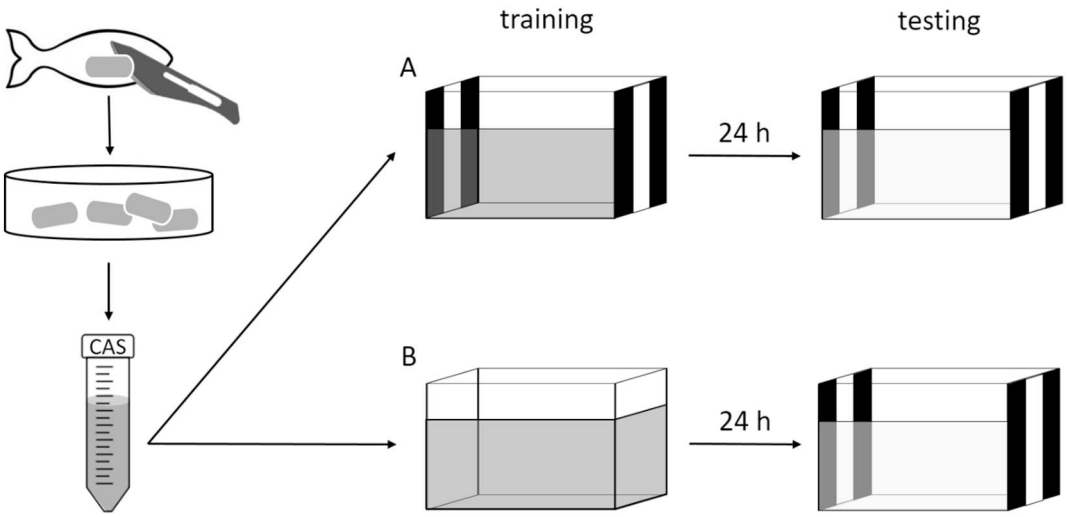

Fig. 2. The protocol scheme of context-dependent fear response in zebrafish (based on Ziani et al., 2018). The protocol assesses contextual fear responses and associative learning using an aversive stimulus - a conspecific alarm substance (CAS), which is obtained from zebrafish epidermal club cells. During the training session, fish are placed in the experimental tanks with (A) or without (B) visual clues in the presence of CAS (darker grey). 24 h later, fish are exposed to the same (A) or a different (B) context in the absence of CAS (light grey).

anxiolytic effect (Singer et al., 2016). This suggests that the form in which nicotine is used has a significant impact on the dosage needed to exert certain effects, in that for pure nicotine the effective doses are much lower than for nicotine tartrate salts (see Section 7).

The zebrafish model is not only useful for studying cognitive properties, but also can serve as a tool for the evaluation of the mechanisms underlying nicotine-induced cognitive enhancement. It has been shown that the positive effect of nicotine on cognition in zebrafish can be reversed by non-selective nicotinic receptor antagonist mecamylamine (Levin et al., 2006a; Braida et al., 2014b). While these two studies revealed activity differences in terms of when mecamylamine was administered relative to nicotine exposure, the more recent report (Braida et al., 2014b) was consistent with previous observations in mice in which nicotine-induced long-term potentiation was suppressed by pre-treatment with mecamylamine, but not affected by post-treatment application of mecamylamine (Matsuyama et al., 2000). Thus, administration of mecamylamine provided evidence that nicotinic receptors are involved in nicotine-induced memory enhancement; and more recent research has contributed to the extension of this knowledge by taking into account different subtypes of nicotinic receptors. Nicotine-induced positive memory performance in zebrafish was reversed by the selective subtype antagonists DhβE (a selective antagonist of α4β2 receptors), MLA (a selective antagonist of α7 receptors) and α-conotoxin (an antagonist of α6β2 receptors) (Braida et al., 2014b). This study confirmed the involvement of different subtypes of nicotinic receptors (mainly α7, α4β2 and α6β2) in nicotine action on memory and learning performance, which is consistent with existing knowledge of nicotine-induced cognitive enhancement in other species (Levin et al., 2006b).

The zebrafish model proved to be useful in studies assessing the role of not only the cholinergic but also the dopaminergic system, which also appears to be involved in the cognitive effects induced by nicotine. A positive correlation was observed between levels of dihydroxyphenylacetic acid (DOPAC, the primary dopamine metabolite) in nicotine-treated zebrafish brain and cognitive function measured in the three-chambered tank test (Eddins et al., 2009).

All of the discussed findings indicate that nicotine has a significant impact on learning and memory performance in zebrafish, and these effects can be demonstrated by means of several different memory-related tests (Table 2).

4. Nicotine impact on addiction

Nicotine is one of the most commonly used drugs worldwide, and the mortality index connected with the abuse of it is extremely high (WHO, 2017). Understanding the mechanisms and factors underlying nicotine dependence should be helpful in searching for new drug candidates for nicotine addiction treatment.

CPP is a well-established behavioural assay for studying addiction and rewarding properties in rodents (Biala et al., 2010), as well as in zebrafish (Klee et al., 2011). CPP is based on the association of drug administration with a particular environment (Fig. 1E). After a conditioning session, animals tend to spend more time in a drug-paired compartment of the apparatus which points out the rewarding properties and the addictive potential of tested drugs. There are two different methods to perform CPP. In the unbiased method, drug- and placebo-paired compartments are chosen randomly by the researcher. In the biased procedure, animals first explore the apparatus and the time spent in both compartments is measured to establish the less-preferred compartment, which is then chosen to be the drug-paired compartment during conditioning (Prus et al., 2009). Development of preference for the initially aversive place after drug administration suggests its rewarding properties; however, reduction of aversion due to anxiolytic effect of the drug also has to be taken under consideration (Kedikian et al., 2013).

Studies show that a 3-day exposure to nicotine induces CPP in zebrafish in a biased procedure for a wide range of drug concentrations (15, 30 and 50 mg/L), providing evidence that zebrafish are an applicable animal model for studying nicotine addiction (Kedikian et al., 2013). Nicotine triggers a preference for an initially aversive place, most likely due to its rewarding properties; however, in biased studies, as mentioned before, nicotine-induced anxiolytic effects cannot be excluded. It has been revealed that zebrafish from a nicotine-paired group spent more time in a non-preferred compartment in comparison to saline and nicotine-unpaired groups, which indicates rewarding properties rather than reduction of anxiety (Kedikian et al., 2013). Nicotine-induced CPP using the biased protocol was also shown after acute intramuscular (i.m.) administration of nicotine ditartrate, performing an inverted U-shaped dose-response curve with the most significant effect at a dose of 0.001 mg/kg (Ponzoni et al., 2014). These results are consistent with those obtained in rodents where nicotine produced CPP in the lower range of doses but conditioned place aversion (CPA) in the

higher range of doses (Le Foll and Goldberg, 2005). Nicotine-induced CPP is antagonized by selective nAChR antagonists (Ponzoni et al., 2014). The previously mentioned selective nAChR antagonists, MLA and DhβE, have their own slight but significant reinforcing effects, but when combined with nicotine, both antagonists blocked nicotine-induced CPP. Similar effects were observed using mecamylamine (non-competitive nAChR antagonist) or the partial nicotinic agonists – varenicline (a partial agonist of α4β2 and α6β2 and full agonist of α7 and α3β4) or cytisine (a competitive partial agonist of α4β2), when administered before the nicotine. Co-administration of these compounds with nicotine (0.001 mg/kg i.m.) caused significant blockage of nicotine induced-CPP (Ponzoni et al., 2014). Moreover, nicotinic partial agonists CC4 (cytisine derivative, a partial agonist of α4β2 and α6β2 with low affinity for α3β4 and α7 subtype) and CC26 (new cytisine derivative) combined with nicotine blocked both the reinforcing and aversive effects of nicotine (Ponzoni et al., 2014).

Another study showed that nicotine induces CPP after a single 20-min treatment (3, 30, 60 μM/L) as well as upon 3 consecutive exposures (6 and 30 μM/L, once daily for 3 days). CPP was also observed after 4 weeks of daily conditioning despite an adverse stimulus (fish were punished by 3s removal from the tank each time they entered the treatment paired side in CPP tank). Moreover, zebrafish withdrawn from chronic nicotine (4 weeks of daily conditioning) exhibited CPP over a three-week period of drug abstinence (Kily et al., 2008).

The larval zebrafish locomotor activation assay can be also considered as nicotine addiction model. This assumption was supported by the data that numerous neural pathways involved in the drug abuse phenomenon are conserved between mammals and zebrafish larvae. This includes dopamine, a neurotransmitter that is engaged in both rewarding and locomotor effects of the drugs. However, larvae have some limitations in terms of fully modelling the addictive potential of psychoactive drugs, such as the lack of withdrawal response and contextual or social behaviours, which can readily be observed in drug-dependent adult zebrafish. It was revealed that nicotine exerts both dose-dependent and biphasic effects on locomotor activity in larvae (Cousin et al., 2014), as has also been observed in mammalian studies (Carey et al., 2004). A larval locomotor model of addiction was used to identify new candidates for nicotine dependence treatment in comparison with the first-choice drugs of the treatment of tobacco dependence, e.g. bupropion or varenicline. The studies revealed that apomorphine (a non-specific D1 and D2 dopamine receptor agonist) and topiramate (an anti-convulsant, anti-migraine, and anti-obesity drug) inhibited nicotine effects on locomotor activity (Cousin et al., 2014).

Based on the studies highlighted here, zebrafish represent a promising model for the evaluation of the rewarding effects of drugs of abuse (Table 3). In particular the CPP paradigm helps understand processes and mechanisms underlying the rewarding effects of nicotine in zebrafish and provides information that would be beneficial for further investigation in mammalian studies. The cited results obtained in zebrafish CPP are supported by CPP findings from rodent studies (Biala et al., 2010).

5. Nicotine impact on social behaviours

Well-established animal models have been used to study social behaviours impacted by nicotine, revealing for example that rodents exposed to nicotine showed significantly less social interactions (e.g. Irvine et al., 2000, Pentkowski et al., 2011). However, there are only a limited number of studies to date on the impact of nicotine on social behaviours in zebrafish. However, one recent study investigated the effects of acute nicotine on zebrafish shoaling. In their natural environment, zebrafish tend to spend the majority of time in loose groups (shoals), which reduces predation risk and enhances reproductive success (Engeszer et al., 2004). Nicotine ditartrate (4 and 8 mg/L) affects shoaling behaviour by increasing the distance between the fish, decreasing their swimming speed and disrupting their polarization

Table 3
Effects of nicotine on addiction in a zebrafish model.

Test	Nicotine ditartrate dose	Immersion time	Apparatus	Effect	Comments	References
CPP	15, 30, 50 mg/L	20 min for 3 days	One half of a tank was coloured lightbrown and the other half was white with two black spots placed at the bottom	+	Nicotine-treated adult zebrafish showed a clear preference for the aversive environment associated with nicotine; the effect was observed for all used concentrations of nicotine	Kedikian et al., 2013
	0.0001–0.1 mg/kg	i.m.	Tank divided into two halves with a perforated wall, containing distinct visual cues (two black polka dots)	+	After a single injection of nicotine, fish were placed in a less preferred side of a tank for 30 min; nicotine elicited CPP in a characteristic inverted U-shaped (dose-effect) manner	Ponzoni et al., 2014
	acute 0–300 μmol/L, repeated (3 days) 0–300 μM/L, chronic (4 weeks) 30 μM/L*	20 min	Rectangular tank with each end distinguished by visual cues (black spots versus vertical black and white stripes)	+	Acute (3, 30 and 60 μM/L) and repeated (6 and 30 μM/L) nicotine induces CPP; CPP response persists for 3 weeks following the chronic administration of nicotine, also despite an adverse stimulus (3-s removal from the tank each time the fish entered the drug treatment side)	Kily et al., 2008
Larval locomotor model of addcition	10–130 μM*	5 min	–	+	Nicotine exerts both dose-dependent and biphasic effects on locomotor activity in larvae; varenicline and bupropion attenuated locomotor activation induced by 20 μM nicotine	Cousin et al., 2014

+ indicates addictive properties; i.m. – intramuscular injection.
* Nicotine form (free base or salt) was not specified.

(defined as the directional synchronization of the shoal). The anxiolytic effects of nicotine seem to be the most probable explanation underlying its effects on zebrafish shoaling; however, further investigations in this field are required (Miller et al., 2013). Considering the fact that zebrafish are highly social animals, they could provide a useful model to study deficits in social interactions.

6. Concentrations and routes of administration of nicotine in the zebrafish model

When designing a new experiment not only a proper form and dose of nicotine should be taken under consideration, but also an appropriate route of administration. In the presented studies, researchers chose nicotine immersion, i.p. or i.m. injections; however, it appears that different methods of administration did not alter the primary effects of nicotine (see Table 2). Nevertheless, we believe that some advantages of specific routes of administration can be pointed out. Although immersion is a quick and easy method which does not require any specific equipment or skills and does not appear to be stressful to the fish, it is difficult to assess how much of the dissolved substance is absorbed or taken up by the fish. This is however of great importance, especially considering differences in active concentrations of free base nicotine and nicotine ditartrate. Taking into account the relevant conversion of nicotine concentrations from free base to salt, 1 mg/L of free base nicotine still differs substantially from standard concentrations of nicotine ditartrate used in zebrafish studies (50–100 mg/L). As free bases are generally more lipophilic than salts and lipophilic compounds cross membranes more readily, free base nicotine would be better absorbed by fish and would cross the blood-brain barrier more rapidly, thereby triggering its psychoactive effects in much smaller concentrations than nicotine ditartrate (Levine, 2006).

In contrast, i.p. or i.m. injections serve as a more controlled way to administer even small doses of the substance, based on the fish weight, and can be used to administer substances non-soluble in water. However, the experience of the operator is crucial to perform a proper injection. It is also more time-consuming and requires several steps, such as 24 h fasting prior to i.p. injections, weighing, chemical or cold-water anaesthesia, the proper performance of the injection and recovery (Kinkel et al., 2010). Each of these manipulations may contribute to elevated stress levels when performed improperly, leading to discrepancies in the observed results. Although there are pros and cons of both immersion and injections, in general when designing a new experiment the route of administration should be chosen based on the chemical properties of the substance to be tested, its chemical form, the researcher's skill level, and financial considerations.

7. Nicotine effects on zebrafish embryos and larvae

Although the primary focus of this review is the impact of nicotine on behaviour in adult zebrafish, several studies have been performed to elucidate the effects of embryonic nicotine exposure on early vertebrate development. It has been shown that exposure to nicotine during early embryogenesis causes paralysis in zebrafish embryos and larvae, effecting secondary motor neurons (SMN) and causing errors in axonal pathfinding (Svoboda et al., 2002; Menelaou and Svoboda, 2009). Fluorescence microscopy analysis of *Tg(isl1:GFP)* zebrafish, which express green fluorescent protein (GFP) in a subtype of spinal secondary motor neurons, revealed changes in motor neuron pathfinding that were still seen in juvenile and adult fish, suggesting that embryonic nicotine exposure can cause permanent changes in motor neuron functions (Menelaou and Svoboda, 2009). Analysis of the mechanisms underlying nicotine-induced motor neuron changes revealed that prolonged overactivation of AChRs can lead to motor axons defects and muscle degeneration. Interestingly, when embryos of a zebrafish mutant lacking skeletal muscle AChRs were exposed to nicotine, these zebrafish developed SMN pathfinding errors without muscle degeneration, further implicating muscle AChRs in nicotine-induced degeneration (Welsh et al., 2009). Nicotine's impact on embryonic development also can be used to elicit specific effects. Although zebrafish larvae do not exhibit spontaneous swimming until 4 dpf, it has been shown that acute embryonic nicotine exposure evokes a robust swimming response already at 36 hpf, and this effect is termed the nicotine-evoked locomotor response (NLR) (Thomas et al., 2009). This finding contributed to the development of an NLR protocol in toxicological screening which allows the rapid assessment of different compounds on locomotor activity in zebrafish embryos pre-treated with nicotine at earlier development stages (Mora-Zamorano et al., 2016). In another study, researchers considered the fact that in developing vertebrate spinal cord cholinergic neurotransmission is associated with locomotor output and tried to evaluate the role of α2A nAChRs in nicotine-induced locomotor activity in zebrafish embryos. Although it was successfully shown that α2A subtype of nAChRs is expressed in spinal cord neurons in embryonic zebrafish, blocking their expression by using morpholino antisense nucleotides had no impact on spontaneous locomotor activity. However, this blockage reduced embryonic nicotine-induced motor output suggesting that α2A subtype of AchRs may be involved in nicotine-evoked locomotor response (Menelaou et al., 2014).

Although zebrafish embryos and larvae have been used in several studies on nicotine, this model remains relatively underexploited in comparison with adult models. To date, only a limited of reports have been published regarding the effects of nicotine on anxiety-related behaviours and addiction in larval zebrafish (see Sections 2 and 4, respectively).

8. Conclusions

There is a significant need for further research on nicotine – in particular (Ackerman et al., 2009) to investigate in more depth the impact of this compound on neurophysiology and behaviour, (Avdesh et al., 2012) to better understand its addictive potential, (Bencan and Levin, 2008) to further elucidate the molecular mechanisms of its bioactivities, and (Benowitz, 2009) to more efficiently explore the therapeutic potential of both nicotine derivatives and other compounds designed to counteract nicotine addiction. The urgency of this research is underscored not only by the continuing worldwide epidemic of tobacco-related illnesses but also by the much more recent and still rapidly expanding health crisis of vaping-related illnesses.

As highlighted above, zebrafish behavioural models represent a robust in vivo bioassay platform that has already proven useful in the investigation of nicotine activity. Zebrafish models encompassing a wide range of complex behaviours – including aggression, anxiety, long- and short-term memory, object discrimination and colour preference – have been successfully used over the past 15 years to evaluate the physiological effects and molecular mechanisms of nicotine. In many cases, findings in zebrafish substantiate earlier findings in rodents and humans, demonstrating the biomedical relevance of these zebrafish models for further research on nicotine.

While most of the studies reviewed here have made use of behavioural assays in adult zebrafish, many of the behaviours relevant for nicotine activity can also be assessed in zebrafish larvae, thereby realizing the key advantages of zebrafish in terms of throughput and size. Interesting areas for further research using zebrafish larvae will likely include pharmacological screening approaches, and focused chemical genetic studies e.g. the analysis of nicotine agonist/ antagonist interactions with a different type of nicotinic receptors (mainly α4β2 and α7). Such zebrafish-based experiments will fill the gap between in vitro and preclinical studies on more advanced mammalian models, and will help facilitate the discovery of new pharmacological tools and drug leads for nicotine addiction and/or targeting nicotinic receptors and signalling pathways for other CNS disorders.

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