

Subchronic effects of plant alkaloids on anxiety-like behavior in zebrafish

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ARTICLE INFO

Keywords:

Anxiety
Zebrafish
Alkaloids
Nicotine
Novel tank diving test

ABSTRACT

Zebrafish provide a valuable emerging complementary model for neurobehavioral research. They offer a powerful way to screen for the potential therapeutic effects of neuroactive drugs. A variety of behavioral tests for zebrafish have been developed and validated for assessing neurobehavioral function. The novel tank diving test is a straightforward, reproducible way of measuring anxiety-like behavior in zebrafish. When introduced into a novel tank, zebrafish normally dive to the bottom of the tank and then gradually explore the higher levels of the water column as time progresses. Buspirone is an effective anxiolytic drug in humans, which has been found, with acute administration, to reduce this anxiety-like response in zebrafish. The current study used the zebrafish model to evaluate the potential anxiolytic effects of alkaloids, commonly found in Solanaceae plants, with known neuropharmacology relevant to mood regulation. In line with previous findings, acute treatment with anxiolytic positive controls buspirone and the plant alkaloid nicotine reduced the anxiety-like diving response in the zebrafish novel tank diving test. Further, both buspirone and nicotine continued to produce anxiolytic-like effects in zebrafish after 5 days of exposure. In the same treatment paradigm, the effects of five other alkaloids—cotinine, anatabine, anabasine, harmaline, and norharmaline—were investigated. Cotinine, the major metabolite of nicotine, also caused anxiolytic-like effects, albeit at a dose higher than the effective dose of nicotine. Nicotine's anxiolytic-like effect was not shared by the other nicotinic alkaloids, anabasine and anatabine, or by the naturally present monoamine oxidase inhibitors harmaline and norharmaline. We conclude that nicotine uniquely induces anxiolytic-like effects after acute and subchronic treatment in zebrafish. The zebrafish model with the novel tank diving test could be a useful complement to rodent models for screening candidate compounds for anxiolytic effects in nonclinical studies.

1. Introduction

Zebrafish has become a powerful tool for investigating the effects of compounds in neurobiology, and it holds great potential for translational research (Kalueff et al., 2014; Khan et al., 2017; Levin et al., 2007; Papke et al., 2012; Stewart et al., 2012; Stewart et al., 2014). Its relative ease of maintenance, expansion, and genetic manipulation and genetic homology to humans have made the zebrafish a popular model organism in biomedical research. Both larval and adult zebrafish are increasingly used for studying the neurobiology of brain function and dysfunction, including depression, anxiety, and cognition (Bailey et al., 2015; Cachat et al., 2010; Stewart et al., 2012; Stewart et al., 2010; Stewart et al., 2011). Many behavioral paradigms can be designed similarly between mammals and zebrafish, indicating the evolutionary conservation of these behaviors across species. The novel tank test, for

instance, is a relatively efficient test of anxiety, with some translational relevance to humans (Levin et al., 2007; Papke et al., 2012; Stewart et al., 2012a). This behavioral paradigm takes advantage of the innate behavior of zebrafish to dive and dwell at the bottom of a body of water to avoid danger or stress. Many anxiolytic drugs, such as diazepam and buspirone, as well as nicotine have been shown to reduce this anxiety-driven behavior (Bencan and Levin, 2008; Bencan et al., 2009; Levin et al., 2007; Stewart et al., 2012). However, the benefits of naturally occurring compounds are less well characterized in this test system.

Various components of Solanaceous plants have been investigated for their efficacy and safety in nonclinical and clinical studies (Afroz et al., 2020; Chowański et al., 2016; Govindan et al., 1999). Alkaloids—natural organic compounds that contain nitrogen—are widely distributed throughout the plant kingdom and are of great interest owing to their ability to regulate mood and anxiety (Perviz et al., 2016).

Abbreviations: nAChR, nicotinic acetylcholine receptors; MAO, monoamine oxidases; DMSO, dimethyl sulfoxide.

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<https://doi.org/10.1016/j.pbb.2021.173223>

Received 21 April 2021; Received in revised form 18 June 2021; Accepted 25 June 2021

Available online 28 June 2021

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In particular, alkaloids that target nicotinic acetylcholine receptors (nAChR) and monoamine oxidases (MAO) have been investigated for the critical roles they play in neuropsychopharmacology (Finberg and Rabey, 2016; Picciotto et al., 2015; Terry Jr. et al., 2015). Clinical and nonclinical studies have indicated that abnormalities in cholinergic signaling are associated with major depressive disorder or anxiety and that drugs that alter nAChR activity can affect behaviors related to mood and anxiety (Mineur et al., 2016; Mineur et al., 2013; Perera et al., 2007; Picciotto et al., 2015; Picciotto and Zoli, 2002; Yu et al., 2014). Several $\alpha 4\beta 2$ nAChR agonists, such as TC-2216 and sazetidine-A, have been shown to induce antidepressant- and anxiolytic-like effects in rodents, for example (Romanelli et al., 2007; Turner et al., 2013; Yu et al., 2014). Also of interest, MAO is a mitochondrial enzyme that catalyzes the oxidative deamination of a variety of monoamines and has a major role in metabolizing released neurotransmitters and thus regulating neural activity (Edmondson and Binda, 2018; Finberg and Rabey, 2016). In fact, MAO inhibitors were the first class of antidepressants discovered and have also been indicated for social anxiety because of the critical role they play in psychopharmacology (Menkes et al., 2016; Sabri and Saber-Ayad, 2020; Tipton, 2018; Youdim et al., 2006).

The current study was conducted to determine the effect of a 5-day administration of the alkaloids nicotine, cotinine, anatabine, anabasine, harmane, or norharmane on anxiety-like behavior by using the zebrafish novel tank test in order to better understand the potential psychoactive properties of these alkaloids. Nicotine, cotinine, anabasine, and anatabine are nAChR-activating alkaloids, and harmane and norharmane are MAO inhibitors, previously identified to be present in Solanaceous plants (Andersson et al., 2003). Based on prior studies implicating nAChR and MAO in mood regulation, it was hypothesized that the aforementioned alkaloids which target one or the other of these systems may produce anti-anxiety-like effects in laboratory animals. Specifically, we hypothesized that these compounds would inhibit the anxiety-like diving response in zebrafish, leading to a greater pattern of exploration away from the bottom of the tank, separate from any effects on locomotor activity. The effects of these alkaloids were compared with that of an anxiolytic reference compound, buspirone. We confirmed that both nicotine and buspirone exhibited anxiolytic-like effect in this zebrafish behavioral paradigm under both acute and subchronic administration conditions. Among the other five alkaloids, only cotinine, a major metabolite of nicotine, induced anxiolytic-like effect in the zebrafish at the highest dose tested. It is worth noting that the effective doses of these compounds were well above what would be naturally present in plants, so the present study may best represent potential benefits for single-alkaloid administration, rather than a plant-derived supplement. Concentrations were scaled based on the tolerability of these compounds within zebrafish to generate well tolerated concentration ranges without adverse side effects. As such, they represent ideal ranges for zebrafish.

2. Methods

2.1. Animal subjects

The studies were conducted in a local colony of AB* wildtype strain of zebrafish, maintained and bred at Duke University (Durham, NC, USA). The experimental procedures were approved by the Duke University Institutional Animal Care and Use Committee (A258-18-11, A259-18-11) in accordance with state and federal regulations. Prior to exposure, the adult zebrafish were housed in mixed (female and male) groups at a density of ≤ 5 fish/L in 3- or 10-L tanks maintained on a recirculating flowing water system (Aquatic Habitats, Inc., Apopka, FL, USA; Aquatic Enterprises, Inc., Bridgewater, MA, USA). The system water was a mixture of sea salt (0.5 parts per thousand of Instant Ocean, Spectrum Brands, Blacksburg, VA, USA) and buffer (125 mg/L of Seachem Neutral Regulator, Seachem Laboratories, Madison, GA, USA) in deionized water. Water chemistry, salinity, and temperature were

monitored weekly. Illumination was set to 14:10 h light:dark cycle, and the temperature of the water was kept at 28 ± 1 °C. The fish were fed three times daily — morning and afternoon with brine shrimp (*Artemia salina*) hatched in-house over 24 h (eggs from Brine Shrimp Direct, Ogden, UT, USA) and at noon with solid pellet food (GEMMA Micro 300 micro-pellets, Skretting USA, Tooele, UT, USA).

2.2. Drug administration

The test compounds included the anxiolytic drug buspirone (1–100 μ M), as a positive control, as well as the alkaloids nicotine (0.3–30 μ M), cotinine (1–100 μ M), (+)-anabasine (0.3–30 μ M), (±)-anatabine (0.3–30 μ M), harmane (0.03–3 μ M), and norharmane (0.03–3 μ M). The concentration ranges were selected on the basis of preliminary tolerability analyses, whereby groups of 5 untreated adult fish were acutely exposed to a selected compound for 20 min and observed for adverse outcomes (data not shown). Adverse outcomes included motor disruption (e.g., sinking and tremor), acute intoxication (e.g., inability to stay upright), and death. The highest selected concentration was a $\frac{1}{2}$ log unit below the lowest adverse concentration, except in the case of cotinine, for which such a level was not reached within biologically relevant levels. The cotinine concentrations were, therefore, set for a $\frac{1}{2}$ log unit above that of nicotine, because the highest tested dose (100 μ M) did not cause toxicity. Buspirone – HCl (CAS 33386-08-2), nicotine ditartrate (CAS 65-31-6), cotinine (CAS 486-56-6), harmane (CAS 486-84-0), and norharmane (CAS 244-63-3) were obtained from Sigma Aldrich (St. Louis, MO, USA). anatabine (CAS 581-49-7) free base was custom-synthesized by WuXi AppTec (purity $\geq 95\%$; Shanghai, China). anabasine (CAS 13078-04-1) was purchased from Tocris Bioscience (CAS No. 53912-89-3; Bio-Techne®, Minneapolis, MN, USA). Harmane and norharmane were administered in 0.1% dimethyl sulfoxide (DMSO) via the system water, while all other compounds were mixed directly into the system water. For each experiment, controls were exposed to the vehicle used for the compound under study, saline with 0.1% DMSO for harmane and norharmane and saline for the other compounds.

For the subchronic dosing procedure, male and female zebrafish (~ 10 zebrafish per sex per concentration per compound) were allowed to freely swim in 1 L of system water with the respective compounds or vehicle in 1-L glass beakers for 20 min per day for 5 days. Male and female fish were identified by their color and shape. The treatment started at 10 am every day. Over the first 4 days of the sequence, cohorts of fish were group-exposed at a density of 10 fish per liter of dosing solution. On the fifth day, pairs of fish were dosed for 20 min immediately before their novel tank dive test. Separate groups of fish were acutely exposed (i.e., a single 20-min treatment) to buspirone or nicotine and tested immediately as controls to confirm the validity of the novel tank test for detecting anxiolytic compounds.

Two cohorts (10 fish per cohort) were generated per treatment group for each compound, except for the acute nicotine control, for which three cohorts were run to allow evaluation of sex-specific effects on the basis of preliminary analyses. Initial studies with harmane/norharmane and anabasine/anatabine were conducted with mixed cohorts; so, these pairs shared a single control group. Each compound was tested at three concentrations.

2.3. Behavioral assessment

In the novel tank diving test, the adult zebrafish were tested for novel environment response and recovery as previously described, but with minor modifications (Bencan et al., 2009; Levin et al., 2007). The experimental setup consisted of two adjacent 1.5-L plastic tanks (Aquatic Habitats, Apopka, FL, USA) filled with system water to a depth of 10 cm. Each tank was a trapezoid: 22.9 cm along the bottom, 27.9 cm at the top, 15.2 cm high, and 15.9 cm along the diagonal side. The test tank was 6.4 cm wide at the top and tapered to 5.1 cm at the bottom. The tanks were video-recorded by a camera placed 50 cm from the side of the

tanks. At the beginning of each trial, two fish were rinsed briefly in fresh system water without compounds and then individually placed in the testing tanks (one in each tank) and recorded for 5 min. The measurements extracted were the total distance traveled in cm, mean distance from the tank floor in cm (see Results section), and time spent at the bottom 1/3rd of the tank (see supplemental data) for each minute, as recorded by a video tracking software (EthoVision XT, Noldus Information Technology, Wageningen, The Netherlands). Each fish was tested only once.

2.4. Statistical methods

The data were evaluated by analysis of variance by using SPSS v.26 (IBM Corp, Armonk, NY). The principal between-subjects factor for hypothesis testing was the concentration of the drug treatment. Sex was the other between-subjects factor. As multiple exposure cohorts were needed, cohort identity was included as a cofactor. The within-subjects factor was the minute of the 5-min test. Dunnett's tests were used to correct for potential alpha slippage with comparisons of each concentration group with the vehicle-treated control. A threshold of $p < 0.05$ (two-tailed) was used for establishing statistical significance.

Greenhouse–Geisser correction was used to adjust for violations of the homogeneity of variance assumption. This correction results in degrees of freedom which are not whole numbers.

3. Results

3.1. Acute treatment with buspirone and nicotine

The effects of acute treatment with buspirone and nicotine were tested as a positive reference (Fig. 1; also see supplemental data). For buspirone, the main effect of treatment and the one-min time bin $\times$ treatment interaction effect were significant for distance from the floor of the tank ($F(3, 71) = 7.06, p < 0.05$ and $F(5.42, 128.31) = 2.73, p < 0.05$, respectively). The middle concentration of 10 μM buspirone led to an increase in the distance from the floor during minutes 1–4, while the highest concentration of buspirone (100 μM) caused an increase in the distance from the floor during all one-min time bins (Fig. 1C; $p < 0.05$; Dunnett's test). The lowest concentration (1 μM) had no detectable effect. Acute buspirone did not significantly alter locomotor behavior, measured as the total distance moved (Fig. 1A). Locomotor behavior did show a main effect of one-min time bin ($F(3.10, 220.30) = 2.80, p <$

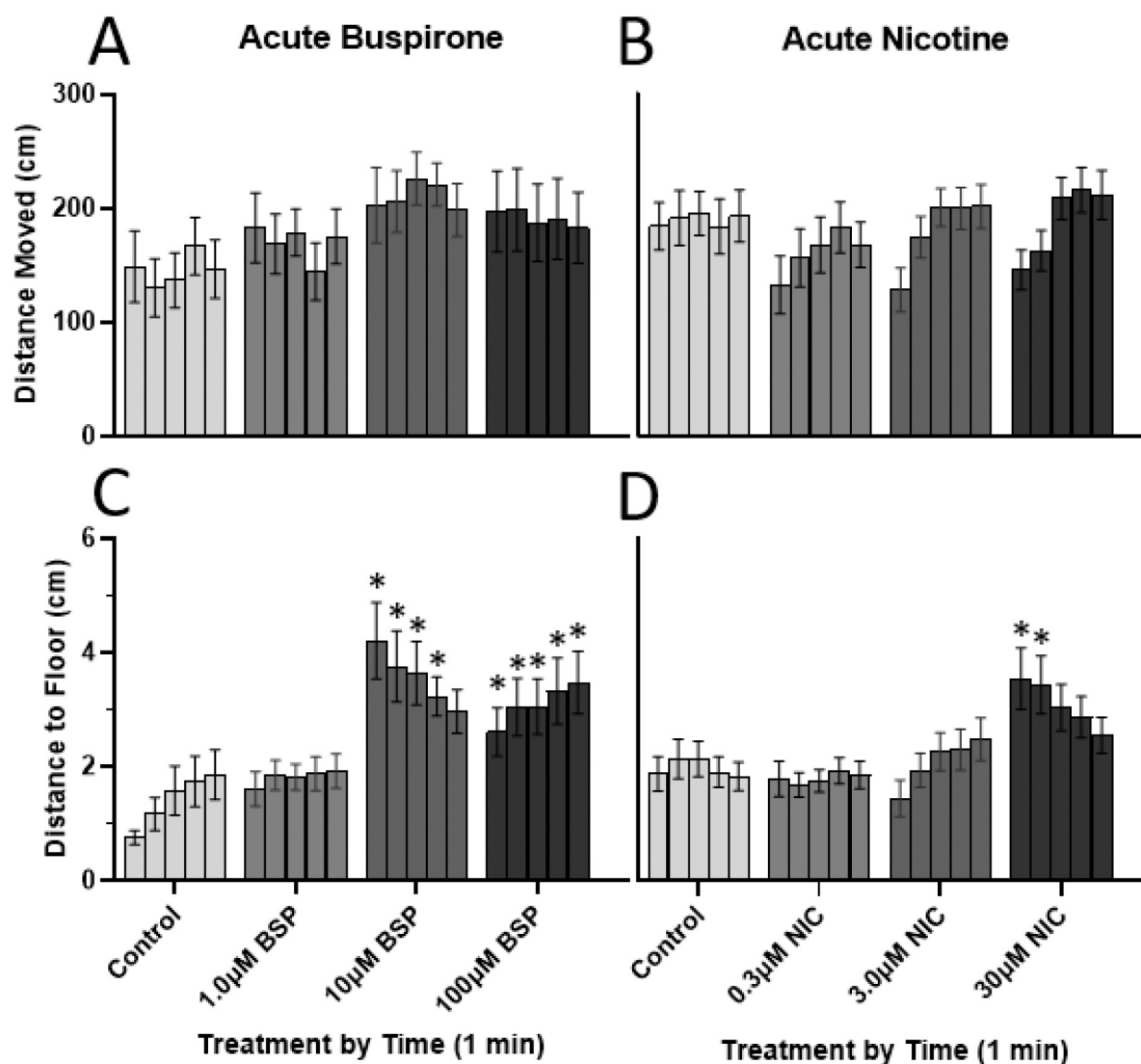


Fig. 1. Acute dosing controls. Locomotor activity, measured as distance moved in cm/min (mean $\pm$ SEM), was not affected by acute exposure to (A) buspirone or (B) nicotine. Diving, measured as distance (cm) from the floor (mean $\pm$ SEM), was altered by acute exposure to (C) buspirone and (D) nicotine in a dose-dependent manner. Asterisks (*) indicate significant differences (Dunnett's test) relative to controls. Buspirone (BSP); nicotine (NIC). $N = 20$ per treatment condition.

0.05), whereby the activity increased over time, as well as a main effect of sex ($F(1, 71) = 9.90$, $p < 0.05$), whereby male zebrafish were hyperactive relative to female zebrafish.

For nicotine, the main effect of treatment and the time $\times$ treatment interaction effect were significant for distance from the floor of the tank (Fig. 1D; $F(3, 103) = 2.85$, $p < 0.05$ and $F(6.08, 208.83) = 4.83$, $p < 0.05$, respectively). The highest concentration (30 μM) caused an increase in the distance from the floor during the first two one-min time bins of the session ($p < 0.05$) (Fig. 1D). Acute nicotine did not significantly alter locomotor behavior, measured as distance moved (Fig. 1B). Locomotor behavior did show a main effect of one-min time bin ($F(2.63, 270.78) = 3.93$, $p < 0.05$), whereby the distance moved gradually increased over time, as well as a main effect of sex ($F(1, 103) = 9.45$, $p < 0.05$), whereby male zebrafish were hyperactive relative to female zebrafish (Fig. 2).

These results of acute treatment confirmed the previously reported anxiolytic effects of buspirone and nicotine and thus supported the reproducibility of the novel tank test. Additionally, they showed that the novel tank test is sensitive to anxiolytic drug effects without affecting the general swimming behavior and is suitable for testing the novel subchronic treatments.

3.2. Subchronic effects

3.2.1. Buspirone

For subchronic buspirone treatment, the main effect of treatment and the one-min time bin $\times$ treatment interaction effect were significant for distance from the floor (Fig. 3A; $F(3, 69) = 5.10$, $p < 0.05$ and $F(6.69, 153.92) = 5.01$, $p < 0.05$, respectively). Post hoc Dunnett's tests revealed that the low (1 μM), middle (10 μM), and high (100 μM) concentrations of buspirone led to an increase in the distance from the floor at minutes 1, 1–3, and 1–5, respectively, relative to the controls ($p < 0.05$). For locomotor activity, main effects of treatment ($F(3, 69) = 3.01$, $p < 0.05$) and sex ($F(1, 69) = 6.62$, $p < 0.05$) as well as a one-min time bin $\times$ treatment interaction effect were observed for distance from the floor ($F(8.70, 200.03) = 2.25$, $p < 0.05$) (Fig. 4A). Post hoc Dunnett's tests revealed that the lowest (1 μM) concentration of buspirone led to increased locomotor activity at minutes 2–3 relative to the controls (Fig. 4A; $p < 0.05$). Male zebrafish were hyperactive relative to female zebrafish ($p < 0.05$).

3.2.2. Nicotine

For subchronic nicotine treatment, main effects of treatment and time were observed for distance from the floor of the tank ($F(3, 69) = 4.89$, $p < 0.05$ and $F(2.94, 202.90) = 3.27$, $p < 0.05$, respectively). Post

hoc Dunnett's tests showed that the lowest (0.3 μM) and highest concentrations (30 μM) caused increased distance from the floor overall ($p < 0.05$) (Fig. 2B). Subchronic nicotine treatment did not significantly alter locomotor behavior (Fig. 3B). Locomotor behavior did show a main effect of one-min time bin ($F(2.16, 149.18) = 2.97$, $p < 0.05$).

3.2.3. Cotinine

For subchronic cotinine treatment, main effects of treatment and time were observed for distance from the floor of the tank ($F(3, 68) = 3.67$, $p < 0.05$ and $F(3.20, 217.85) = 3.27$, $p < 0.05$, respectively). Post hoc Dunnett's tests showed that the highest concentration (100 μM) induced an increased distance from the floor overall ($p < 0.05$) (Fig. 2C). Subchronic cotinine treatment did not significantly alter locomotor behavior (Fig. 3B). Locomotor behavior did show a main effect of one-min time bin ($F(2.87, 195.33) = 6.34$, $p < 0.05$).

3.2.4. Anatabine

For subchronic anatabine treatment, no main effects of treatment, sex, or time block were observed for distance from the floor (Fig. 2D). For locomotor activity (Fig. 3D), there was a significant main effect of one-min time bin ($F(2.65, 185.28) = 4.22$, $p < 0.05$), whereby the distance moved increased over time, indicating acclimation to the novel apparatus.

3.2.5. Anabasine

For subchronic anabasine treatment, no main effects of treatment, sex, or time were observed, either for distance from the floor (Fig. 2E) or locomotor activity (Fig. 3E).

3.2.6. Harmane

For subchronic harmane treatment, no main effects of treatment, sex, or time block were observed for distance from the floor (Fig. 2F). For locomotor activity (Fig. 3F), there was a significant main effect of sex ($F(1, 70) = 7.01$, $p < 0.05$), whereby male zebrafish were hyperactive relative to female zebrafish, as well as a significant one-min time bin $\times$ treatment interaction ($F(10.02, 233.82) = 4.22$, $p < 0.05$). However, none of the post hoc Dunnett's test comparisons of controls with the treatment groups reached significance.

3.2.7. Norharmane

For subchronic norharmane treatment, a sex $\times$ time $\times$ treatment interaction effect was observed ($F(9.32, 214.28) = 1.97$, $p < 0.05$). However, none of the post hoc Dunnett's test comparisons of controls with the treated groups reached significance (Fig. 3G). For locomotor activity, there were significant main effects of treatment ($F(3, 69) = 5.75$, $p < 0.05$), sex ($F(1, 69) = 9.67$, $p < 0.05$)—whereby male zebrafish were hyperactive relative to female zebrafish—as well as one-min time bin ($F(3.48, 240.12) = 3.89$, $p < 0.05$), whereby activity increased across the session, showing acclimation to the novel apparatus. Post hoc comparisons showed no significant effects relative to the controls, although the effect at the low concentration (0.03 μM) approached significance ($p = 0.07$).

4. Discussion

In this study, we were able to confirm the acute anxiolytic-like effects of buspirone and nicotine in zebrafish, which further supported the robustness and reproducibility of the zebrafish novel tank test for detecting anxiolytic compounds, as in the case of buspirone, and compounds that have previously been shown to have anxiolytic-like effects, as in the case of nicotine. We further extended these findings to demonstrate that subchronic buspirone treatment also successfully suppressed the anxiolytic-like effect at the same doses as those used for the acute effect. Furthermore, when applied subchronically, nicotine and its major metabolite cotinine caused a significant decrease in the novel tank diving response early in the test session. These effects were

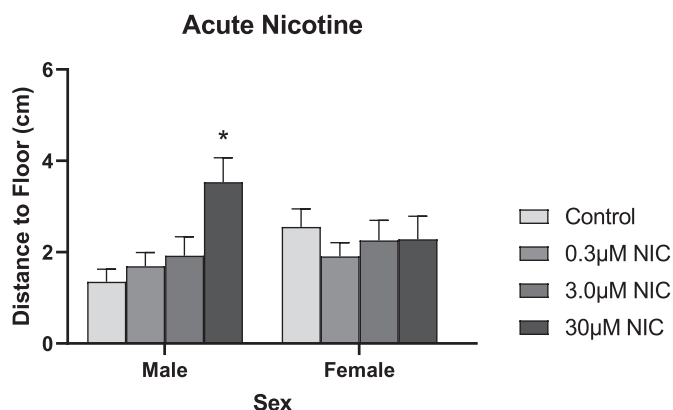


Fig. 2. Acute nicotine effects by sex. Diving responses, measured as distance (cm) from the floor (mean $\pm$ SEM), were significantly altered by 30 μM nicotine treatment in male but not in female zebrafish. Asterisks (*) indicate significant differences relative to controls. Nicotine (NIC). $N = 20$ per treatment condition.

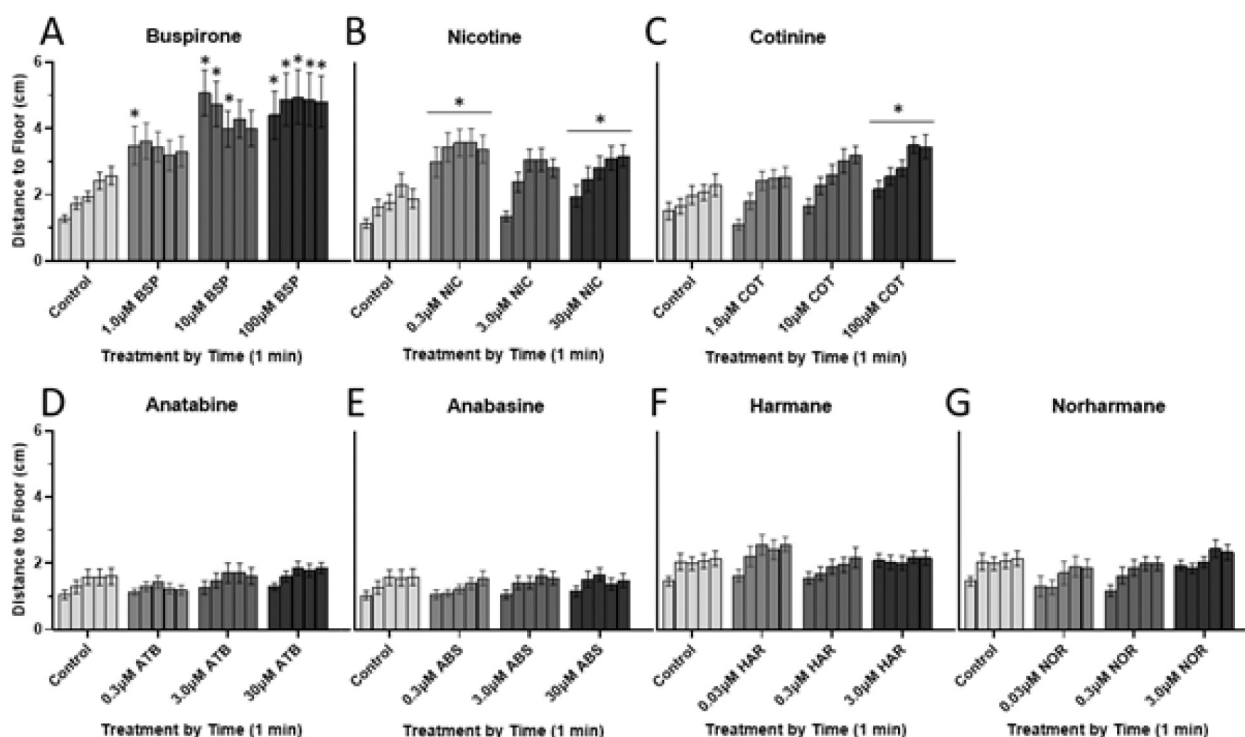


Fig. 3. Effects of compounds on diving responses. Diving responses, measured as distance (cm) from the floor (mean $\pm$ SEM), were altered in a dose-dependent manner by subchronic exposure to (A) buspirone, (B) nicotine, and (C) cotinine but not (D) harmane, (E) norharmane, (F) anabasine, or (G) anatabine. Asterisks (*) indicate significant differences relative to controls. Buspirone (BSP); nicotine (NIC); cotinine (COT); anabasine (ABS); anatabine (ANT); harmane (HAR); norharmane (NOR). $N = 20$ per treatment condition.

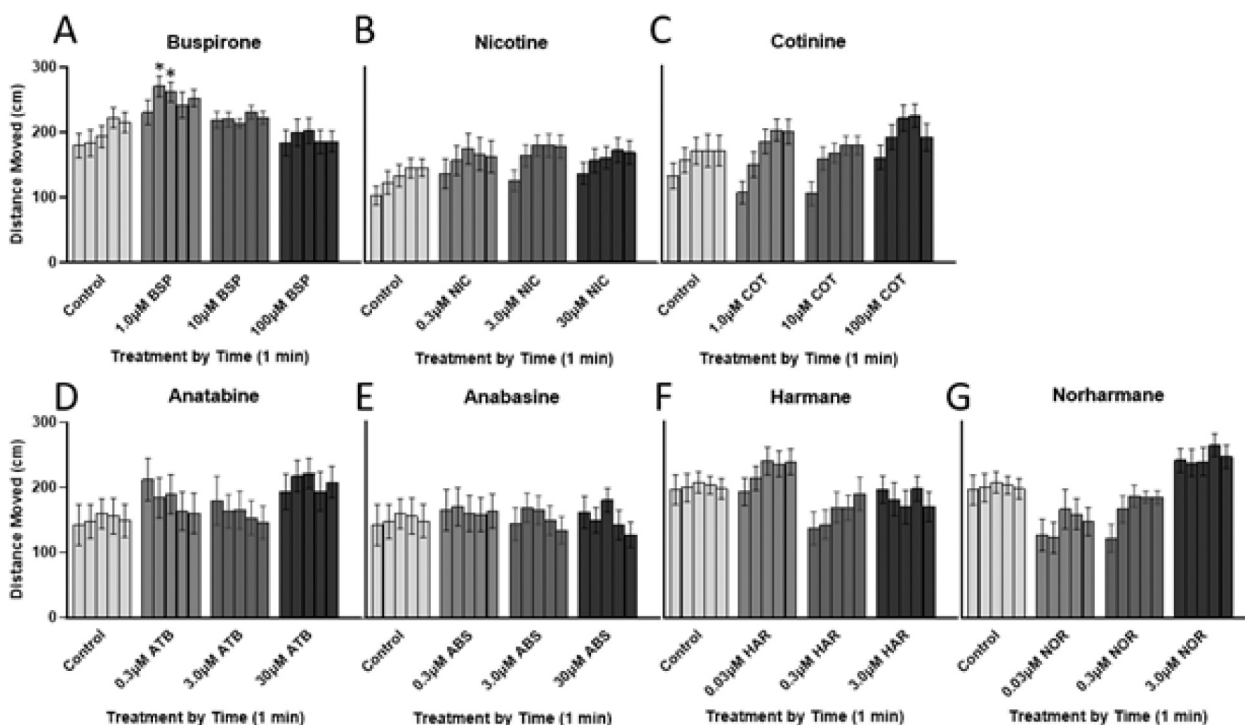


Fig. 4. Effects of compounds on locomotor activity. Locomotor activity, measured as distance moved in cm/min (mean $\pm$ SEM), were altered in a dose- and time-dependent manner by subchronic exposure to (A) buspirone but not (B) nicotine, (C) cotinine, (D) harmane, (E) norharmane, (F) anabasine, or (G) anatabine. Asterisks (*) indicate significant differences relative to controls. Buspirone (BSP); nicotine (NIC); cotinine (COT); anabasine (ABS); anatabine (ANT); harmane (HAR); norharmane (NOR). $N = 20$ per treatment condition.

observed in the absence of changes in general locomotor activity, indicating that they are not an artifact of any stimulant effects produced by cholinergic activation. However, nicotine appears to be substantially more potent in reducing diving, as these effects reached significance at 0.3 μ M and 30 μ M, while cotinine required a concentration of 100 μ M to show an anxiolytic-like effect.

In prior studies, acute nicotine treatment has been shown to cause a decrease in anxiolytic-like behavior in zebrafish in the novel tank diving test (Bencan and Levin, 2008; Levin et al., 2007). The anxiolytic effect of acute nicotine treatment is mostly associated with its effects on nAChRs, as these effects are reversed by $\alpha 7$ or $\alpha 4\beta 2$ nicotinic antagonists (Bencan and Levin, 2008). The present findings that nicotine is anxiolytic-like in zebrafish fits well with these prior studies. The effects induced by the acute vs. subchronic administration schedules, however, were not entirely identical. In the acute study, nicotine led to an enhanced pattern of swimming away from the floor of the tank, but in a time-dependent manner. This effect was observable at the beginning of the session, but quickly attenuated over time. This led to an unusual pattern, where the treated fish swam closer to the surface at the beginning of the trial than at the end of the trial, whereas non-treated control fish normally show the opposite trajectory in this task (swim closer to the floor at the beginning than at the end). The effects of subchronic nicotine exposure were more pervasive, with the fish maintaining a visibly stable (0.3 μ M) or ascending (30 μ M) pattern of exploration away from the floor across the session. It is notable that the subchronic nicotine schedule produced an anxiolytic-like effect at the lowest dose tested (0.3 μ M), which was not the case under the acute condition, perhaps indicating the potential for sensitization—that is enhanced effect—to low doses with subchronic treatment.

The relatively higher doses of cotinine (relative to nicotine) needed to produce an anxiolytic effect somewhat mirror the lower affinity of cotinine with nAChRs relative to nicotine (Riah et al., 1999; Vainio and Tuominen, 2001) and provides support for the effect of nicotine being due mainly to its own activity and not that of cotinine, its main metabolite. At first glance, the need for increased levels of cotinine and the lower overall efficacy of cotinine may appear to negate the potential for nicotine/cotinine interactions in anxiety-like functions. However, among human nicotine users, it has been noted that internal cotinine levels are generally elevated relative to internal nicotine levels, with prior reports noting a cotinine:nicotine ratio of 1.5:1 to over 10:1, depending on the subjects and assessment methods used (Benowitz and Jacob, 1993; Hatsukami et al., 2010; Hecht et al., 2014; Mooney et al., 2008; Yamazaki et al., 2010; Zarth et al., 2014). This is complemented by animal data showing elevated serum cotinine levels relative to nicotine levels in nicotine-self-administering rats (Ding et al., 2021; Shoaib and Stolerman, 1999). This difference is due to the slow clearance of cotinine relative to nicotine (half-lives of nicotine vs. cotinine = 2.62 h vs. 17.45 h in humans) (Tutka et al., 2005) and the potential for accumulation of cotinine over continued use. Matching pharmacokinetic data are needed for zebrafish treated under chronic or subchronic conditions, although the potential for cotinine to produce a similar response at higher doses may indicate possibilities for future studies concerning the anxiolytic effects of cotinine. Cotinine has the advantages of low toxicity, slow kinetics, and absence of nAChR upregulation, stimulant effects, and effects on cardiovascular function and stress/sex hormones (Grizzell and Echeverria, 2015; Grizzell et al., 2014; Terry Jr. et al., 2015; Terry Jr. et al., 2005; Wang et al., 2020).

In contrast, the other alkaloids—anabasine, anatabine, harmaline, and norharmaline—failed to show any effects in the present study. There may be a couple of logical explanations for the discrepancies among the $\alpha 4\beta 2$ nAChR-targeting alkaloids. First, the functional activities of the tested compounds on the $\alpha 4\beta 2$ nAChR vary. Nicotine and anabasine show the highest activity among the compounds, while cotinine and anatabine are at least 10-fold weaker as $\alpha 4\beta 2$ nAChR agonists. Furthermore, anabasine may not fully activate the $\alpha 4\beta 2$ nAChR. Second, a study by Papke et al. suggests that many nAChR subunits, including $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 7$, $\beta 2$, and $\beta 4$,

can be cloned from zebrafish, and the activities of key pharmacological tools like nicotine on these zebrafish nAChRs seem sufficiently similar to those on mammalian receptors (Papke et al., 2012). However, there are some differences between zebrafish and humans in this regard. For example, nicotine seems to be a potent $\alpha 7$ nAChR agonist in zebrafish, which is not the case in humans (Alijevic et al., 2020). This effect is also supported by previous studies showing that antagonism of either $\alpha 7$ or $\alpha 4\beta 2$ nAChRs can suppress the anxiolytic-like effect induced by nicotine in the novel tank diving test (Bencan and Levin, 2008). Lastly, the brain bioavailability of the compounds may not be the same.

The reason why subchronic treatment of zebrafish with the MAO inhibitors harmaline and norharmaline failed to show any effect is rather puzzling in light of the fact MAO inhibitors have been reported to suppress anxiety (Jaka et al., 2021; Tyrer and Shawcross, 1988). In fact, harmaline and norharmaline have been shown to reduce anxiety in rodents in previous studies (Aricioglu and Altunbas, 2003). Interestingly, these studies also used acute treatment of harmaline and norharmaline, which, therefore, suggests that chronic treatment may perhaps induce tolerance and does not result in any detectable effect. However, this is not the effect we observed with nicotine. In addition, we cannot eliminate the possibility that MAO inhibitors have different potency in zebrafish compared to mammals in light of the fact that there are some differences between mammalian and zebrafish MAO systems. For example, zebrafish MAO can be inhibited by most MAO A selective reversible inhibitors but not by all MAO B inhibitors (e.g., deprenyl but not farnesol or safinamide) (Aldeco et al., 2011; Setini et al., 2005). Thus, further characterization of the MAO pharmacology in zebrafish is necessary to understand the lack of effect we observed with harmaline and norharmaline.

The present data also provide insight into locomotor behavior in zebrafish and the locomotor effects of nicotine. Nicotine is an established stimulant compound in vertebrate species, although prior studies in zebrafish have provided only mixed evidence for this. In our two previous publications, we found that a 3-min exposure to 100 mg/L (216 μ M) nicotine led to locomotor hyperactivity in one case (Bencan and Levin, 2008), while in another case, it produced hypoactivity late in the 5-min testing session (Levin et al., 2007). Duarte et al. (2019) found no locomotor effects following a 3-min exposure to 1 mg/L (2.2 μ M) nicotine, while Singer et al. (2016) found locomotor hypoactivity with this same dose. Dean et al. (2020) failed to find either stimulatory or sedative effects of 3-min exposure to 25–400 mg/L (54–865 μ M) nicotine on distance moved, although they did find that 50 mg/L (108 μ M) nicotine led to increased immobile time.

Prior and current results highlight an important limitation in the zebrafish model, which is that the substrates and neurology of zebrafish and mammalian nervous systems are similar in key respects, but not all respects. In order to effectively use zebrafish to predict mammalian outcomes, it will be necessary to characterize the presence and sensitivity of underlying substrates across species and to focus on neurochemical systems with well conserved molecular, cellular and neurobehavioral functions. A related limitation is in the pharmacokinetics of these drug exposures. Unlike rodent models, zebrafish passively uptake these compounds from the environment, so the precise internal dose of each compound is unknown for most compounds, with the exception of one recent study reporting the brain bioavailability of harmaline and norharmaline after an acute treatment in zebrafish (Jaka et al., 2021). This makes direct correlations of concentrations used in zebrafish to mammalian doses difficult to extrapolate. The doses were chosen on a phenomenological basis, with the highest tested dose falling just below the threshold for adverse behavioral effects or increased lethality, so comparisons with other species, such as rodents, may require a similar approach. Additionally, although all fish were allowed an equal length of time to uptake the compound of interest, it is not known how the timing of testing related to the peak internal doses achieved (peak occurred prior to, during or after the test) and the degree to which this may differ between compounds. Uptake and

pharmacokinetic studies may be needed to refine this model and to individually select ideal exposure durations and testing latencies.

It is important to note that there were sex differences in behavior in the current study. Sex differences were seen, with males swimming faster than females within data on acute nicotine or bupirone treatment, as well as subchronic bupirone, harmaline and norharmaline. Sex differences in locomotion have been seen previously, with adult male zebrafish swimming faster than females (Conradsen and McGuigan, 2015; Philpott et al., 2012). Sex differences have also been seen with the diving response in the novel tank diving test. It has been found that female zebrafish have a lower anxiety-related response compared with males (Ampatzis and Dermon, 2016). This is similar to what was seen in the current study for acute nicotine treatment. Overall, however, these sex differences tended not to interact with treatment. Overall, sexual determination and the sex-dependency of neurodevelopment and behavior operate very differently in zebrafish than mammals (Liew and Orbán, 2014), therefore it is not simple to extend comparisons concerning sex differences in zebrafish and mammals such as rodents or humans.

In conclusion, our findings suggest that subchronic bupirone, nicotine, and cotinine treatment can induce anxiolytic-like effects in zebrafish and highlight the usefulness of the zebrafish model as a tool for neurobehavioral research for investigating various aspects of anxiety-related states (Clemente et al., 2004; Khan et al., 2017; Kysil et al., 2017; Parker et al., 2013; Stewart et al., 2012). However, the lack of an observable effect by other alkaloids suggests the need for further optimization of the experimental design, including pharmacokinetic-derived parameters for exposure duration and testing latency for each test compound, as well as methods to increase the throughput of the test with greater numbers of fish tested at the same time and methods to minimize freezing responses upon entry into the novel tank. The present data also emphasizes the need for further characterization of the nicotinic and MAO systems in zebrafish to understand the potential differential factors between humans and zebrafish.

Declaration of competing interest

MCP, JH, and KK are employed by Philip Morris International. AH and EL received funding from Philip Morris International. The study was fully funded by Philip Morris International.

Acknowledgement

This work was funded solely by Philip Morris International. This study was a collaboration between scientists at Duke University and Philip Morris International who are authors of the article. The design of the studies was done by consensus of the authors, the dosing and data collection were done at Duke University by the Duke authors who also conducted the statistical analysis. The interpretation of the data followed directly from the analysis. The writeup of the studies was done by consensus of all authors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pbb.2021.173223>.

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