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Augmented Reinforcing and Anxiolytic Effects of Nicotine and Ethanol in Zebrafish

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Abstract - Epidemiological studies have revealed a high incidence of alcohol and nicotine co-dependence. Alcohol and nicotine can act on the brain to release dopamine and produce reward. It is possible that the prevalence of alcohol and nicotine co-abuse is simply due to the combined rewarding effects of each. However, another commonality of both substances is that they are taken in conjunction with stressful events. Since the neurobiological mechanisms that contribute to alcohol and nicotine co-abuse are not fully understood, the aim of this study was to investigate the combined effects of ethanol and nicotine by studying a repertoire of zebrafish behaviors exhibited during rewarding and stressful conditions. We hypothesized that combining alcohol and nicotine might produce enhanced behavioral effects, which could contribute to their co-abuse. In conditioned place preference (CPP) studies, ethanol and nicotine produced inverse U-shaped dose response curves demonstrating both rewarding and aversive effects in zebrafish. Combining low doses of ethanol and nicotine significantly increased CPP compared to the effects produced by the individual substance. In novel-elicited stress response tests, zebrafish were exposed to acute or chronic regimens of ethanol, nicotine or, a combination dose. Significant drug effects were observed following chronic exposure, in which nicotine and ethanol + nicotine significantly attenuated species-typical stress responses, including diving to the bottom of the test tank. Although the augmentation of reinforcing and anxiolytic effects was evident only when low doses were combined, the findings suggest that combining ethanol and nicotine could enhance both the rewarding and anxiolytic effects to promote co-dependence.

Introduction

Alcohol and nicotine are the two most abused licit drugs in the United States (Jamal et al. 2016, Johnston et al. 2006, Pesta et al. 2013, SAMHSA 2014) and epidemiological studies over several decades indicate a high incidence of alcohol and nicotine co-abuse (Adams 2017). It has been estimated that over 83% of alcoholics also smoke and that alcoholism is approximately 10 times more prevalent in smokers than in non-smokers (Batel et al. 1995). Similarly, people who are dependent on nicotine appear to be 4 times more likely than non-smokers to become alcoholics (Eckhardt et al. 1994). Although both drugs of abuse appear to be highly rewarding based on the protracted history of co-abuse, the precise neurobiological mechanisms underlying the popularity and co-dependence of this specific drug combination are still unclear.

Although alcohol and nicotine act via different receptors in the central nervous system, both substances can activate the mesolimbic dopamine pathway that mediates reward. Nicotine, structurally similar to the neurotransmitter acetylcholine, binds to nicotinic acetylcholine receptors (nAChRs) (Simmons and Gould 2014). In the mesolimbic dopamine system, nicotine can stimulate nAChRs on dopamine neurons originating in the ventral tegmental area (VTA) to stimulate dopamine release into the nucleus accumbens and produce rewarding effects (D'Souza and Markou 2011). Pharmacological studies have provided evidence that nAChRs are conserved in zebrafish (Eddins et al.

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2009) and that stimulation of these receptors by nicotine can release dopamine in regions homologous to the dopaminergic mesolimbic pathway (Klee et al. 2011). Ethanol can activate two different neurotransmitters in the brain called gamma-aminobutyric acid (GABA) and glutamate, and it is the disinhibition (inhibition of GABA receptors) of VTA neurons that leads to dopamine release in the mesolimbic system (Vengeliene et al. 2008). Since both drugs of abuse produce rewarding effects via activation of the mesolimbic dopamine system, this circuit has been proposed as a common neural substrate for alcohol and nicotine interactions (Adams 2017, Clark and Little 2004, Deehan et al. 2015, Doyon et al. 2013).

Another common factor shared by alcohol and nicotine is the correlation of high use of these substances following stressful events, suggesting that nicotine and ethanol could exert anxiolytic effects in humans, rodents, and zebrafish. Nicotine has been shown to alleviate stress behaviors in zebrafish, rodents, and humans (Anderson and Brunzell 2015, Balfour 1991, Doyon et al. 2013, Leão et al. 2015, Levin et al. 2007). Most relevant to this study is the finding by Levin et al. (2007) in which a regimen of nicotine treatments attenuated species-typical stress responses in zebrafish. Alcohol also has anxiolytic effects in the central nervous system of several species (Clark and Little 2004, Vengeliene et al. 2008). In zebrafish studies, it has been shown that specific doses of alcohol reduced stress responses, including reducing erratic swim patterns and diving to the bottom of the test tank, under anxiogenic situations (Egan et al. 2009, Gerlai et al. 2000, Tran and Gerlai 2014).

Although there are copious reports investigating the effects of either alcohol or nicotine, the combined effects of these substances have not yet been investigated in zebrafish. In a recent study, Xu et al. (2017) examined DNA from a cohort of more than 200 alcohol and nicotine co-abusers and found intriguing biomarkers that might play a role in epigenetic changes of addiction-related genes. Because of the versatility of zebrafish in scientific research, zebrafish models of ethanol and nicotine co-dependence could be useful in behavioral testing, genetic screening, and developmental and epigenetic studies to further our understanding of the underlying interactions of these prevalent substances of abuse.

Thus, the goal of our study was to investigate the combined effects of ethanol and nicotine combinations on a repertoire of zebrafish behaviors that reflect reward and anxiety. Pre-clinical studies in rodents have found that speedballing (simultaneously using heroin and cocaine) produces synergistic increases in dopamine release in the mesolimbic system (Hemby et al. 1999). This is reminiscent of the high incidence of the nicotine and alcohol co-dependence described above, and could potentially involve heightened rewarding and anxiolytic effects when ethanol and nicotine are combined. Based on the high incidence of nicotine and alcohol co-dependence described above, we hypothesized that combining ethanol and nicotine might produce heightened rewarding and anxiolytic effects compared to the effects produced by the individual drugs. Such a finding would provide a putative explanation for the high prevalence of alcohol and nicotine use. Our research examined the well established phenotypes for addiction, stress, and anxiety, and utilized behavioral paradigms common to behavioral pharmacology studies with nicotine or ethanol (Kalueff et al. 2014). To investigate the combined rewarding effects of alcohol and nicotine, zebrafish were examined in drug-induced conditioned place preference (CPP) experiments using a range of doses of ethanol and nicotine. In a second series of experiments, novelty-elicited stress response tests were conducted following acute and chronic treatment with nicotine, ethanol, and a combination of both drugs.

Methods

Animals and maintenance

Adult zebrafish (*Danio rerio*) were obtained from a local commercial distributor (Petco, Lawrenceville, GA, USA). Fish obtained from the local pet store were quarantined by housing in a 10-gallon tank for 14 days. These tanks were never used for experiments, and fish were observed daily for signs of illness. Sick or dead fish were immediately removed. Fish nets and syphons were dedicated to quarantine tanks and never used for housing tanks. In housing and experimental tanks, the tap water was conditioned by sitting in open tanks and checked daily with Tetra Easy-Strips 6-in-1 Test Strips to maintain parameters of water hardness at 75–150 mg/l, chlorine at 0 mg/l, and nitrates at 0–40 and nitrites at 0.5 mg/l or less. They were then housed in a zebrafish vivarium and maintained on a 12-hour day/night cycle with a water temperature of 23 °C. All fish underwent a 7-day acclimation period in 20-gallon housing tanks in groups of 10–15 fish per tank. All experiments were conducted during the light phases, between 8 a.m. and 5 p.m. All protocols for animal care standards were inspected and approved by the Institutional Animal Care and Use Committee (IACUC) of Georgia Gwinnett College.

Conditioned place preference (CPP)

Nine different drug combinations were prepared. Three concentrations of ethanol were dissolved in the water: 0.25%, 0.50%, and 1.0%. Three concentrations of nicotine were also dissolved in water: 0.01%, 0.03%, and 0.05%. Preliminary studies were conducted in which a wider range of ethanol and nicotine doses was tested in CPP experiments. A range of doses was selected to capture the peak dose that produced a maximum effect and lower doses that engendered the minimally rewarding and aversive properties of these substances at low and high concentrations, respectively. Further, these doses are comparable to doses used in previous CPP experiments with zebrafish (Kily et al. 2008). Finally, three combination concentrations of ethanol and nicotine were prepared: nicotine (0.01%) was held constant and combined with ethanol (0.25%, 0.50, and 1.0%). All drugs were diluted in 20 liters of tank water.

The testing apparatus was a 20-liter fish tank, formatted with plexiglass tracks to insert 2 sliding plexiglass dividers. Thus, the test tank contained 3 chambers: the outer chambers were designed with distinct visual cues (large black spots versus alternating black and white stripes), and the middle chamber was clear with no pattern. Patterns were visible on 2 walls and the floor of each chamber (Fig. 1A). A small camera was placed 3 feet in front of the tank to record zebrafish activity. A new batch of zebrafish was acquired for each CPP experiment, and no fish participated in more than one trial.

Preference test. Prior to inducing CPP with nicotine or alcohol, fish were tested to determine which chamber of the test tank was preferred. Each test began by placing a fish in the middle compartment and then simultaneously removing the 2 dividers allowing fish to swim freely between the 3 chambers. Following a 5-minute acclimation period in the test tank, zebrafish behaviors, including the time spent and number of entries into each chamber, were monitored with a Dynex 1.3MP webcam and recorded using the video tracking features of Noldus EthoVision XT 10 (Noldus, Information Technology, Wageningen, The Netherlands) during the 10-minute preference test (pre-test). The purpose of this preference test was to assign subjects to a drug-paired chamber (conditioned with drug) and vehicle-paired chamber (conditioned with vehicle) for drug-induced place conditioning. The chamber in which more time was spent was assigned the vehicle-paired chamber, whereas the chamber in which less time was spent

was subsequently assigned the drug-paired chamber. Thus, using the experimental conditions we attempted to switch the chamber preference as a function of the reinforcing effects of the test drugs. Similar preference tests have been utilized in other CPP studies (Achat-Mendes et al. 2005 and 2007, Kedikian et al. 2013).

Conditioning. For each conditioning session, fish were placed in a specific chamber for 20 minutes and the divider was inserted to restrict fish to the specific visual cues of that chamber. When restricted to the preferred chamber (assigned vehicle-paired), no drug was present in the water, whereas the test drug (nicotine (0.01%, 0.03%, or 0.05%), or ethanol (0.25%, 0.30%, or 0.5%), or nicotine (0.01%) + ethanol combinations) was diluted in the test tank water during restriction in the less-preferred (assigned drug-paired) chamber. In this way, fish were conditioned in the drug-paired and vehicle-paired chambers in alternating sessions for a total of 8 conditioning sessions with 4 drug sessions and 4 vehicle sessions. Following each session, fish were netted into recovery tanks to wash off residual treatment, and then returned to housing tanks in the vivarium. A range of drug doses were tested for nicotine (0.01%, 0.03%, 0.05%), ethanol (0%, 0.25%, 0.5%, and 1%), and ethanol + nicotine (0.01+%) combinations. Each drug concentration was tested in 8–15 fish for each of the 10 different experiments, and no fish were used in more than one experiment. In order to investigate the rewarding potential of ethanol and nicotine when taken together, a low nicotine dose that was found to produce no CPP was selected for combination experiments. This was done in contrast to combining high doses of each drug, which would instead create a ceiling effect and limit the analysis of the combined effects of drugs.

Post-preference testing. To determine the reinforcing effects of ethanol and nicotine, the place preference of each fish was determined on the 10th day, after the last conditioning session. The procedure for this test was the same as the initial preference test (pre-test) described above in which the divider was removed to allow exploration of all chambers of the tank. Time spent in each chamber was recorded and compared to behavior in the pre-test. Any change in place preference was determined by subtracting the baseline time spent on the drug-treatment side from the final time spent on the drug-treatment side expressed in seconds.

Drugs. Given that the average blood plasma nicotine from smoking commercially available cigarettes is 1mg (National Cancer Institute, 2001), and that nicotine was dissolved in 20 liters of water of a 10-gallon test tank, the nicotine percentages (0.01%, 0.025%, and 0.05%) used in this study were calculated to approximate 0.2, 0.5 and 1 whole cigarette, respectively.

In tests involving drug combinations, a selected dose of nicotine (0.01%) that did not engender CPP when tested alone was combined with the known range of ethanol doses (0.25%, 0.5%, 1.0%) by mixing the drugs in a flask before adding to the experimental tank. The reason for selecting the lowest dose was the expectation that drug combinations would produce greater effects compared to effects produced by one drug alone. Thus, the lowest drug dose was selected to prevent any ceiling effect that might result from combining high doses of each drug.

Novelty-elicited stress response test

Acute drug treatments. Zebrafish were individually netted from their 20-gallon housing tanks into a 3-liter pretreatment tank and were acutely exposed to 1 of 3 doses of ethanol, nicotine, ethanol + nicotine, or drug vehicle: 0.03% ethanol, 0.01% nicotine, or 0.03% ethanol + 0.01% nicotine. Acute treatments lasted for 5 minutes in the pretreatment tank, after which stress testing using the procedure described below was conducted.

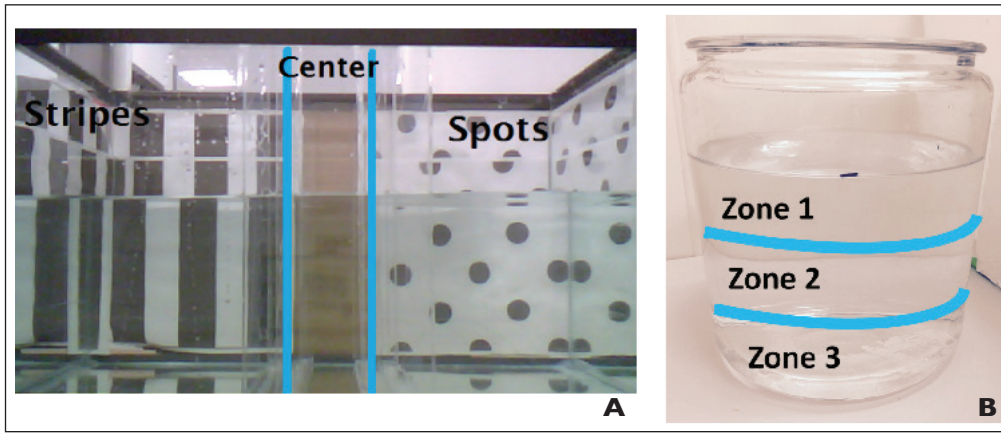

Figure 1. Testing apparatus used in A) conditioned place preference and B) novelty-elicited stress response tests showing the different chambers and zones in which specific zebrafish behaviors were monitored. In place preference, the tank was covered with paper printed with wither stripes or spots so that the drug-paired and vehicle-paired chamber has distinct visual cues. Each fish began each session in the center of test tank. The blue lines highlight grooves in the tank in which guillotine doors were placed to confine fish to one tank during conditioning. Guillotine doors were removed during preference and post-conditioning tests. The novel test tank for measuring stress responses contained three zones of which zone 3, the lowest zone, is frequented by zebrafish during stressful situations.

Chronic drug treatments: Zebrafish were netted as a group from their 20-gallon housing tanks into the 3-liter pretreatment tank in which ethanol (0.03%), nicotine (0.01%), 0.03% ethanol + 0.01% nicotine, or drug vehicle was administered depending on the group. For chronic treatments, exposure lasted 60 minutes and was conducted for 6 consecutive days. Novelty testing began on the 6th day, following the last chronic treatment. After each drug treatment, fish were netted into a recovery tank with clean water and then returned to home tanks.

The protocols for acute and chronic treatments were selected based on the validation studies of chronic administration of the anxiolytic drug fluoxetine (Egan et al. 2009) and the finding that the 5-minute acute exposure was sufficient for ethanol accumulation in the zebrafish brain in the time course study by Tran et. al (2015). Control groups for acute and chronic drug exposure received no drug as the experimental tank water served as the vehicle. They were handled and tested using the same procedure as that used with their experimental counterparts.

Novelty-elicited stress test: In order to investigate the effects of nicotine and ethanol on anxiety, zebrafish novelty-elicited motor responses were recorded using the video tracking features of Noldus EthoVision XT 10. Following both acute and chronic treatments, the novelty-elicited stress test was conducted in which fish were moved, for the first time, into a 10-gallon cylindrical tank (Fig. 1B). After 30 seconds of acclimation, tests were conducted for 10 minutes in which species-typical responses indicative for zebrafish stress were monitored and recorded by EthoVision.

Typically, zebrafish dive to the bottom of a novel environment reminiscent of an escape mechanism from predators and suggestive of stress or anxiety. Zebrafish also exhibit increased episodes of erratic swimming behaviors and periods of irregular locomotor activity leading to increased swimming during stressful conditions (Klee et al. 2011, Levin et al. 2007). Thus, the measurements taken to assess zebrafish stress or anxiety included entries into the lowest zone of the tank, total distance swum, and episodes of high mobil-

ity (≥ 60% in 6 seconds). Using EthoVision, the tanks were zoned into 3 sections (Fig. 1B) and time spent in the lowest zone (zone 3) was analyzed while other behaviors were monitored throughout the test tank.

Statistical Analysis

Motor responses of the zebrafish were tracked and recorded by Noldus EthoVision XT 10. The data were analyzed using GraphPad Prism 5 for Windows (GraphPad Software, San Diego, CA, USA). Two-way ANOVA with Bonferroni's post-hoc test were used to compare effects of drug and drug doses on CPP. The magnitude of CPP was calculated as (time spent in drug-paired chamber after conditioning)—(time spent in that same chamber before conditioning). Comparisons between two groups in the novelty-elicited stress tests were done with Student's t-test. In instances where two groups with unequal number of subjects were compared, data were analyzed using two-sample unequal variance Student's t-test.

Results

Conditioned place preference

To investigate the reinforcing effects of ethanol and nicotine, the effects of drug conditioning on changing zebrafish place preference was measured. Thus, the magnitude of place preference reported in Figures 2, 3, and 4 represents the degree to which drug conditioning changed the preference toward the drug-paired chamber in zebrafish. Results in Figure 2 show that conditioning with increasing doses of ethanol produced an inverted U-shaped dose-response curve in which the intermediate dose (0.5%) engendered the largest magnitude of CPP (161 ± 38) that was significantly different from the control group. Conditioning with the low (0.25%) and high (1%) doses of ethanol resulted in lower magnitudes of CPP (21 ± 10 and 91 ± 42 sec, respectively) that were not significantly different from controls.The lowest ethanol dose tested produced effects similar to the control group (Veh) that received no drug during conditioning. The magnitude of CPP induced by the low dose and the control group was -18 (± 21) sec and 21 (± 10) sec, respectively. The intermediate dose (0.5%) significantly increased the magnitude of CPP, compared to the control group ($p < 0.05$), as the time spent in the drug-paired chamber was greater by an average of 147 (± 17) sec than the time spent in the vehicle-paired chamber. CPP robustly declined following conditioning with the highest ethanol dose tested (1%). A 2-way ANOVA revealed a significant effect of ethanol conditioning ($p < 0.005$, $F (1, 50) = 7.765$) and a significant effect of ethanol dose $p < 0.05$, $F (2, 50) = 4.361$) and Bonferroni t-tests revealed a significant effect of 0.5% ethanol to induce CPP compared to the control group.

Similarly, the results in Figure 3 for nicotine-induced place preference revealed a similar dose-response relationship in which the intermediate dose (0.03%) engendered the largest magnitude of CPP (227 ± 68 sec), compared to the low (0.01%, 83 ± 90 sec) and high doses (.05%, –85 ± 89) of nicotine. A one-way ANOVA revealed a significant effect of nicotine dose ($p < 0.05$, $F (3, 68) = 2.252$) and nicotine conditioning ($p < 0.05$, $F (3, 68) = 2.866$) on the magnitude of CPP engendered and the Bonferroni t-test revealed significantly increased place preference for 0.03% nicotine compared to controls ($p < 0.05$).

To examine the combined reinforcing effects of ethanol and nicotine, the low dose of nicotine (0.01%) that produced minimal CPP effects was combined with increasing doses of ethanol (0.25%, 0.5% and 1.0%). Figure 4 shows that ethanol conditioning in a second group of zebrafish reproduced the inverted U-shaped dose response of the magnitude of CPP (gray line) as was observed with the ethanol dose-response curve in Figure 2. In the

ethanol + nicotine experiments, combining the low nicotine dose (0.01%) with ethanol produced a significant trend in which the CPP decreased as ethanol doses increased (black line). A one-way ANOVA revealed a significant effect of dose on the magnitude of CPP induced by ethanol + nicotine combinations ($p < 0.005$). Notably, the CPP engendered by ethanol (0.25%) + nicotine (0.01%) was significantly higher (141 ± 35 sec) than the magnitude of CPP engendered by 0.25% ethanol alone (22 ± 39, Student's t-test $p < 0.05$). Furthermore, while the intermediate ethanol dose (0.50%) produced the maximum CPP effect in the ethanol dose-response curve, combining this dose with nicotine significantly decreased CPP (39 ± 43 sec) compared to when administered alone (161 ± 38 sec, Student's t-test $p < 0.05$). A similar effect was observed when 0.5% ethanol was combined with nicotine. This combination dose significantly decreased CPP (–86 ± 49 sec) compared to effects produced by 0.5% ethanol alone (91 ± 42 sec, Student's t-test $p < 0.01$).

Novelty-elicited stress response

In order to examine the effects of ethanol and nicotine on species-typical stress responses, zebrafish behaviors during the novelty tank test were measured following administration of acute or chronic regimens of ethanol and nicotine. Figure 5 results reveal the entries into the lowest zone (zone 3) of the novel test tank following acute and chronic drug administration. Acute exposure to ethanol and nicotine produced similar effects (114 ± 15 and 82 ± 9, respectively) compared to control (88 ± 7) zebrafish that received no drug treatment (Fig. 5A). For all three groups, the mean frequency of entries into the lowest zone was between 88 ± 7 and 114 ± 15 times during the test. The ethanol + nicotine combination resulted in a significantly increased frequency of entries into the lowest zone (155 ± 22) compared to

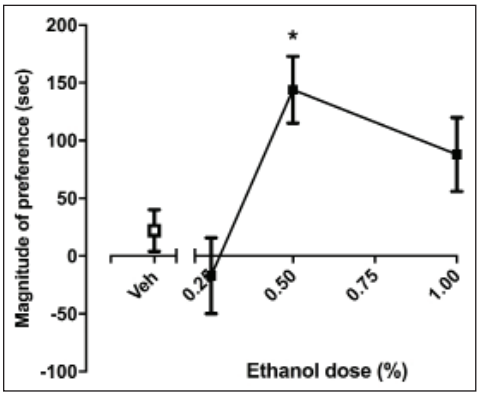

Figure 2. Dose-response relationship of ethanol-induced conditioned place preference in zebrafish. The magnitude of CPP is calculated as the difference between time spent in the drug-paired chamber before and after conditioning with ethanol (n = 12–15 per dose). 2-way ANOVA: Effect of conditioning ($p < 0.005$), Effect of dose ($p < 0.05$). Bonferroni t-test, $p < 0.05$ for 0.5% ethanol.

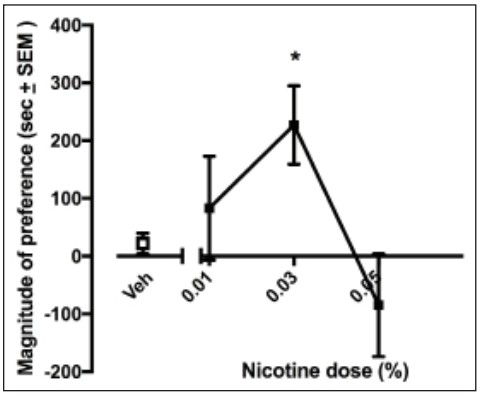

Figure 3. Dose-response relationship of nicotine-induced conditioned place preference in zebrafish (n = 10–15 per dose). 2-way ANOVA: effect of conditioning ($p < 0.05$), effect of dose ($p < 0.05$). Bonferroni t-test, $p < 0.05$ for 0.03% nicotine.

control fish (Student's t-test $p < 0.05$). Following chronic treatments, animals' baseline frequency to enter the lowest zone of the test tank was approximately double in controls and in fish exposed to chronic ethanol treatments, averaging 203 ± 19 entries into the lowest zone (Fig. 5B). In contrast to acute treatments, however, chronic administration of nicotine alone and of the ethanol + nicotine combination significantly attenuated the frequency of entries into the bottom of the tank to 122 ± 15 and 141 ± 13 entries, respectively and by approximately 70% of the frequency of entries of controls.

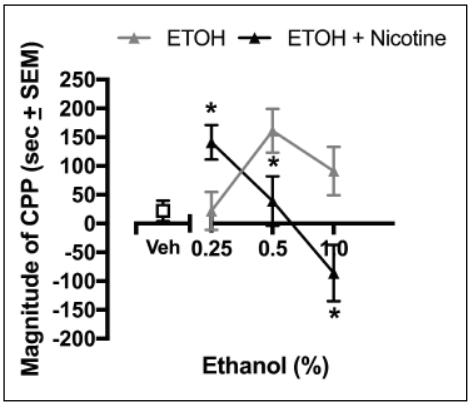

Figure 4. Comparison of the magnitude of CPP between responses engendered following ethanol alone versus combinations of ethanol + nicotine in which increasing doses of ethanol were combined with the low non-reinforcing dose of 0.01% nicotine (n = 12–15 per dose), * $p < 0.05$ comparing effects of respective doses of ethanol to ethanol + nicotine.

Results on the total distance swum in the novel test tank are presented in Figure 6. Acute treatment with ethanol or nicotine produced no differences in the total distance swum compared to controls (Fig. 6A). However, the ethanol + nicotine combination significantly reduced the total distance traveled (3880 ± 206 cm) compared to the distance traveled by controls (3173 ± 337 cm), Student's t-test $p < 0.05$). Chronic treatment with both nicotine and the ethanol + nicotine combination significantly decreased total distance swum compared to controls ($p < 0.05$, Fig. 6B).

High mobility results revealed that compared to controls, the frequency of high mobility episodes exhibited following acute treatments was similar, ranging from a minimum of 490 ± 29 to a maximum of 557 ± 65 episodes across all groups (Fig. 7A). The chronic control and chronic ethanol groups showed similar effects as acute groups in which high mobility episodes ranged from 328 ± 33 to 476 ± 66 (Fig. 7B). In contrast, compared to chronic controls, chronic treatment with either nicotine or the ethanol + nicotine combination significantly decreased the frequency of high mobility swimming to 202 < 25 and 131 < 16 episodes, respectively ($p < 0.05$ and $p < 0.01$). Furthermore, statistical analysis showed a significant difference in high mobility behavior between groups exposed to nicotine alone and ethanol + nicotine combinations ($p < 0.05$).

Discussion

In this study, the behavioral effects of the same doses of ethanol and nicotine were compared in conditioned place preference and novelty-elicited stress response tests. Our results demonstrate that ethanol and nicotine alone, and in combination, can engender reinforcing and anxiolytic effects in zebrafish. Moreover, data from both CPP and novelty-elicited stress tests show that, when combined, low doses of nicotine and ethanol produce augmented reinforcing and anxiolytic effects greater than that observed when administered alone. These findings suggest a synergistic interaction between the rewarding and anxiolytic effects of nicotine and ethanol and provide evidence of potentially two neurobiological systems that may underlie their co-dependence.

In the CPP experiments, the range of doses tested for ethanol and nicotine experiments produced inverted U-shaped dose-response curves. This classical relationship between drug

dose and effects is a common phenomenon in pharmacology studies (Calabrese and Baldwin 2001). In this study, the curves reflect an increase in CPP at lower doses and a decrease in CPP at higher doses. This indicates, for example, that as nicotine doses increase from 0.2 (0.01%) to 0.5 (0.025%) of a cigarette, its reinforcing effects increase such that zebrafish spend more time in the drug-paired chamber. However, after the maximum CPP effect is achieved for each curve, as nicotine's doses increase to a dose equivalent to a whole cigarette (0.05%), the magnitude of CPP begins to decrease. In Figures 3 and 4, the descending limbs of each curve extend below the x-axis, showing that at these higher doses, zebrafish begin to spend more time in the vehicle-paired chamber. Although these data are not significantly different to controls, the trend reveals that at these drug doses and combinations, zebrafish avoided the drug-paired chamber.

The exact mechanism by which a drug switches from reinforcing to aversive properties is not well understood and may depend on a variety of factors. For example, a single nicotine treatment can induce CPP (Belluzi et al. 2004) and high doses of nicotine fail to produce aversion in adolescent but not adult mice. The difference between nicotine's rewarding and enhancing effects may be due to sensitivity of nicotinic receptors and not dose as these behaviors in adolescent mice correspond to increased capacity compared with adult receptors (Kota et al., 2007). Although the mechanism by which these drugs can engender or preclude CPP are unknown, these findings suggest that the higher nicotine and ethanol + nicotine doses were aversive or not rewarding to zebrafish.

Most notably, the CPP experiments revealed that combining low doses of ethanol and nicotine produced reinforcing effects that were greater than the reinforcing effects engendered by either drug alone. Figure 4 shows that administration of nicotine shifted the ethanol dose-response curve to the right such that the ethanol dose (0.25%) that produced the least CPP at the base of the ascending limb of the ethanol curve produced the maximum effect on the curve when combined with nicotine. Subsequently, doses greater than 0.25% (including the dose that produced the maximum CPP when administered alone) produced significantly lower CPP in the descending limb of the curve. Since this low dose of nicotine was unable to produce CPP when administered alone, these results suggest that ethanol can potentiate the rewarding effects of nicotine, and is consistent with results from human studies in which ethanol was found to enhance the subjective rewarding effects of nicotine, e.g. smoking satisfaction (Rose et al. 2004).

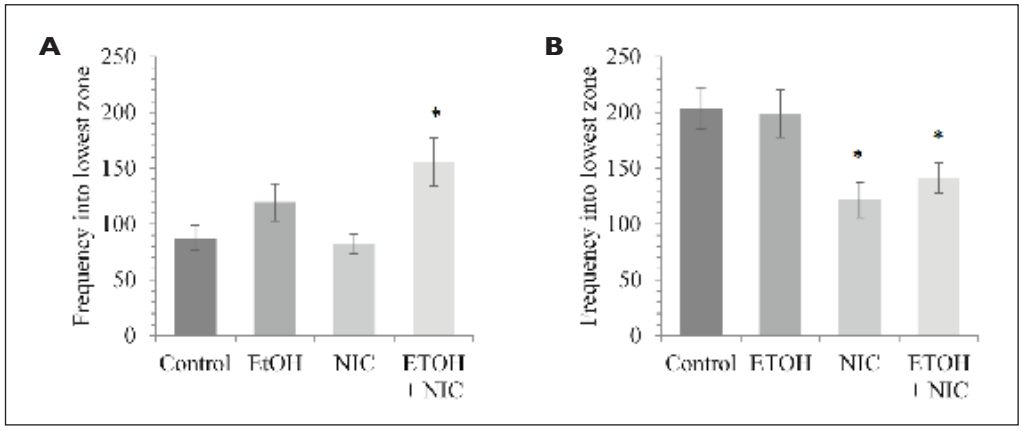

Figure 5. In the novelty-elicited stress test, the number of times zebrafish entered the lowest zone of the novel test tank following A) acute and B) chronic treatments (n = 10–15 per dose). Student's t-test, * $p < 0.05$ compared to control.

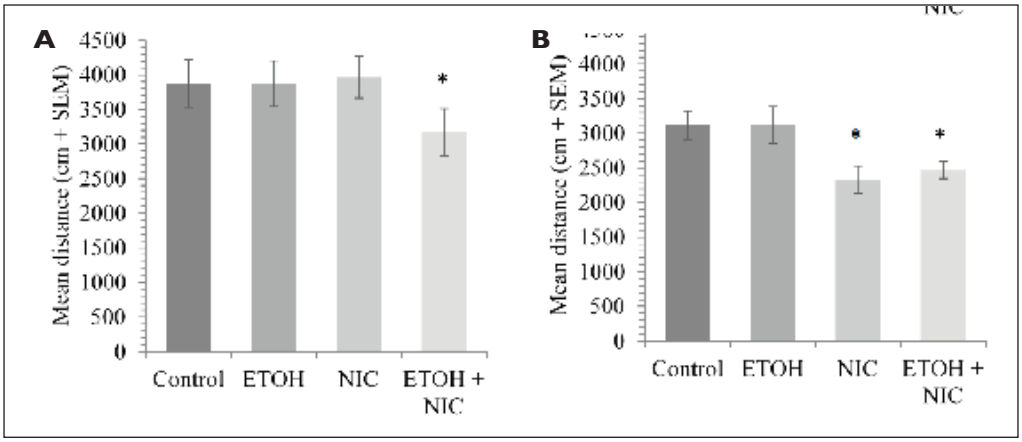

Figure 6. Total distance swuam in the novel test tank following A) acute and B) chronic treatments (n = 10–15 per dose). Student's t-test, * $p < 0.05$ compared to control.

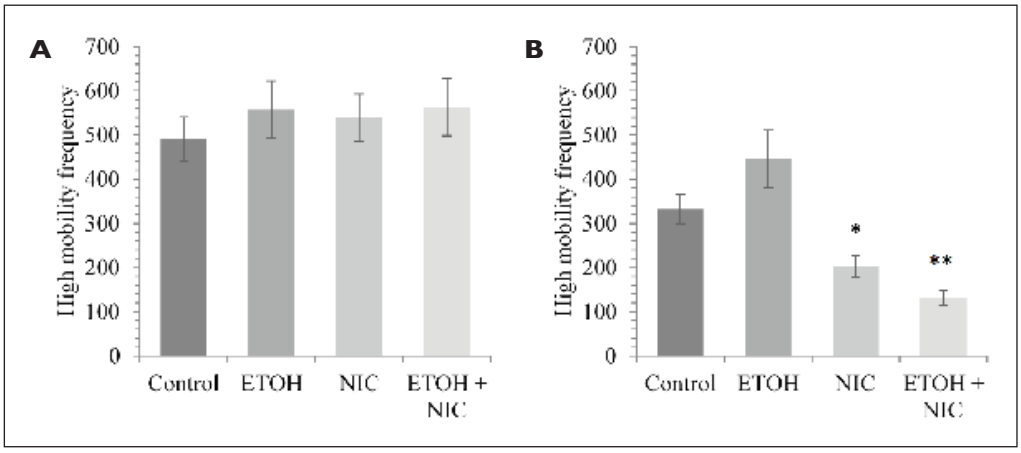

Figure 7. Number of times zebrafish exhibited high mobility (>60% in 6 seconds) in the novel test tank following A) acute and B) chronic treatments (n = 10–15 per dose). Student's t-test, * $p < 0.05$, ** $p < 0.01$ compared to control.

In the novelty-elicited stress response tests, chronic but not acute exposure to ethanol + nicotine combination significantly reduced species-typical stress responses in zebrafish compared to controls and to the same doses of ethanol alone. In our acute experiments, it was found that exposure to nicotine or ethanol produced stress responses that were not different from controls (Figs. 5A, 6A, and 7A). This finding is not surprising given that a low dose of each drug was selected in order to assess the drug combination effects and to prevent a ceiling effect when drugs were combined. Interestingly, administration of the drug combination significantly increased the frequency of entries into the lowest zone (Fig. 5A) suggesting that acutely, the drug combination potentiated anxiety in zebrafish. It is possible that the effects of the drug combination were stronger that individual drugs and thus inherently anxiogenic in addition to exposure to the novel test tank.

In contrast, the chronic experiments in which fish were exposed to drugs for six consecutive days revealed that exposure to nicotine alone and ethanol + nicotine combinations produced significant decreases in all of the stress responses examined—entries into the

lowest zone, high mobility episodes and distance swum (Figs.5B, 6B, and 7B). Furthermore, chronic exposure to the drug combination had an even more attenuated effect than all other drugs, decreasing high mobility episodes to a greater degree than nicotine and controls (Fig. 7B).

The finding that repeated exposure to nicotine and the ethanol + nicotine combination can alleviate stress responses suggests that these drug doses and combination may be anxiolytic. The finding that nicotine produces anxiolytic responses in zebrafish has been reported (Levin et al. 2007), whereas it has been found that high doses of ethanol can sometime produce anxiogenic responses (Gerlai et al. 2000). In light of this, since chronic ethanol + nicotine combinations reduced stress responses, it is possible that nicotine may have a role in attenuating ethanol's anxiogenic responses. Recent studies support our findings that exposure to ethanol + nicotine drug combination could produce overall anxiolytic effects due to the synergistic interaction of both drugs in low doses and that this synergy may be a result of prior exposure to ethanol (Truitt et al. 2015). If prior exposure is a factor in the anxiolytic effects of the drug combination, this may explain why the reduction in stress responses was observed following only chronic, but not acute combination treatments in this study. Considering the novelty-elicited stress test results and to the extent that the species-typical behaviors measured in this study are indicators of anxiety, the evidence suggests that the ethanol + nicotine drug combination may exert anxiolytic effects following long-term, repeated exposure.

Taken together, the CPP experiments and novelty-elicited stress tests provide evidence that in zebrafish, combining nicotine and ethanol can potentiate each other's effects to heighten reward and alleviate stress and anxiety compared to when taken individually. These findings are consistent with previous findings in rodents (Truitt et al. 2015) and demonstrate that zebrafish can serve as a suitable model to investigate the synergistic interactions between ethanol and nicotine.

Future studies will investigate potential mechanisms for alcohol-nicotine co-abuse. In particular, it will be informative to explore the interaction of these molecules at the mesolimbic dopaminergic system and the hypothalamic-pituitary-adrenal (HPA) axis in the brain. The mesolimbic dopaminergic system consists of the VTA, the nucleus accumbens, and the prefrontal cortex and has been implicated in the motivation to obtain a variety of rewards, including the positive reinforcement of alcohol and nicotine (Wise 1988). This is thus a neural substrate of importance for alcohol and nicotine interactions (Adams 2017, Deehan et al. 2015, Doyon et al. 2013). Intriguingly, it has been shown that dopamine neurons in the VTA spontaneously fire at increased rates when specific doses of ethanol and nicotine are present in vitro (Clark and Little 2004). While it is possible that the enhanced rewarding effects of alcohol and nicotine, due to enhanced dopamine release, could work in concert to alleviate stress, another possibility is the mutual recruitment of the hypothalamus-pituitary-adrenal (HPA) axis, which is associated with stress hormone signaling. The HPA axis is a strong candidate for drug interactions since it can be activated by drugs of abuse, including nicotine and alcohol during drug intake, withdrawal, and relapse to drug use (Sarnyai et al. 2001).

Beyond the common neural substrates at which these drugs act, two candidate molecules where ethanol and nicotine converge are nAChR and glucocorticoids, stress hormones. Human genetics studies with twin subjects have revealed that neuronal nAChR genes may contribute to the susceptibility to both ethanol and nicotine dependence (Lesson-Schlaggar et al. 2006). Both ethanol and nicotine can also trigger the release of stress hormones, corticosterone, which can in turn modulate dopamine release (Burke and Miczek 2014). In future studies we will be scouring the libraries of zebrafish mutants to utilize zebrafish

that have genetically engineered components of the glucocorticoid signaling system or varieties of nAChR subunit mutations. These tools will allow us to dissect existing genetic contributions to ethanol and nicotine co-dependence. Thus, using zebrafish to investigate these neurobiological substrates could reveal genetic and molecular mechanisms that act in concert to potentiate reward and regulate stress. Such investigations will elucidate shared interactions between alcohol and nicotine.

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