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Effects of Nicotine on Contextual Fear Conditioning in Adult Zebrafish (Danio rerio)

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Abstract

The goal of this experiment was to investigate the effects of nicotine in fear-conditioned zebrafish. Fear was analyzed by the presence of freezing, which was defined as the absence of movement for at least two consecutive seconds, excepting gill-related or ocular motions. Zebrafish have been used as subjects in many experiments to understand the effects of drugs on behavioral mecahnisms, but their memory processes have not been extensively studied. The present experiment investigated whether or not nicotine, given immediately after contextual fear conditioning, enhanced memory consolidation. Zebrafish were randomly categorized into two groups: placebo and high-dose nicotine (100mg/L). Individual fish were placed into the conditioning apparatus, where a Rigol DP832 programmable power supply administered shocks in a distinct environment. After conditioning, subjects were transported back to their home tanks. Reliable measurements were taken after conditioning to assess the effects of nicotine on emotional (fear) memory. Freezing was predicted to show a decrease in nicotine-exposed subjects in the original, yellow context. AquaScan behavioral tracking software and webcams measured behavioral correlates of fear, i.e. freezing. It was hypothesized that nicotine would decrease conditioned fear, resulting in shorter durations of observed freezing, when compared to placebo-treated subjects. The data suggested that there was a trend related to drug exposure and conditioning context, but the significance (p-value) was not great enough to conclude that there was a direct relationship between nicotine exposure and memory consolidation in this experiment.

Introduction

Memory is the ability to process information, store it, and recall it at a later time; these are respectively referred to as memory encoding, storage, and retrieval. In the brain, memory is first regulated by the thalamus and frontal lobe, which fire neurons that increase intensity of memories. The amygdala then uses emotion to increase attention to details. The stimuli are decoded by the cortex and hippocampus. Unfortunately, the way that things are remembered is not always accurate. Elaboration of a memory, time, re-consolidation, and environmental factors can influence the way a memory is encoded and consolidated. Memory can be stored short-term or long-term depending on the learning method: chunking, rehearsing, etc. Memory consolidation simply means a short-term memory has been coded into a long-term memory. Usually memories that are often needed or used frequently are stored as LTM, but this is not the case with individuals with PTSD. People with PTSD incorrectly transfer a STM event into LTM. One experiment that aimed to shed light on how nicotine affects PTSD memory issues involving the retrieval portion of traumatic memories was Gould and Nader's *Nicotine and Extinction of Fear Conditioning* paper. Retrieving memory should become more difficult as the memory fades over time, but those who have experienced trauma fail to let these flashbacks do so. One problem is that the role of neuroreceptors, specifically nicotinic acetylcholine receptors, on fear is not well understood. It was hypothesized in Gould's study that the nicotine-exposed subjects would exhibit less fear-like behavior: freezing. Their results supported this as they concluded that "acute nicotine's impairing effects on fear consolidation are associated with ventral hippocampal disinhibition. Therefore, these results further our understanding of the interaction between nicotine addiction and anxiety and stress disorders by describing novel neural mechanisms mediating fear consolidation" because previously the roles of nAChRs on fear were not well

mapped. Their study also found that several different brain regions were involved in fear consolidation.

Since freezing has been found to be a reliable behavioral correlate of fear in previous studies, our study went forth analyzing it as well. It was predicted that freezing would be significantly greater in the placebo group in the original conditioning context, where shocks occurred on the first day of testing, when compared to the nicotine-exposed novel context environment. Both groups should show more freezing in the novel conditioning context than in the conditioning tank, but the nicotine-treated group should still display the lowest amount of freezing. The novel blue tank was predicted to have more freezing because this color has been proven to stress the fish, while the yellow conditioning tank has no known stressful impacts on the fish (Wehner 2004). Contextual fear conditioning the zebrafish puts them in a stressful situation that should invoke PTSD symptoms. Once fish have been shocked in an environment, the fear behavior should appear when exposed to that environment again even when no shocks are administered. Exposing fish to high doses of nicotine after the traumatic event should block the fear behavior, however, because it disrupts the neurons in the brain that are attempting to store, or consolidate, information into long-term memory. This means that the nicotine treated subjects should display less freezing.

It is estimated that one in eleven people will be diagnosed with PTSD in their lifetime. Post-Traumatic Stress Disorder is a mental illness that can develop after an individual is exposed to a life-threatening or disturbing event or stimulus. PTSD is the result of incorrect storage of memories. The traumatic incident is consolidated and stored in various neurons that are primed together, or connected, throughout the brain (Elias et al 2010). Therefore, long-term memories are not stored in one single part of the brain, but rather are distributed throughout the cortex. An

individual with PTSD is unable to forget these memories that are dispersed throughout the brain in the form of neurons, which leads to an increase in fear-memory consolidation. When you cannot forget a memory, it is said that consolidation of the trauma is stored as a LTM. Memory consolidation is the opposite of memory retrieval difficulty. These stored consolidations appear as recollections or flashbacks, mood alterations, hyperarousal, and behavioral deficits. These are all common signs of post-traumatic stress disorder, too. Treatment options usually involve exposure therapy, cognitive-behavioral therapy, and eye movement desensitization and reprocessing. Combination therapy involving these methods, in addition to drug treatment, would be predicted to have the best results for patients. It is important to note that nicotine does not have the same effect as MDMA or steroids on the brain; they each use different pathways when administered (Fuduccia 2018). The most common way to study PTSD is through contextual fear conditioning of animals.

Contextual fear conditioning is a behavioral paradigm where subjects learn to associate certain environments with an aversive experience, resulting in the expression of fear responses. Fear conditioning mainly relies on the amygdala, which stores emotional memories, and the hippocampus, which regulates learning and memory. Fear conditioning is conducted in order to mimic the effects of individuals who suffer from post-traumatic stress disorder. Contextual fear conditioning can be influenced by prescription and nonprescription drugs, including nicotine.

Nicotine ($C_{10}H_{14}N_2$) is one of the most addictive and poisonous substances known to humans. Nicotine is a plant-based stimulant that causes ganglionic effects in the central nervous system, depending on the dosage; high dosages can block receptor signals, whereas low doses stimulate neurological effects such as transcriptional activity. Transcriptional activity is the process where DNA is converted into RNA, which is responsible for coding and regulating gene

expression. Nicotine acts as an agonist that interacts with nicotinic cholinergic receptors in the brain that are composed of RNA. When nicotine binds to nicotinic receptors, it causes cell depolarization, which triggers a calcium influx that triggers a release of adrenaline in the body. This is why an increase in blood pressure and elevation of heart rate are common side effects of nicotine ingestion. Nicotine can activate several different pathways in the brain, but most commonly it binds to nicotinic acetylcholine receptors (nAChRs), which can cause the release of dopamine through the SNARE complex (Whirl-Carrillo et al 2012). Even though this substance is known for its toxicity, research has shown that nicotine may have potential healing effects on the brain. Studies that focus on the relationship of smokers versus non-smokers and the development of Parkinson's disease, Alzheimer's, ADHD, and even schizophrenia are of increasing interest. These studies have found that increased exposure to nicotine after experiencing a traumatic event can influence memory consolidation.

Cotinine, which is the predominant metabolite of nicotine, has mixed findings. In some studies, it has been found to decrease consolidation of fear memory, while others have found it to increase consolidation. Scientific researchers, Kumari, Echeverria, studied the effects of cotinine in humans and mice. Kumari's research found that 12microg/kg of body weight of cotinine altered neural activity in humans and led to an arousal or enhancement of attention and consolidation. On the other hand, Echeverria's research found that cotinine decreased consolidation in fear-conditioned mice. In Echeverria's experiment, mice were fear-conditioned and had their corticosterone levels measured to establish a baseline. Corticosterone is a steroid-based hormone involved in the regulation of stress responses, including anxiety. "Cotinine did not affect corticosterone levels, but decreased the consolidation of contextual fear, decreased anxiety and the stability and/or retrievability of contextual fear memory.

Cotinine-treated mice showed higher levels of the active forms of ERK1/2 than vehicle-treated mice after FC. This evidence suggests that cotinine is a potential new pharmacological treatment to reduce symptoms in individuals with PTSD" (Echeverria 2012). This is believed to be the reason why smoking rates in individuals suffering from depression, anxiety, and posttraumatic stress disorder (PTSD) are much higher than in the general population. A normal person's memory would fade, but a person who has experienced trauma will continuously experience flashbacks and react with fear-like behaviors. Memory, therefore, does not fade as it normally would in traumatized individuals. Nicotine does seem to help reduce flashbacks, which is why this increase in usage is held by individuals suffering from PTSD, and other mental disorders, compared to the general population (Harvard Health 2014). Nicotine decreases consolidation of fear-memories differentially, which results in the renewal of fear being blocked (Kutlu 2015)**.** Since nicotine blocks fear-related memories and consolidation, it was predicted that in our study the results would indicate the high dose nicotine exposed fish would show less fear behaviors than the control group. Zebrafish were contextual fear conditioned over the course of three days to mimic effects of an individual who suffers from post-traumatic stress. After subjects were conditioned, the appropriate nicotine-exposed group was immediately immersed in 100mg/L of water for five minutes. The freezing duration was then analyzed and timed for all subjects.

Methods

Subjects

This experiment was approved by the Institutional Animal Care and Use Committee (IACUC) on October 09, 2019. The approval code is #2019-6. Naive adult male and female zebrafish (*Danio rerio*) were purchased from the Pet & Aquatic Warehouse (PAW) in

Lynchburg, Virginia. After their arrival in the behavioral neuroscience lab at the University of Lynchburg, zebrafish acclimated to their new environment for approximately two weeks. Forty-eight fish were housed in 10-gallon aquarium tanks (6 fish/tank) containing de-chlorinated, bacteria-treated water and kept at 78°F. Fish were fed daily with Tetra tropical flakes, and the light cycle was kept at 16 light:8 dark. Fish were randomly assigned to placebo (N=24) and to nicotine (N=24) groups.

Contextual Fear Conditioning

Following acclimation, subjects were placed individually into a 9.13" x 6.00" x 6.63" dimensioned conditioning tank containing 1000 mL of aquarium water at 78°F. Nicotine tartrate salt, purchased from Sigma-Aldrich, was added into the aquarium water for ionization. The inside walls and floor of the contextual fear conditioning chamber were painted in a matte canary yellow color with an acrylic, non-toxic paint. Stainless steel mesh-wire grids were attached to each side of the conditioning tanks and were submerged in the aquarium water. Electrodes were attached to the grids in the tanks and were connected to a Rigol programmable power supply. The conditioning tanks were surrounded by large sound-attenuating chambers (Colbourne Instruments) that had a hole cut in the top for video recording. Two overhead lights were turned on, the doors of the sound-attenuating chambers and conditioning room were shut to minimize noise, and shocks were administered: three separate shocks (20mA, 3-sec duration) were delivered one minute apart. An HD Logitech webcam (C920) recorded the subjects' movements and reactions. Subjects were in the conditioning tank as briefly as possible. Once they were placed in the tank, the doors were closed, video recording turned on, and shocks administered after 60 seconds. The water in the contextual fear conditioning tanks was changed after each conditioning session to decrease the effects of pheromones on behavior.

Drug Exposure

Immediately following contextual fear conditioning, subjects were transported via a net and plastic measuring cup to either placebo or nicotine-containing (100 mg/L) tanks for five minutes. Then, zebrafish were placed back into their home tanks.

Behavioral Testing

Twenty-four and forty-eight hours following conditioning and drug exposure, zebrafish were individually placed back into the fear conditioning context or a novel context. Behavioral testing, freezing, was counterbalanced with 24 and 48 hours following conditioning to prevent order effects. Half of the fish (n=24) were tested in the conditioning context first after 24 hours, while the other half (n=24) were tested in the novel context first. After 48 hours, the opposite group was tested first in the novel context, and the other group was tested in the conditioning context.The novel context consisted of a matte white painted tank with a light blue, textured flooring (kitchen shelf liner), which was filled with 1000mL of ionized aquarium water. Webcams monitored the precise movements of fish for two minutes because conditioned fear responses are generally seen within this timeframe (Kenney et al. 2017). Video recordings of the behavioral data were analyzed without the experimenter knowing the identifications of the two treatment groups. Time was recorded by the same researcher using a stopwatch. Subjects were recorded for the entirety of the experiment in both the novel and conditioning contexts. Their freezing was monitored and measured (see Figure 1 for differences in duration of freezing). For this experiment, the freezing fear-behavioral response was analyzed and compared to nicotine-exposed and placebo individuals. Freezing was defined as the absence of all movements, except for ocular and respiratory ones for at least five seconds (Curzon 1970). There was only one behavioral bout shown for each subject that was recorded, but the length of time

for each subject's bout varied. Fish show signs of fear behavior within the first few minutes of being placed in a stressful environment; therefore, only the first few minutes of swimming were analyzed for each fish in each conditioning chamber (n=96). Fish only showed one bout of freezing, which means that multiple bouts were not shown within the three minute analyses.

Results

A two-way, between-subjects design ANOVA test was run to statistically analyze the effects of nicotine exposure on freezing in adult *Danio rerio*. It was predicted that the nicotine-exposed subjects would show less fear-like behavior, or freezing, than the placebo group; however, no significant main effect of drug ($F= 2.180$, $p= .143$; nicotine vs. placebo) or context ($F= 1.55$, $p= .216$; conditioning vs. novel) was observed. Moreover, no significant interaction ($F= 3.63$, $p= .06$; drug x context) was found. The interaction of drug x context approached significance ("marginally significant"), which suggests that there was a tendency for freezing to be higher in the drug and placebo group in one context. As shown in Figure 1, the mean freezing in the placebo group was greater to the conditioning context compared with the novel context (18.1 vs. 3.7, respectively). Furthermore, the mean freezing to the conditioning context was greater in the placebo group compared with the nicotine treated fish (18.1 vs. 2.7, respectively). Because the drug x context interaction was not significant ($p= .06$), post hoc tests were not conducted (see Figure 1). Freezing was determined for both the control and experimental groups in the novel and conditioning contexts 24 and 48 hours after exposure. Since the p-value was not less than 0.05, the data were not statistically significant. No interaction effects seemed to be present. Error bars are indicative of the preciseness present when the experiment was conducted.

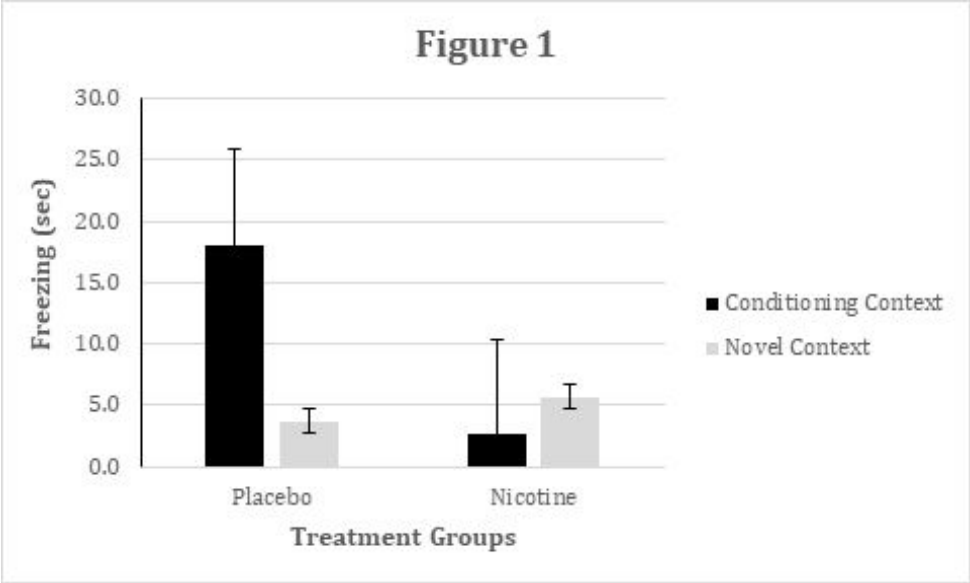

Figure 1. Average Duration of Freezing in Nicotine Exposed (n=24) vs. Placebo Subjects (n=24)

Discussion

The purpose of this study was to examine the effects of high-dose nicotine exposed zebrafish in both novel and conditioning contexts. It was predicted that the high dosage of nicotine exposure would correlate with a decrease in freezing duration: however, the data were not statistically significant and, therefore, could not support this hypothesis.

The present study supported the idea that nicotine does play a role in fear memory consolidation, but was only marginally significant. Previous research has found that nicotine has a differential effect on fear, meaning that the timing in which it is administered changes the results. When nicotine is given immediately after conditioning, less freezing is observed, but when it is given during exposure, more freezing is present. Prior studies found this information by rapidly assessing spatial discrimination in zebrafish that were either receiving a high-dose, low-dose, or no nicotine. The high dose groups showed improved accuracy in spatial awareness (Levin 2006).

There were limitations surrounding this experiment that could have impacted the data and results found. Only one experiment was conducted with fewer than 100 fish. Increasing the number of times the experiment was completed would have decreased the risk for Type II errors, and increasing the sample size would have led to a greater power (power =1-β) of the test performed. Other factors such as light exposure in the testing laboratory, temperature in the room, and variability in fear-behaviors (such as distance traveled) could have played a role in how the subjects responded to stimulus. The extraneous variables of temperature and lighting were kept as constant as possible for both the experimental and control group, so the variation seen in the conditioning context is not known. Additional experiments should be conducted to assess causes for the variation, as well as analyses of the distance travelled. Distance travelled and freezing would be predicted to have similar results, since both are common fear behaviors that can be measured. A baseline testing of fear for all subject groups was not conducted, which could have presented beneficial information about pre-existing fear in zebrafish. Baseline measurements should be measured, and other unknown confounding variables should be taken into consideration when studying fear in zebrafish in future studies.

In conclusion, fear is a natural response that occurs in all animals. The factors that impact fear, however, still remain open for interpretation because the p-value for our experiment was only marginally significant. Nicotine, trauma, and other elements (MDMA) can exacerbate the severity of duration of fear-like behavior, but the specific nAChRs that mediate nicotine and ethanol's effects on learning remain unknown. This study's hope was to investigate the findings of nicotinic effects in zebrafish that were contextual fear-conditioned. Eventually, there may be connections associated with how nicotine affects humans that suffer with PTSD in the same way that it affects fear-conditioned zebrafish. Rodents have been used in experimentations to draw

connections to humans for decades, thanks to Joseph and Charles Vacanti. Their experiment, which used a mouse to study plastic and reconstructive surgery, sparked the lab rat's journey as the 'go-to' scientific subject to use for testing. Unfortunately, there have not been many connections drawn between humans and fish. Zebrafish are more than 70% similar to humans genomically, so the paradigm developed between fish and humans would be a more helpful way to examine learning and memory than to compare mice and humans. Results from other studies, including Curzon, Kenney, Kutlu, Levin and Gould, have demonstrated that nicotine interferes with acquisition and consolidation of fear, as well as anxiety, through the modulation of specific subtypes of nicotinic acetylcholine receptors (nAChRs) in brain regions involved in emotion processing, such as the hippocampus. The direction of nicotine's effects on these behaviors is determined by several factors that include the length of administration, hippocampus-dependency, and source of anxiety. Overall, studies suggest that nicotine alters behaviors related to fear and anxiety and that nicotine contributes to the development, maintenance, and reoccurrence of anxiety disorders. Nicotine has the ability to desensitize and upregulate these receptors that affect memory, which is why those who suffer from PTSD have an inability to forget certain memories. Failure of consolidation can be treated, but is patient specific. Timing between intervention and the triggered event and the number of therapeutic sessions following, can either be helpful or harmful in the individual's recovery. Immediate, high-dose nicotine exposure was thus conducted to further grasp the relationship between timing, dosage, and responses in subjects who were exposed to trauma (high-voltage shocks). It was hypothesized that nicotine would decrease conditioned fear, resulting in less observed freezing, when compared to placebo-treated subjects. The data suggested a trend that less frequent freezing correlated with the drug-exposed zebrafish in the original context more often than the

control group in the novel context. However, the significance (p-value) was not great enough to conclude that there was a direct relationship between nicotine exposure and memory consolidation in this experiment.

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