HIGH SCHOOL EDITION
Journal of Student Research
Volume 11 Issue 3 (2022)

Attenuation of Alcohol and Nicotine Induced Behaviors in *Drosophila melanogaster* by Sinomenine

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ABSTRACT

The purpose of this experiment is to determine the efficacy of Sinomenine, an opioid receptor antagonist, in attenuating ethanol and nicotine induced behaviors in Drosophila melanogaster. While ethanol and nicotine are quite different in their direct mechanism of action, current research suggests that opiate reward, CREB-mediated transcription, and long-term potentiation are common pathways implicated in the development of various addictions. Due to its ability to antagonize the mu-opioid receptor, Sinomenine prevents endorphin binding, drug-induced CREB transcription, and consequent increase in dopamine production by tyrosine hydroxylase that leads to euphoria and addiction. Sinomenine may be particularly promising over other opioid receptor antagonists for sedative addictions due to its opioid-receptor independent anxiolytic effects. In a CAFE assay performed to measure preference through consumption of ethanol or nicotine, Sinomenine reverses both naive and conditioned preference for ethanol, as well as conditioned nicotine aversion. Measuring locomotion, Sinomenine attenuated both the ethanol and nicotine induced decreases in negative geotaxis exhibition in Drosophila melanogaster. Quantifying olfactory preference, Sinomenine reversed conditioned odor preference in a Y-maze, but not naive preference. Reflecting its effects on the CREB pathway, Sinomenine inhibited a nicotine-induced increase in cAMP, but did not significantly affect cAMP levels in ethanol treated Drosophila. According to these results, Sinomenine is particularly effective in attenuating conditioned ethanol or nicotine-induced behavior and produces mixed results in terms of naive exposure. The natural chemosensory preferences or aversion of ethanol and nicotine may be independent of the CREB pathway, while developed preference may be directly dependent on it.

Statement of Purpose

The purpose of this experiment is to determine the efficacy of Sinomenine in attenuating specific ethanol and nicotine induced behaviors. In each assay, the negative control is 1-5 day old wild type *Drosophila* which have been exposed to ethanol or nicotine through various means, and the independent variable is whether a group is treated with Sinomenine or a positive control (naltrexone or 3iY). In the CAFE assay, the dependent variable is the amount of yeast, sucrose, and ethanol medium consumed. In the negative geotaxis assay, the dependent variable is the number of *Drosophila* which cross a 5cm line after acute exposure to volatilized nicotine or ethanol. In the y-maze assay, the dependent variable is the percentage of Drosophila in the ethanol or nicotine odor compartment. In the secondary messenger ELISA assay, the negative control are supernatants of Drosophila fed ethanol or nicotine containing medium for two days and not treated with Sinomenine, while the dependent variable is the amount of cAMP (cyclic adenosine monophosphate) pmol/50ul of supernatant, which was converted from absorbance units by the microplate reader.

Background

Drugs of addiction include depressants such as alcohol and benzodiazepines, stimulants such as nicotine, methamphetamine, and cocaine, and opioids such as morphine and fentanyl. Approximately 22.7 million Americans (8.6%) have an addiction to drugs or alcohol which will only continue to worsen throughout the opioid crisis. Over the course of the last six years, the synthetic opioid-involved death rate increased 1,040%, from 1.0 to 11.4 per 100,000 American citizens (Mattson, 2021). These various types of drugs elicit a direct response through different pathways. Alcohol addictions are caused by multiple pathways including GABA receptor activation as an agonist, endogenous opioid release, and glutamate receptor inhibition as an antagonist. Cocaine addiction involves the blocking of presynaptic dopamine transporters, as well as a neuroinflammatory response caused by activation of TLR4 and opioid receptors (Northcutt, 2015). Nicotine causes an increase in dopamine in the nucleus acumbens and is associated with endorphin release and upregulation of the transcription factor CREB (Walters, 2005). Whether it is through direct binding or an indirect release of endogenous opioids, many drug addictions involve the activation of opioid receptors secondary to dopamine release to cause euphoria. Currently, treatments for various addictions often overlook the role of opioid receptors involved in depressant and stimulant addictions. Additionally, various addictions have few pharmacotherapy treatments at all such as nicotine, and those which do, such as cocaine, do not have treatments that inhibit the ability to achieve euphoria but rather rely on reducing withdrawal symptoms to prevent relapse, which depends on a certain degree of will (Kampman, 2005). In the case of opioid addictions, certain treatments such as methadone are addictive themselves, and hence are problematic in light of the current opioid epidemic.

Development of substance use disorders and physiologic dependence is based on neurological memory and reward systems. In humans, the Mesolimbic Dopamine Pathway primarily consists of dopaminergic and glutamatergic transmissions between the nucleus accumbens, the ventral tegmental area, and the hippocampus (S. Blaess, 2020). The main receptors involved in these transmissions are NMDA and AMPA (glutamate) and D1-D5 (dopamine) receptors. Glutamate is essential to most processes as it is the most prevalent excitatory neurotransmitter and is involved in other neurotransmitter releases. It is especially important in studying addiction as it plays a role in memory, learning, and the excitation during dopamine release associated with drug euphoria as well as long term potentiation (Meldrum, 2000). The pathways in which different drugs create different stimulations in the brain are distinct in response, yet have overlapping preliminary pathways that result in the ability for the drug to access that specific system and cause a response. Opioids bind to MOR (mu opioid) receptors on dopaminergic neurons to induce dopamine release (Shang, 2015). Current medications to treat opioid addiction and relapse focus on competitive antagonists such as methadone and naloxone which bind to mu-opioid receptors (MOR). Similarly, Sinomenine, an alkaloid derived from an east asian root, has been shown to reduce relapse by decreasing levels of tyrosine hydroxylase and NMDA receptors by inhibiting mu-opioid receptors through the CREB phosphorylation pathway (Jinying, 2018). TH is an enzyme which catalyzes tyrosine into L-dopa, a precursor to dopamine. Recent studies indicate that the down regulation of TH occurs through decreased activation of a transcription factor known as CREB. Increased levels of cAMP allows for the activation of PKA (protein kinase A). This is important for CREB, a transcription factor, as PKA phosphorylates CREB, allowing it to bind to the enhancer region, CRE (Kida, 2012). This process enhances the genes downstream of CRE, including TH. By downregulating TH, drugs that cause addiction by activating dopaminergic neurons will not be able to stimulate a euphoric response due a decrease in available dopamine, causing decreased propensity for relapse. Additionally, Sinomenine has analgesic properties as evident by its current primary use in treating rheumatoid arthritis (Wei, 2020). This could be beneficial for patients suffering from sedative addictions, as it may simultaneously help treat their addictions as well as subside their anxiety.

The effect of Sinomenine on the CREB pathway is tied to its inhibitory effect on opioid related LTP. In LTP, synapses become stronger through the frequent activation of neurons (Lüscher, 2012). In glutaminergic neurons, NMDA receptors allow calcium to pass through when a glutamate ligand is bound. The increase in intracellular calcium concentration will cause a cascade of compensational reactions to increase the amount of NMDA and AMPA receptors, allowing for more calcium to enter the cell as it is activated more frequently. This process is involved across

different drug addictions such as opioid and alcohol dependence, and the increase in the amount of receptors is what causes the increase in synaptic strength and ability to create action potential. While opioids generally do the opposite in the spinal cord in response to pain as they inhibit calcium receptors, in the brain, LTP results in increased receptors and synaptic strength, leading to hyper polarization rather than depolarization of neurons (Evans, 2016). In the past two decades, various studies have shown that the increase in calcium and NMDA receptors in LTP causes the activation of calmodulin-dependent protein kinase II (CaMKII), which also phosphorylates CREB, allowing for the upregulation of downstream genes such as TH.

Another common factor underlying drug addictions are secondary messengers and transcription factors, particularly cAMP response element-binding protein (CREB). Both dopamine receptors and Mu-opioid receptors belong to the family of G-protein coupled receptors and their stimulation results in the activation of the effector adenylyl cyclase, a cAMP producing enzyme (Chan, 2016). Cyclic amp activates protein kinase A, which can phosphorylate multiple transcription factors, including CREB. The transcription factor CREB is an enhancer which binds to the CRE promoter region and upregulates downstream genes. The binding of CREB causes the upregulation of various proteins, enzymes, and receptors, primarily the enzyme tyrosine hydroxylase and NMDA receptors (Wang, 2018). Tyrosine hydroxylase is an enzyme which catalyzes the amino acid tyrosine into the preliminary catecholamine for dopamine, L-Dopa. Tyrosine hydroxylase has been implicated in different pathologies and healthy pathways alike for its regulation of dopamine such as in Schizophrenia and Parkinson's Disease. More relevantly, dopamine is the primary neurotransmitter implicated in all drug addictions and its increase or transporter inhibition causes feelings of euphoria. NMDA receptors are less directly involved in stimulating euphoric aspects of addictions and are more related to memory pathways such as long term potentiation (LTP), which plays a role in the memory of the euphoria elicited by drugs.

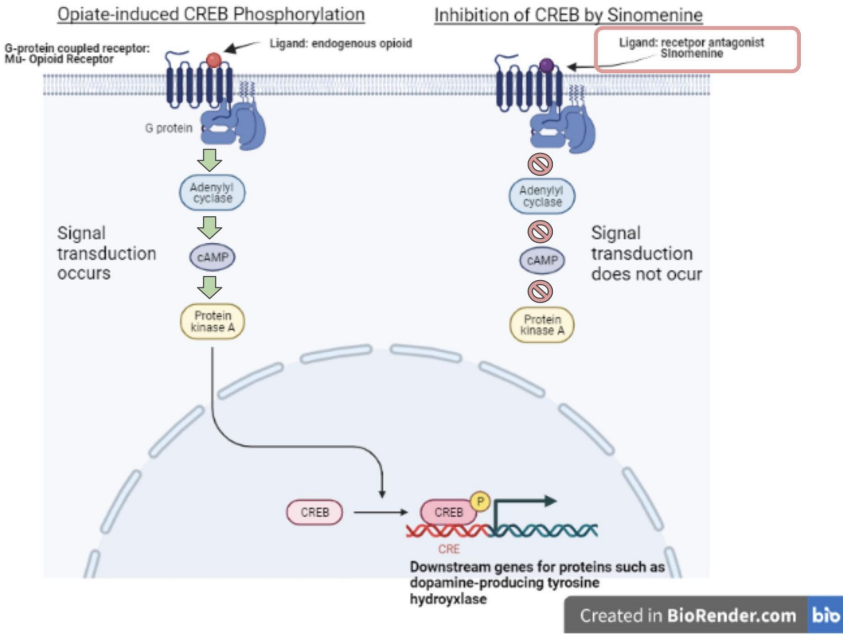

Figure 1. cAMP/PKA/CREB pathway and inhibition by Sinomenine. Endorphins bind to the mu-opioid receptor, causing a signal transduction resulting in increase in tyrosine hydroxylase synthesis. Receptor antagonist by Sinomenine prevents upregulation of p-TH by CREB.

Many treatments for psychostimulant and depressant addictions are specific to the direct pathway that is unique to the addiction. Nicotine, a psychostimulant, exerts its effects on the brain by binding to neuronal acetylcholine nicotinic receptors (nAChRs), allowing for an influx of calcium, which depolarizes the neuron. Depolarization activates ion-gated channels, increasing the amount of intracellular calcium, which contributes to an intracellular cascade

that will increase dopamine production, causing the rewarding effect of nicotine (Timwari, 2020). Ethanol binds to multiple receptors to induce its effect including GABA, acetylcholine, serotonin, and NMDA receptors. Most research pertaining to current drug treatments for addictions function on the direct surface level in which drugs interact with their receptors. For example, the current treatment for nicotine is bupropion, which can bind to 3 types of acetylcholine receptors and inhibit nicotine's bond to these receptors (Fernandez, 2007). This has its limitations as bupropion does not bind to all 7 nAChRs as nicotine does, and in alcohol addictions, until recently, the case for pharmacotherapy has been relatively low considering there are multiple pathways in which ethanol can induce its effect. However, recent research has revealed that secondary messenger systems persist across various types of drug addictions involving endogenous opioid reward pathways as a factor contributing to the addiction. Referring back to opioid reward pathways, protein kinase A is a cAMP-dependent kinase that is involved in synaptic plasticity and gene transcription. Cyclic AMP is produced by adenylyl cyclase, which can be activated by many different g-coupled receptors including dopamine and opioid receptors. Recent studies reveal that activation of D1 receptors, which are involved in acute drug reward and conditioning, stimulate cAMP signaling, while D2 receptors, which are not implicated in drug reward and conditioning, do not affect cAMP signaling (Koob, 2018). These last studies reveal the critical role of the secondary messenger cAMP in the development of addiction. Further, PKA-mediated transcription, which begins with the activation of PKA by cAMP, is also responsible for calcium permeability in NMDA receptors, which would affect the formation of LTP between neurons (Murphy, 2014). Drugs such as methamphetamine, ethanol, nicotine, cocaine, and opioids are associated with varying increases in cAMP levels throughout prolonged exposure. New terminology has even been created to describe the superactivation of adenylyl cyclase dubbed as "cAMP overshoot," and is a primary marker in determining opioid addiction in research models (Chan, 2016).

When studying treatments that may be applicable to multiple addictions, understanding interactions between different pathways caused by binding at different receptors, and how receptor antagonists may halt these pathways is key to inhibiting the effects of drugs of addiction. Recent studies propose that across various addictions, the activation of endogenous opioid reward systems and long-term potentiation are the hallmark pathways of addiction. The activation of both of these pathways involve multiple transcription factors, primarily CREB. What is promising about these findings is that pharmacotherapies which target opioid receptors may be applicable to other addictions including stimulants and depressants. Two such drugs are naltrexone and Sinomenine.

Naltrexone was initially approved by FDA in 1984 as a medication to treat opioid use disorder (OUD), but recently in 2006 became approved for treatment of alcohol use disorder (AUD) as well. Naltrexone can bind to all three types of opioid receptors: mu-opioid, delta-opioid, and kappa-opioid receptors. Mu-opioid receptors are the primary receptors implicated in various addictions and cause analgesia, euphoria, sedation of the central nervous system, and respiratory depression and constipation (Law, 2013). Delta-opioid receptors cause hallucinogenic effects and kappa-opioid receptors cause dysphoria. While naltrexone had historically been administered on an empirical means of successfully attenuating alcohol addictions rather than an in depth understanding of its pathway, studies revealed that alcohol induces the release of endogenous opioids which then bind to opioid receptors to cause euphoria and addiction (Swinford, 2012). Additionally, nicotine also stimulates the release of endogenous opioids, primarily nociceptin (Xue, 2007). While many years have passed since its completion, a study conducted in the 1980's by Karras and Kane revealed that the opioid receptor antagonist naloxone reduced nicotine and tobacco cravings (Karras and Kane, 1980). A follow up study revealed that naltrexone acted similarly to naloxone in decreasing cravings for nicotine and tobacco in chronic smokers (King, 2000). Previous studies have shown that mu-opioid antagonists such as naltrexone and naloxone have been effective in attenuating substance addictions that did not involve exogenous opioids.

Sinomenine, a far less researched substance, is an alkaloid derived from the plant *Sinomenium acutum* and has immunosuppressive, analgesic, sedative, and anxiolytic-like effects. Studies have shown that it binds to the mu-opioid receptor, acting as an antagonist (Wang, 2008). Additionally, Sinomenine is not addictive (Zhou, 2004). The analgesic, sedative, and anxiolytic effects of Sinomenine are independent of its action of mu-opioid receptors (Wang, 2008). Similar to other treatments for opioid use disorders, Sinomenine is an antagonist of mu-opioid receptors. This means that it will competitively inhibit endogenous opioids from binding to opioid receptors to produce euphoria. As

previously mentioned, ethanol causes a release of endorphins (opiates) to produce euphoria and nicotine as well to a lesser degree. The inhibition of mu-opioid receptors would prevent the pleasurable feelings associated with alcohol and nicotine addictions. Additionally, through the inhibition of mu-opioid receptors, treatment with Sinomenine should regulate the increase in cAMP caused by ethanol or nicotine exposure. The promising aspects of Sinomenine as an alternative to current drug treatments involve its properties as an analgesic and anxiolytic effects independent of opioid receptors, as well as its ability to regulate the effects of drug addiction in both the euphoric dopamine reward pathways as well as long term potentiation, which most drugs are limited to one. In a recent study conducted on morphine dependent mice and morphine treated neuroblastoma cells, Sinomenine inhibited morphine induced conditioned place preference, the expression of genes coding for NMDA receptors, CAMKII, and CREB, decreased levels of cyclic AMP in morphine treated SH-SY5Y cells, and decreased intracellular calcium in the SH-SY5Y (Jinying, 2018). Even more recently, a study involving zebrafish yielded similar results as Sinomenine decreased conditioned place preference by morphine in zebrafish by mediating tyrosine hydroxylase and NMDA receptors, specifically the NR2B subunit, in the zebrafish brain (Lin, 2021). These new studies support the theory that Sinomenine, traditionally a treatment for rheumatoid arthritis, could not only be a viable treatment for opioid addictions as an antagonist to mu-opioid receptors, but also attenuate other drug addictions by its ability to regulate tyrosine hydroxylase and NMDA receptor upregulation by the cAMP/PKA/CREB pathway.

In the present study, wild type *Drosophila melanogaster* is used as the model organism for alcohol and nicotine addiction. While seemingly distant from humans, the *Drosophila* genome is 60% homologous to the human genome and 75% of genes responsible for human diseases have homologs in *Drosophila* (Mirzoyan, 2019). *Drosophila* are an optimal model organism for their fast reproductive maturation, short generations, and relatively simple maintenance requirements. *Drosophila* have four life stages: embryo, larva, pupa, and adult. The embryonic stage lasts about two days while the larval and pupal stage take about four days each (Hales, 2015). The larval stage is divided into first, second, and third instar stages. In the first and second instar, the larva is developing and burrows into a medium for nutrients. In the third instar, they prepare for pupation and climb the side of the vial (Hales, 2015).

Regarding neurological studies, adult *Drosophila melanogaster* have a developed nervous system consisting of 100,000 neurons (Scheffer, 2020). Their nervous system consists of many of the same neurotransmitters in humans including serotonin, dopamine, GABA, glutamate, octopamine, tyramine, which are all implicated in alcohol-induced behaviors (Chvilicek, 2020). More importantly, the cAMP/PKA/CREB pathway is fully preserved in *Drosophila*, as well as long term potentiation, which has been identified as being localized within the mushroom body of the adult *Drosophila* brain (Ueno, 2013). Tyrosine hydroxylase is preserved and coded by the *ple* gene, and the cAMP Response Element-Binding Protein is shortened to *Drosophila* homolog of *dCREB2* (Brody, 2018). Regarding Drosophila's natural sensory preferences, which could affect behavior in acute drug exposure assays, *Drosophila* have a natural affinity to ethanol as fermentation of fruits, their primary food source, increasingly releases ethanol as they ripen (Pohl, 2012). Previous studies have shown that *Drosophila* will seek ethanol consumption even when needing to overcome an electric shock to do so (Kaun, 2014). While Drosophila's aversion to the taste of nicotine makes self-administration assays difficult, various studies reveal that nicotine induces hyperlocomotion, which is regulated by dopamine and the cAMP/PKA/CREB pathway (Morris, 2018). One limitation of *Drosophila melanogaster* as a model organism for this experiment is the lack of established research confirming opioid induced behavior. However, previous studies reveal that naltrexone can dose-dependently reverse ethanol preference in *Drosophila* through a CAFE assay (Koyyada, 2018). Established research demonstrates that naltrexone's mechanism of action in alcohol addictions is directly related to its antagonism of mu-opioid receptors. The significance of the study reveals *Drosophila* as having certain responses to mu-opioid receptor antagonists. Therefore, naltrexone is a proper control for this experiment to parallel Sinomenine's efficacy as they are both mu-opioid antagonists and naltrexone has effectively attenuated alcohol preference in *Drosophila*.

A number of assays to quantify addictive and drug-induced behaviors in *Drosophila* have been developed and peer reviewed as consistent means of determining the effects of pharmacotherapies on conditioned *Drosophila* subjects. The CAFE assay is a quantitative assay which involves using capillaries to administer liquid medium to

Drosophila test subjects in a vial (Diegelmann, 2017). Capillaries of different mediums can be used to determine preference, and exposure to certain mediums prior to transfer to capillary feeding vials can be used to induce developed preference.

Negative geotaxis is a behavioral trait exhibited by *Drosophila melanogaster* in which they have the natural tendency to fly against gravitational pull (Ali, 2011). This is a particularly useful trait when studying drug-induced sedation and locomotor impairment. The knock-down assay consists of conditioning *Drosophila*, in this case to ethanol or nicotine, tapping a test vial three times against a lab bench to knock the *Drosophila* to the bottom, then counting the number of flies which cross a 5cm line in ten seconds as standard exhibition of negative geotaxis (Ali, 2011). Dopamine modulates many locomotor activities in drosophila. In an early study which involved nicotine and ethanol volatilization prior to a knockdown assay, 3iY effectively attenuated the decrease in negative geotaxis induced by ethanol and nicotine, and hence, is a suitable positive control for negative geotaxis assays involving ethanol and nicotine (Bainton, 2000).

Y-maze assays quantify olfactory memory and learning by isolating odors which *Drosophila* subjects had previously been exposed to. *Drosophila* are exposed to volatilized ethanol or nicotine, then placed in a Y-maze with two compartments, one with a control and the other with the odor they were previously exposed to. After being acclimated within the apparatus for one day, the number of *Drosophila* in each compartment are counted. Y-mazes are placed in a room only lit by Far-red LED lights to control visual cues (Simonnet, 2014).

Hypotheses

Across the four assays, *Drosophila* pre-treated with Sinomenine will exhibit reduced preference for ethanol or nicotine, corrected negative geotaxis, and basal levels of cyclic AMP as opposed to those not treated with Sinomenine. In conditioned trials for the CAFE and Y-maze, *Drosophila* are exposed to either ethanol or nicotine containing medium or vapor whereas in naive trials they are not.

If *Drosophila* are placed in vials containing two capillaries, one with a control solution and the other with ethanol or nicotine, then naive and conditioned Drosophila treated with Sinomenine will have decreased preference for the ethanol or nicotine containing solution.

If *Drosophila* are pre-treated with Sinomenine prior to exposure to volatilized ethanol or nicotine, Drosophila pretreated with Sinomenine will exhibit negative geotaxis in greater numbers than those not pretreated with Sinomenine.

If *Drosophila* are exposed to volatilized ethanol or nicotine and later placed in a Y-maze, those pretreated with Sinomenine will exhibit decreased olfactory preference for the ethanol or nicotine odor.

If *Drosophila* are placed in vials with ethanol or nicotine containing medium, transferred to vials containing Sinomenine, flash frozen, decapitated, and used in an ELISA assay, drosophila treated with Sinomenine will exhibit lower levels of cAMP than those exposed to ethanol or nicotine and not treated with Sinomenine.

Materials

Drosophila melanogaster wildtype; Formula 4-24® Instant Drosophila Medium; Nutri-Fly® Drosophila Agar, Gelidium; Sinomenine; Naltrexone solution; 3-Iodotyrosine; Ethanol (190 proof); Nicotine; Digital; milligram scale; Hot plate; Stir bar; 25, 100, and 250ml graduated cylinders; 80ml, 100ml, and 500ml beakers; Y-maze kits designed and constructed for *Drosophila melanogaster:* Polypropylene y-connector, Micropipette tips, Foam flugs, Cotton flugs,

Drosophila vials; Capillary feeder apparatus, Drosophila vials, Cotton flugs, Capillary tubes, Micropipette tips, Parafilm wrap; Volatilization and geotaxis apparatus, Conical flask, High temperature tote box, ¼" Plastic tubing (x2), Battery powered water pump, 10ml glass pipette tips; Digital caliper; Cold centrifuge; Microcentrifuge; Pellet Pestle; 1X Phosphate buffer saline; Liquid nitrogen; cAMP Assay Kit (Competitive ELISA); Colorimetric microplate reader**.**

Methods

General Procedures

Safety Protocols

The procedural process is separated into preparation of mediums and treatments, three behavioral assays, and a cAMP ELISA assay. Proper BSL-2 and COVID-19 guidelines regarding social distancing, face coverings, hand washing, and disinfected benches to name a few will be followed in a BSL-2 lab, as well as proper ventilation of volatilized ethanol and nicotine under a fume hood. 190 proof ethanol and nicotine were stored in the BSL-2 lab.

The following chemicals's MSDS sheets describe certain hazards: Nicotine, Ethanol, Hydrochloric Acid, Sinomenine Hydrochloride, Naltrexone, Acetic Acid, Propionic Acid, Rabbit Anti-cAMP polyclonal Antibody, Acetylating Reagent A, Acetylating Reagent B

The following hazards are listed pertaining to the above chemicals:

- Acute Toxicity - Dermal
- Eye Damage/Irritation
- Flammable Liquids
- Corrosive to Metals
- Aquatic Hazard (Long-Term)
- Skin Corrosion/Irritation

The following safety precautions are taken:

- A long-sleeved lab coat, long pants, and nitrile gloves will be worn to prevent any contact between the chemicals and the researcher's skin.
- Face shields will be worn during the volatilization of ethanol and nicotine, and the procedure will be carried out under a fume hood to ventilate the vapor.
- To prevent eye damage, lab goggles will be worn at all times
- Flammable liquids will be stored in a dry and well-ventilated space, away from any sources of ignition.
- Disposal of aquatic hazards will be carried out by placing in a sealed container in a biohazard bin and disposed of by a registered biohazard waste disposal company.

Use of each chemical:
Ethanol: used in CAFE solution, nutri agar medium vials for conditioning, and volatilization
Nicotine: used in CAFE solution, nutri agar medium vials for conditioning, and volatilization
Sinomenine: used in nutri agar medium vials for treatment and pretreatment
Hydrochloric acid, acetic acid, propionic acid: used in preservatives of nutri agar.
Naltrexone: positive control used in nutri agar medium vials for treatment and pretreatment
Anti-cAMP Polyclonal antibody, Acetylating Reagent A, Acetylating Reagent B: cAMP quantification with ELISA kit.

Model Organism

About 2-5 day old wildtype *Drosophila melanogaster* were used in each of the assays. Age controlling was performed using grape juice agar. *Drosophila* laid eggs in the trap juice agar containing Petri dish, which was then washed with PBS and then the larva were transferred to Formula 4-24® Instant Drosophila Medium vials. Active yeast was added to instant medium vials to promote mating.

Vial Preparation

The CAFE, Y-maze, and ELISA assay require vials of ethanol and nicotine containing medium to established conditioned behavior. For conditioning vials, the concentrations used were 15% ethanol and .01mM of nicotine. These were mixed during the process of making nutri agar, which entails adding 87g of Nutri-Fly® Drosophila Agar to 500ml of water and mixing until even over a hot plate. Additionally, for treatment vials, concentrations of .1% naltrexone, .1% Sinomenine, or 10mg/ml of 3iY were mixed with nutri agar, and flies were transferred either for pretreatment or post-conditioning.

Pretreatment

2-5 day old *Drosophila melanogaster* are transferred to vials containing .1% Sinomenine, .1% naltrexone, 10mg/ml of 3iY, or control vials for 48 hours

CAFE Assay

Apparatus Construction

A damp cotton flug is placed at the bottom of a 25 x 95mm Drosophila vial to maintain humidity. Two holes are then formed in a different fitted cotton flug using a probe. Two 100-300 ul micropipette tips are placed into each cotton flug. Lastly, a 100mm glass capillary is tightly fit into each micropipette tip. After loading with solution, wrap the tip in parafilm to prevent evaporation.

Ethanol solution preparation

To create 100 ml of 15% ethanol, 5% sucrose, and 5% yeast extract solution, pour 75ml of distilled water and mix with 15ml of 190 proof ethanol. Then, weigh, pour, and mix 7.95g of sucrose and 7g of yeast extract until the solution is even.

Nicotine solution preparation

To create 100 ml of 0.01 mM nicotine solution, .1uL of nicotine solution is mixed with 89.5ml of distilled water. Then, Weigh, pour, and mix 7.95g of sucrose and 7g of yeast extract until the solution is even.

Assay Protocol

2-5 day old *Drosophila* are transferred to nutri agar vials for two days containing either ethanol and nicotine solution, or a control (no drug conditioning for the naive groups). *Drosophila* are then transferred to treatment or control vials for 24 hours. Nutri agar contains .1% naltrexone, .1% of Sinomenine, or no treatment for the control. *Drosophila* are then transferred to the CAFE vials; however, the capillary tubes are not inserted for 3 hours to allow for metabolism of the treatment. One capillary containing ethanol solution or nicotine solution is inserted into a pipet while a control capillary only containing 5% sucrose and 5% yeast extract is inserted into the other of each CAFE apparatus. The amount of medium inserted into each capillary is marked with a black line. The tips of each capillary is fixed with parafilm to prevent evaporation. 24 hours after capillary insertion, the amount of medium consumed is calculated using a digital caliper, aligned to the space between what is left of the medium and the black line. To convert to preference, the amount consumed from the ethanol capillary is taken as a percentage of the total amount of medium

consumed between the two capillaries.

Negative Geotaxis Assay

Drosophila are treated with Sinomenine, 3iY, or neither according to pretreatment procedures. A two holed-stopper is placed into a conical flask and situated under a fume hood.Two 10ml glass pipette tips are fixed into the stopper. A small electric air pump is connected to one of the pipet tips using a plastic tube. The pipet tip must reach near the bottom of the flask without touching it. The other pipet tip is connected to a vial containing pretreated or control-medium fed *Drosophila melanogaster* and a black line drawn 5cm from the bottom. *Drosophila* are transferred to vials over ice an hour prior to volatilization exposure. A solution of 190 proof ethanol or a 2% nicotine solution is poured in the conical flask until the solution meets the end of the first pipette. The conical flask is placed in a heat-resistant container of boiling water under the fume hood and turn on the electric pump for 60 seconds. The vial is removed from the apparatus and immediately tapped against a small cloth on the lab bench three times to knock the *Drosophila* to the bottom of the vial. The number of *Drosophila* which cross the standard 5cm line drawn on the vial are counted to evaluate the locomotive effects of ethanol and nicotine.

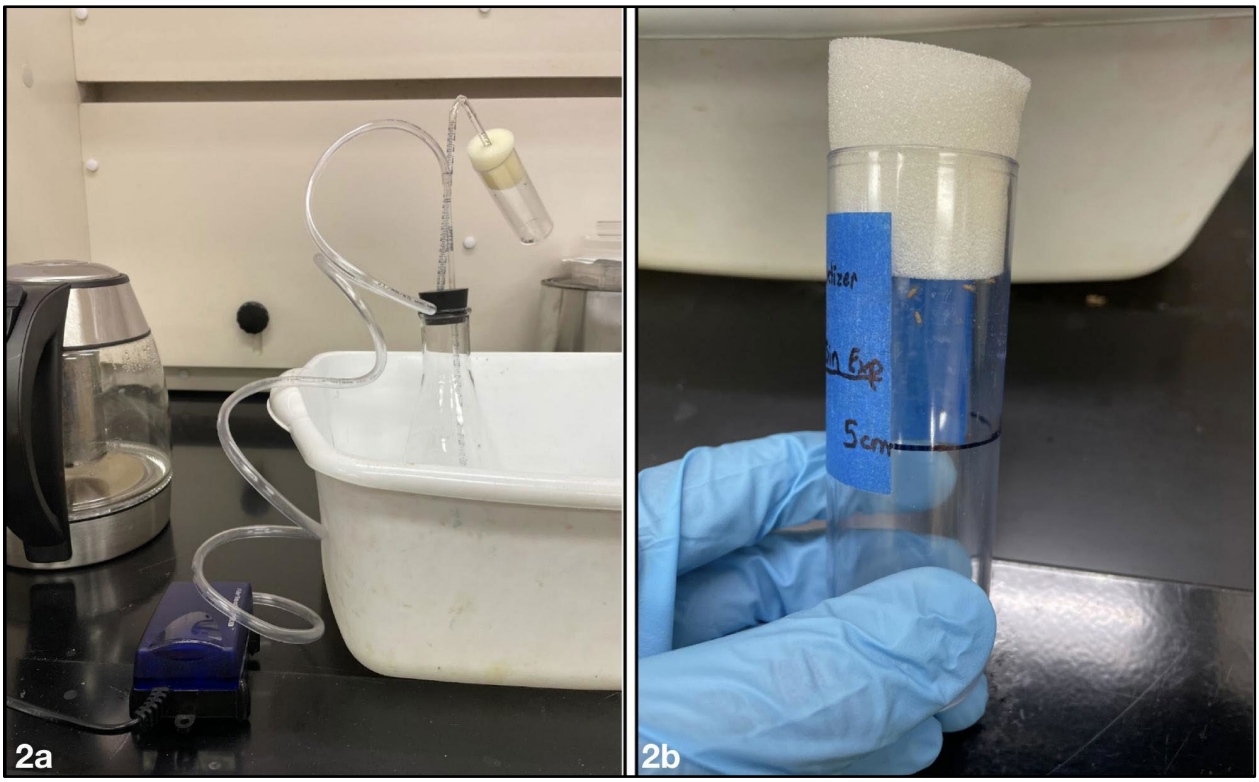

Figure 2. Volatilization apparatus under a fume hood (2a) and a sample vial (2b)

Olfactory Preference Assay

Volatilization Conditioning

Exposure to volatilized ethanol or nicotine was performed under the same procedure listed under Negative Geotaxis steps 1-6. For this particular assay, this procedure is repeated four times over the course of 48 hours.

Apparatus Construction

Three micropipet tips are fixed into each end of a polypropylene y-connector using parafilm. Each pipet is cut roughly 2cm from the tip, or the appropriate distance to allow *Drosophila* to fit through the pipet tip. A foam flug is fit around each pipet tip. The bottom of a cotton flug is soaked for each y-maze with 1ml of either ethanol or nicotine and fixed to the bottom of a vial, soak another with 1ml of water and place in a second vial. The bottoms instead of tops are soaked to prevent toxicity. The vials are labeled as which is the drug and which is the control for each maze.

Assay Protocol

Drosophila are pretreated with Sinomenine, naltrexone, or neither according to pretreatment procedures. Over the course of 48 hours, *Drosophila* are transferred to the volatilization apparatus 4 times. Throughout the 48 hours, *Drosophila* are transferred back into treatment or control vials. After 4 sessions of exposure, *Drosophila* are transferred to the y-mazes. The y-mazes are placed under far-red light to control visual cues. After 24 hours in the y-mazes, the number of *Drosophila* are counted in each compartment. To calculate preference, calculate the number of *Drosophila* in the drug odor vial over the total number which chose a vial (deduct those which remained in the initial vial from total).

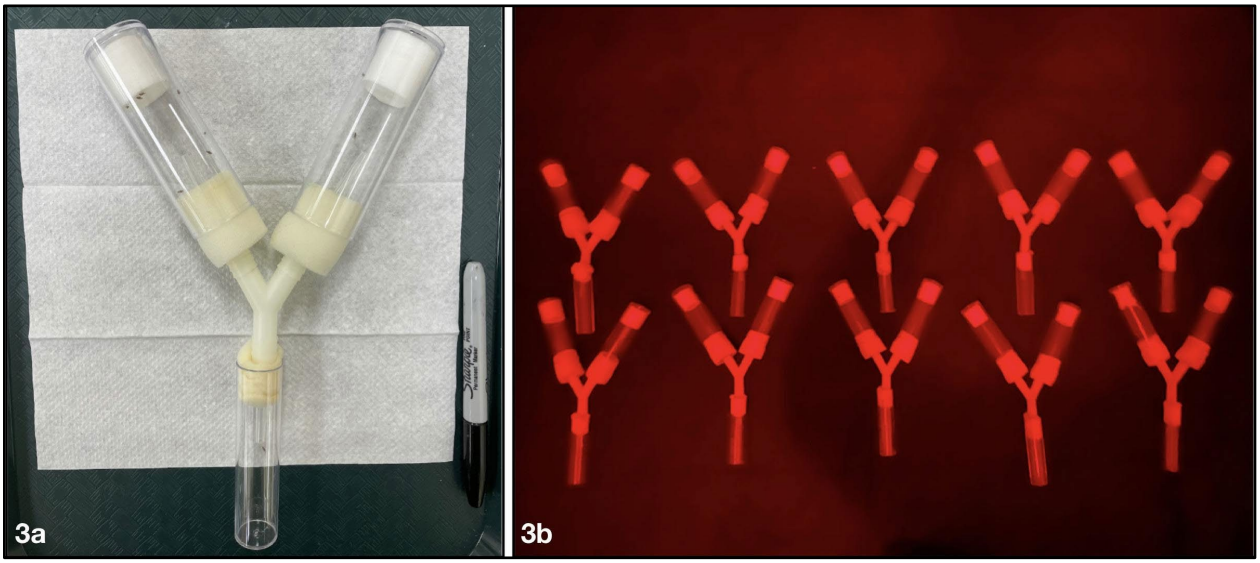

Figure 3. Trial sample from Y-Maze (3a) and a full group under far-red LED light (3b)

ELISA cAMP Quantification Assay

Sample Preparation

2-5 day old *Drosophila melanogaster* are transferred to conditioning vials containing the solutions of ethanol, or nicotine, or control medium for 48 hours. 25-30 *Drosophila* are used per vial. *Drosophila* are then transferred to treatment vials containing 0.1% naltrexone, 0.1% Sinomenine, or control medium for 48 hours. Vials are then placed in a -86 °C freezer to freeze *Drosophila*. Full bodies are then transferred to microPCR tubes and vortexed in liquid nitrogen to knock off heads and appendages. Empty out contents on a paper towel to separate manually. Heads are separated from the rest of the contents and placed back in the microPCR tubes. Drosophila heads are homogenized using a pellet pestle. 300 ul of 1XPBS are pipetted into PCR tubes and vortexes for 10 seconds each. MicroPCR tubes are placed in 50ml centrifuge tubes and fitted using paper towels. PCR tubes are weighed to ensure balanced weight for centrifugation. Centrifuge tubes are placed in a cold centrifuge for 15 minutes at 4 degrees celsius and at 15,000 RPM. Using the micropipette, 200 ul of supernatant are pipet into new PCR tubes.

ELISA Protocol

Directions provided by Abcam for the ab65355 cAMP Direct Immunoassay Kit were followed for reagent preparation, standard preparation, acetylating reaction mix, and cAMP quantification.

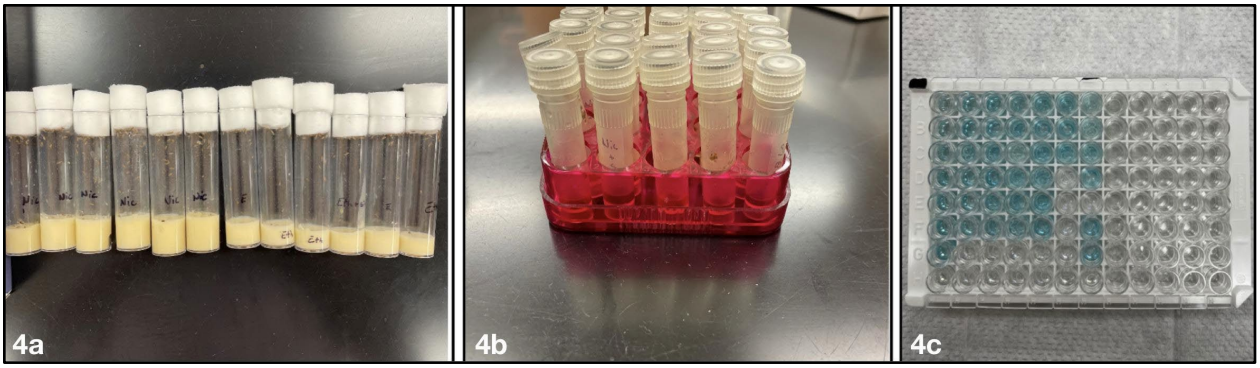

Figure 4. Drosophila drug conditioning in nutri agar (4a), cAMP standards (4b), and cAMP ELISA after HRP Developer (4c). The cAMP ELISA contained two identical standard curves. G6 contains a researcher error so A6, a copy of the same standard, was used instead.

Data Analysis

Two-way anovas are used to determine statistical differences between wildtype, treated with Sinomenine, and positive control groups to analyze if Sinomenine decreased the effects of ethanol or nicotine. To determine whether there are statistical differences between experimental groups (Sinomenine treatment) and the positive control (Naltrexone or 3iY treatment) or experimental groups and the negative controls (Ethanol or Nicotine without drug treatment), one way T-Tests are performed between respective groups.

Statistical Analysis and Graphs

Significance of Sinomenine					
Exposure	Drug	CAFE	N.geotaxis	Y-Maze	ELISA
Naive	Ethanol	1	N/A	0	N/A
	Nicotine	0	N/A	0	N/A
Conditioned	Ethanol	1	N/A	1	0
	Nicotine	0	N/A	1	1
Acute	Ethanol	N/A	1	N/A	N/A
	Nicotine	N/A	1	N/A	N/A

Figure 5. Summary of significance between Sinomenine treatment and both naive and conditioned control groups across the four assays. 1 indicates significance following a one-tailed t-test while 0 indicates no significance. Naive entails no prior conditioning followed by prolonged exposure, conditioned is conditioned prior to prolonged exposure, and acute is no prior exposure and short exposure time to induce behavioral effects.

DISCUSSION

Conclusions

Across multiple assays, Sinomenine was able to attenuate certain behaviors which were a result of conditioning, as well as behaviors induced by acute exposure. These results may indicate Sinomenines efficacy specifically in preventing the pleasurable effect caused by certain drugs, and hence would be used to prevent drug-induced euphoria in relapses. Sinomenine's ability to correct negative geotaxis in Drosophila, specifically in the case of acute drug exposure, may indicate potency for competitive antagonism in cases of overdose. Further research involving fast-action after exposure rather than using Sinomenine pretreatment would support such a hypothesis. The investigation utilizes established frameworks for quantifying drug induced behavior in Drosophila and applies it to a novel combination of Sinomenine as treatment and both a depressant and a stimulant as addictions to demonstrate its efficacy in attenuating various drug induced behaviors as a prospective pharmacotherapy.

The CAFE assay results are depicted on Figure 6. It is able to observe the effects of Sinomenine on both naive (not conditioned) preference as well as conditioned preference of ethanol and nicotine. The *Drosophila* conditioned to ethanol displayed significantly higher preference for the ethanol-containing medium than those not conditioned ($p < 0.05$). Treatment with Sinomenine significantly decreased ethanol preference in the ethanol conditioned group ($p < 0.05$). Additionally, Sinomenine significantly decreased ethanol preference in the naive group ($p < 0.05$).

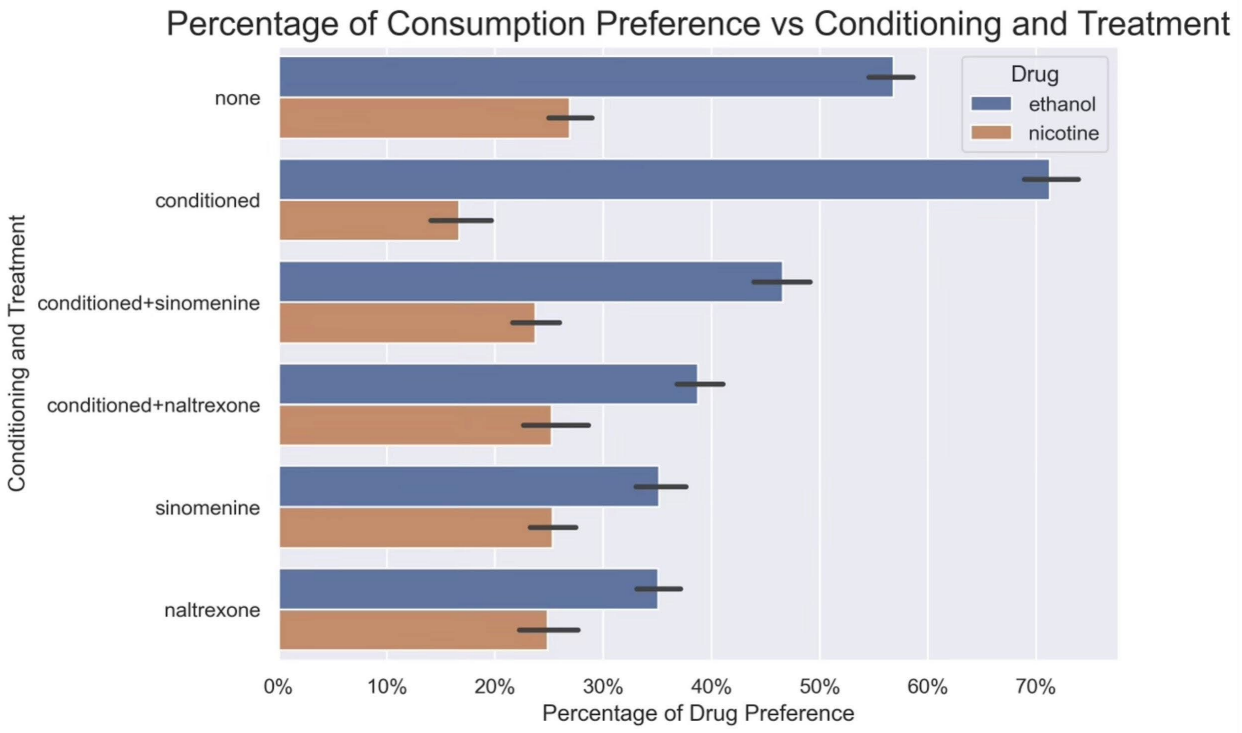

Figure 6. Depicts the results of the CAFE assay. There is a significant difference ($p < 0.05$) within experimental groups: conditioned+Sinomenine (eth) & conditioned (eth), conditioned+Sinomenine (nic) & conditioned (nic), and Sinomenine (eth) & none (eth). These comparisons reveal Sinomenine attenuates conditioned and naive ethanol preference and unexpectedly reverts nicotine aversion.

These results suggest that, as expected, *Drosophila* do have a preference for ethanol, even when not conditioned to it. This is probably affected by various factors, including natural preferences as well as the rewarding effects of ethanol consumption. In nature, *Drosophila* are naturally attracted to the odor and taste of ethanol as certain fruits, their primary food source, ferment, increasingly releasing ethanol. Additionally, the rewarding effects of ethanol can still be implicated in the non-conditioned group to a lesser extent than the conditioned group. The release of dopamine and endorphins following ethanol consumption leads to continuous desire to drink the ethanol containing-medium. The data supports that Sinomenine reverses ethanol preference similar to naltrexone, and in the proposed mechanism that it blocks the binding of endorphins as naltrexone does. What is unexpected is that naive Sinomenine treated flies did not show significant difference in ethanol preference to naive naltrexone treated groups, but there was a significant difference between the two groups when conditioned to ethanol. Sinomenine is effective in attenuating preference; however, possibly to a lesser degree than naltrexone. What is more likely is a concentration of 0.1% is sub-optimal for a Sinomenine treatment solution. Previous studies have revealed that 0.1% concentration of naltrexone in yeast and sucrose medium has been optimal in reversing ethanol preference, and thus using the same concentration of Sinomenine allows for direct comparison of efficacy.

Conversely to ethanol, *Drosophila* conditioned to nicotine displayed significantly lower preference for nicotine compared to naive *Drosophila* ($p < 0.05$). Further, this effect of conditioning leading to further aversion of nicotine was reversed by treatment of Sinomenine as the Sinomenine treated group displayed significantly greater preference than the non-treated group ($p < 0.05$). Similar to ethanol, *Drosophila melanogaster* natural preferences and aversions to certain substances affects self administration. *Drosophila* exhibit an aversion to bitter alkaloids such as caffeine, and as an alkaloid, nicotine as well. What is promising about these results is Sinomenine's ability to reverse the conditioned aversion, yet not affecting naive aversion. The data supports that naive *Drosophila* will avoid nicotine regardless of Sinomenine intervention, however, Sinomenine reversed the levels of aversion similar to the initial aversion without conditioning. This supports the hypothesis that Sinomenine regulates long term potentiation and memory stimulated by drug exposure. The flies conditioned to nicotine were not affected by the learning and memory effects of nicotine exposure and addiction as it was inhibited by Sinomenine treatment.

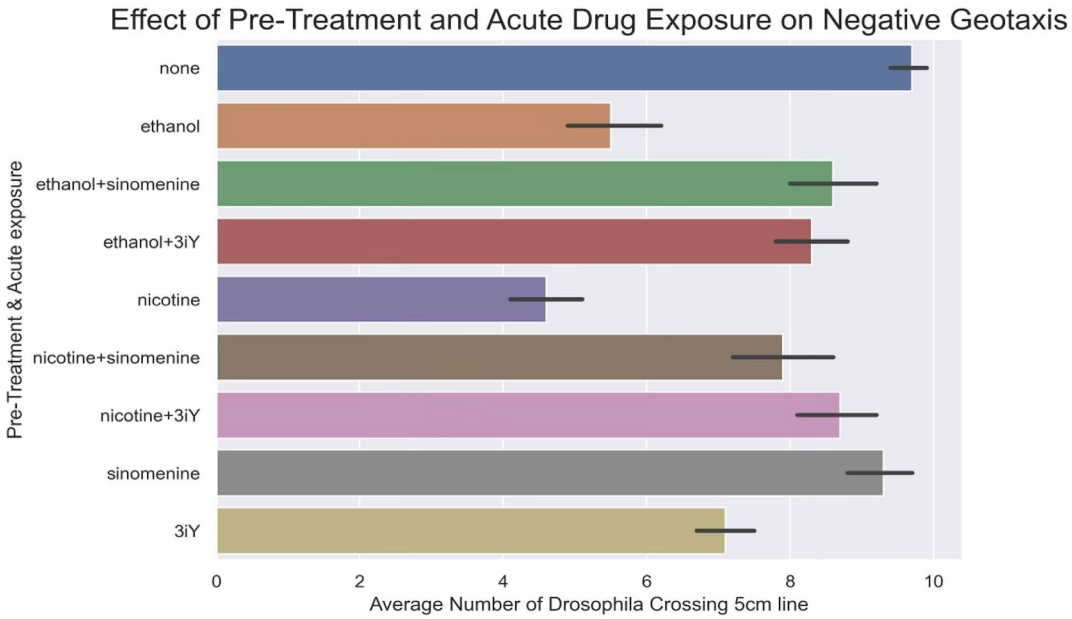

Figure 7. Depicts the negative geotaxis assay. There is a significant difference ($p < 0.05$) within experimental groups: ethanol+Sinomenine & ethanol and nicotine+Sinomenine & nicotine. These comparisons reveal that Sinomenine reverts the inhibition of negative geotaxis by ethanol and nicotine, attenuating their locomotor effects.

Depicting the data collected during the knockdown assay, Figure 7 illustrates the acute effects as opposed to conditioned preference of ethanol and nicotine through vapor exposure using a volatilization apparatus to induce sedation and locomotor impairment by ethanol and nicotine. Acute exposure to both ethanol and nicotine significantly reduced the negative geotaxis response in *Drosophila* subjects as expected ($p < 0.05$, $p < 0.05$). The graph supports the claim that Sinomenine reverses the sedative and locomotor effects of ethanol and nicotine as Sinomenine treated *Drosophila* displayed significantly increased rates of the negative geotaxis as an average of the total number of flies ($p < 0.05$, $p < 0.05$). Further, the difference in Sinomenine's inhibition of both ethanol and nicotine and intervention of 3iY was insignificant ($p > 0.05$, $p > 0.05$). However, anova statistical analysis followed by 1 and 2 tailed two sample t-tests of the effects of Sinomenine and 3iY on negative geotaxis in *Drosophila* independent of acute exposure to ethanol or nicotine reveals that 3iY significantly reduces exhibition of negative geotaxis in *Drosophila* while Sinomenine does not. This supports prior studies which revealed that Sinomenine regulates and prevents the dopamine increase following opioid receptor activation, but does not affect basal dopamine levels. 3iY does in fact affect dopamine levels regardless of drug exposure as it directly inhibits tyrosine hydroxylase function. Regardless of whether it is a stark increase or decrease, irregular levels of dopamine affect locomotor ability in *Drosophila melanogaster*.

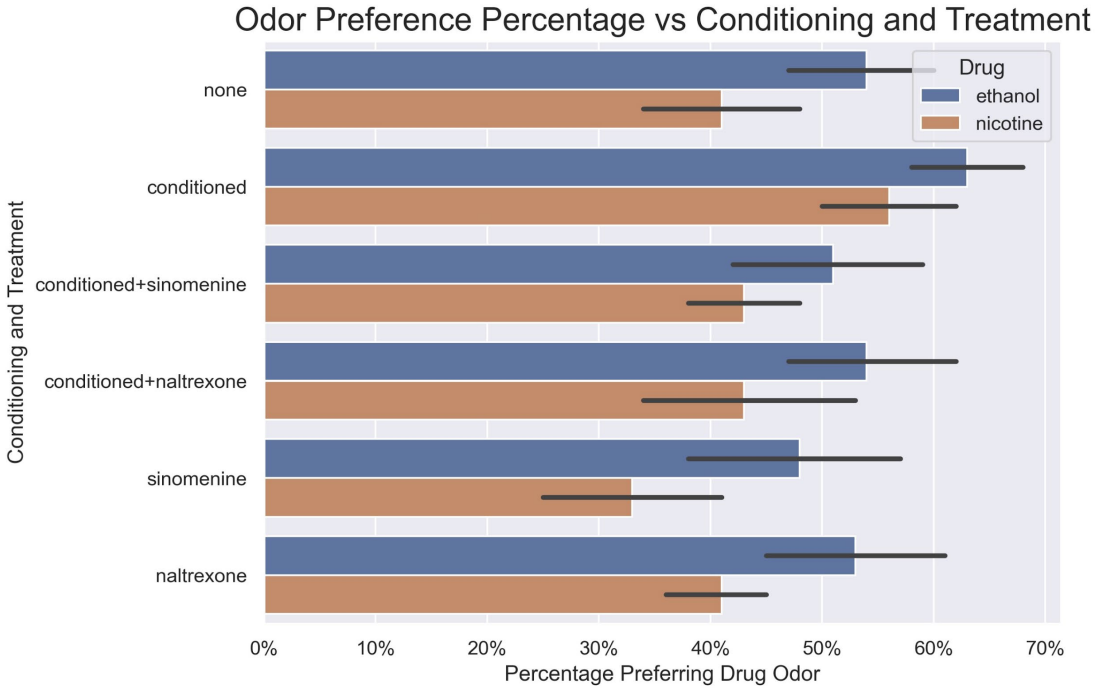

Figure 8. Depicts y-maze assay. There is a significant difference ($p < 0.05$) within experimental groups: conditioned (eth) & conditioned+sinomeine (eth), conditioned (nic) & conditioned+Sinomenine(nic). However, there is no significant difference ($p > 0.05$) within the following groups: none (eth) & Sinomenine (eth) and none (nic) & Sinomenine (nic). These findings indicate that Sinomenine has ameliorative effects on conditioned odor preference to ethanol and nicotine but not naive preference.

To demonstrate Sinomenine-induced inhibition of olfactory learning associated with repetitive exposure to volatilized ethanol or nicotine, Figure 8 displays preference percentage among flies exposed to ethanol or nicotine odor in a y-maze. There is no significant preference for nicotine odor among naive *Drosophila*; however, there is significant preference for ethanol odor among naive flies ($p > 0.05$, $p < 0.05$). Additionally, conditioning of both groups significantly increases odor preference ($p < 0.05$, $p < 0.05$). The chart displays no significant difference between naive *Drosophila* in the ethanol maze and naive *Drosophila* treated with Sinomenine in the ethanol maze.

However, the data does indicate that Sinomenine significantly decreases the conditioned increase in both ethanol odor preference as well as conditioned nicotine preference ($p < 0.05$, $p < 0.05$). A possible explanation for these observations may again lie in natural sensory preferences in *Drosophila*. *Drosophila* encounter ethanol in nature and seek it to find nutrients whereas they are not readily exposed to nicotine. However, nicotine exposure does condition *Drosophila* to seek nicotine odor in a Y-maze, even though they do not naturally have a preference for it. This data is consistent with the negative geotaxis assay in that pretreatment with Sinomenine affects the drug-induced behavior of nicotine and ethanol. While the negative geotaxis assay displays that Sinomenine prevents ethanol and nicotine from inducing their effects in *Drosophila*, the y-maze data displays Sinomenines ability to correct preference as well, and not just induced behavior. This is consistent with consumption preference of ethanol in the CAFE assay, but its effects on the rewarding properties of nicotine were previously limited by sensory taste preference, which is negated by the vapor conditioning and odor preference in the y-maze assay.

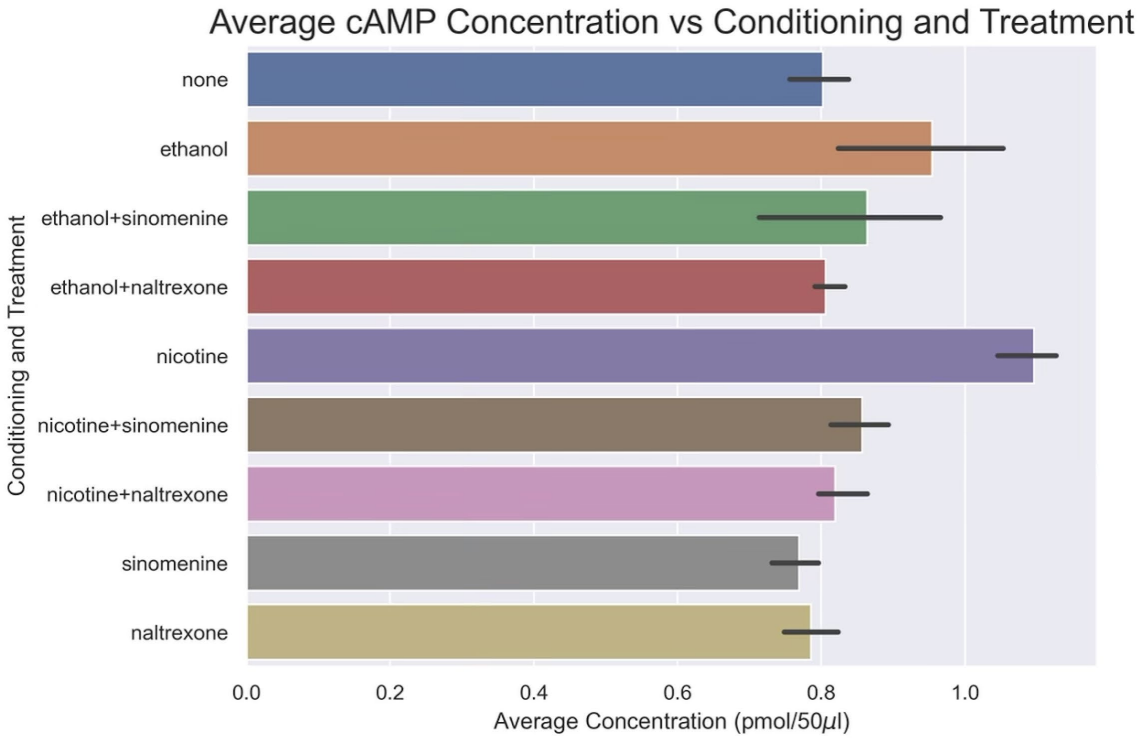

Figure 9. Depicts cAMP ELISA microplate assay. There is a significant difference ($p < 0.05$) between nicotine & nicotine+Sinomenine. However, there is no significant difference ($p > 0.05$) between none & ethanol and ethanol & ethanol+Sinomenine. The former results indicate that Sinomenine decreases the nicotine-induced increase in cAMP while the latter results may have occurred either due to researcher error as indicated by the high standard error or by developed tolerance to ethanol, which is discussed in the conclusions section.

Figure 9 displays the differences in cAMP levels detected in supernatants drawn from *Drosophila* tissue lysate after consumption of ethanol or nicotine containing medium, followed by treatment with Sinomenine or naltrexone. The graph depicts an increase in cAMP levels following untreated ethanol consumption. However, Anova statistical analysis reveals that this increase, along with the Sinomenine and naltrexone treated groups, exhibited no significant difference in cAMP levels ($p > 0.05$). Conversely, nicotine significantly increases cAMP levels comparative to the negative control ($p < 0.05$). Further, Sinomenine reverses this increase and significantly reduces cAMP levels in nicotine-conditioned *Drosophila*. The lack of a significant increase in the ethanol groups may be explained by an increase in tolerance throughout 48 hours of consumption. Cyclic AMP levels are expected to increase among

initial consumption of ethanol, but as tolerance increases, ethanol consumption decreases cAMP levels rather than increase them. However, ethanol did not decrease cAMP levels significantly either as would be expected in the case that tolerance was induced. If *Drosophila* had only recently become tolerant to ethanol within the hours prior to homogenization, then it is possible that the cAMP levels decreased only a small amount before they were homogenized, preventing a significant decrease in cAMP levels from being present in the lysate. Nicotine conditioning increased cAMP levels as expected. Consistent with the finding of the negative geotaxis and y-maze assay, Sinomenine reversed the effects of nicotine conditioning, in this case, on levels of cAMP.

The data collected in the four assays reveals that Sinomenine's ability to attenuate drug induced behavior in *Drosophila* is affected by natural preferences of *Drosophila*; furthermore, when this is controlled for, Sinomenine reverses drug-induced behavior by ethanol or nicotine. In the CAFE assay, Sinomenine attenuated ethanol preference in both naive and conditioned *Drosophila*. While *Drosophila* have a natural aversion to the taste of nicotine, the data collected suggest that Sinomenine disrupts the memory of nicotine exposure, and thus reverts aversion to nicotine to naive levels. The geotaxis assay allows for measurement of drug-induced effects of ethanol and nicotine independent of sensory preference. Sinomenine reversed the effects of acute exposure to ethanol and nicotine and attenuated drug-induced locomotor impairment in Drosophila. Previous studies have shown that negative geotaxis in drosophila is mediated by both cAMP/PKA/CREB and dopamine pathways. These previous studies further support the use of a negative geotaxis assay as a means of identifying a connection between the CREB pathway, drug-induced behaviors, and Sinomenines mechanism of action. The consistency with the results of the negative geotaxis assay and previous studies involving the aforementioned pathways highlights the specific, proposed mechanism of action of Sinomenine, and how it may be effective in treating drug addictions. The y-maze assay includes preference without relying on the sense of taste, and hence is an appropriate assay to pair with the CAFE and knock-down assay. While the data reveals that naive Drosophila still have natural preferences for ethanol and lack preference for nicotine, which is unchanged by Sinomenine pre-treatment, conditioning of both drugs led to increased preference of each. Sinomenine did not affect the lack of preference in naive drosophila in the nicotine maze; however, in groups conditioned to nicotine, Sinomenine pretreatment did reverse the preference caused by conditioning. The same results were reflected in ethanol trials, barring the fact that Drosophila do show preference for ethanol odor, which is unaffected by treatment with Sinomenine. The cAMP ELISA assay attempts to highlight the mechanism in which Sinomenine may reverse drug-induced behaviors in the previous assays. Inhibition of mu-opioid receptors would prevent increases in cAMP following receptor activation by endorphins. Sinomenine significantly reversed the increase in cAMP induced by nicotine consumption. Regulation of cAMP to basal levels affects both the reward system as well as locomotor ability as seen by the geotaxis and y-maze assay. The ELISA further supports the findings of the geotaxis and y-maze assay in regards to amelioration of nicotine-induced preference and locomotor impairment. The insignificant increase in cAMP by ethanol could possibly be due to a mix of factors, most probably related to development of tolerance leading to decrease cAMP levels. However, the lack of a significant decrease comparative to the negative control leaves further uncertainty about the probability that the result was due to development of ethanol tolerance.

Applications

The increase in nicotine abuse following the popularity of e-cigarettes, the exponential increase in opioid-abuse related deaths, and steady increases in alcoholism throughout the past two decades reveal a growing need for improved pharmacotherapies and behavioral treatments for substance use disorders. Certain substance use disorders do not have FDA approved pharmacotherapies and in some cases such as methadone for opioid use disorder, pharmacotherapies can be addictive themselves. Past research has indicated that Sinomenine can be used to inhibit drug induced behavior, particularly opioid addictions in mice and zebrafish. However, this study is particularly novel in that it investigates the efficacy of Sinomenine in attenuating ethanol and nicotine induced behavior rather than that of opioids. This rationale is supported by previous studies which indicate that other substance use disorders are mediated by opiate

reward pathways, as well as common transcription factors, particularly CREB, which are implicated in the development of multiple addictions such as alcohol, nicotine, methamphetamine, and heroin.

The use of a combination of behavioral assays and a secondary messenger quantification assay allows for a comprehensive understanding of the behavioral effects of Sinomenine as a treatment for alcohol use disorder and nicotine addiction, as well as evidence of possibly its mechanism of action. Further, the consistent exacerbation of the responses by Drosophila subjects following conditioning to nicotine and ethanol as opposed to only naive exposure supports the use of Drosophila as a model organism for addictions and reward. In regards to research pertaining particularly to the model organism, this study also confirms the natural naive preferences and aversions to certain substances which could affect self-administration in investigations of addiction.

Sinomenine's ameliorative effects are both implicated between oral consumption and inhalation of ethanol and nicotine. While this study focuses on ethanol and nicotine, the use of opioid antagonists to attenuate the behavior induced by alcohol, a depressant, and nicotine, a stimulant, reveals that Sinomenine may be applicable to a variety of substance use disorders. The degree in which the CREB pathway is involved in each type of addiction and whether CREB-enhanced transcription is induced in this addiction by opioid receptors or other receptors may determine Sinomenine's effectiveness as a treatment.

Limitations

The primary limitations of this investigation involve concentrations of treatments, the limitations of *Drosophila melanogaster* as a model organism for addiction, and the limitations of using a secondary messenger quantification assay. Using varying concentrations of Sinomenine would be proper for a novel study since there has not been previous investigations which establish Sinomenine treatment in *Drosophila*. However, since the study parallels Sinomenine to naltrexone, which has been used in *Drosophila*-modeled studies to attenuate alcohol and nicotine-induced behavior, the same concentration was used for both treatments. The lack of multiple concentrations may explain why Sinomenine was significantly less effective than naltrexone in attenuating ethanol conditioned preference, or also possibly why it didn't affect naive preference to ethanol in the CAFE or y-maze. Mice are generally the standard model organism for studies involving drug addictions. The lack of a culmination of research establishing opioid reward in Drosophila indicates their limitations. However, the assays used have been established and developed for quantifying specific behaviors in Drosophila rather than addiction itself, which cannot currently be modeled in Drosophila. This limits the claim of Sinomenine's efficacy to only drug-induced behavior rather than addiction as a whole. While the use of a cAMP quantification ELISA is appropriate since the claim is that Sinomenine induces its effect via the cAMP/PKA/CREB, it does not have a paralleled assay to then associate CREB with addiction. Previous studies have shown that CREB is a transcription factor which has implications in various addictions; however, it would still be optimal to have an ELISA quantifying the levels of Tyrosine Hydroxylase or dopamine to demonstrate the effects of Sinomenine at the start and end of the CREB mediated pathway.

Error Analysis

The Y-maze and ELISA assays indicate possible errors in procedural execution and preference analysis. To calculate preference in the Y-maze, only *Drosophila* which chose a vial were taken into account while those which remained in the original vial were not. However, a few trials contained drosophila which straddled between the arms of the Y-connector and pipet tip, and not fully in the vial. This indicates that they had chosen the odor to some degree, yet then cannot be counted since they didn't fully enter the vial. Further, it was difficult to keep a consistent level of homogenization among each trial as under 30 seconds of homogenization, some groups of fly heads were more homogenized than others. To correct this, certain vials were homogenized more than once in order to achieve an even level of homogenization among the vials. The lack of an ethanol induced increase in cAMP in the ELISA assay was unexpected. It is possible that the correct protocols were followed and that the insignificant increase in cAMP was due to

a development of tolerance. The uneven homogenization of the *Drosophila* heads causing the inconsistent levels in cAMP, particularly for the ethanol control group, is equally possible.

Future Research

The focus of this study was the efficacy of Sinomenine, traditionally a treatment for rheumatoid arthritis, in attenuating ethanol and nicotine addictions through the CREB and opioid reward pathways. The particularly promising aspect of Sinomenine that may make it more viable than other opioid receptor antagonists is its analgesic and anxiolytic effects which are independent of opioid receptors, as well as the fact that it is not addictive. While this study did include a depressant, it would be very interesting to see the effects of Sinomenine on benzodiazepine and barbiturate addictions. Benzodiazepines and barbiturates are depressants which are used to treat patients suffering from anxiety and are highly addictive. The fact that Sinomenine is effective in attenuating behaviors induced by ethanol, a depressant, in drosophila is promising. Sinomenines anxiolytic properties may allow patients addicted to sedatives to have relief from their anxiety as well as ameliorate their addiction. Further studies could involve assays including both those which quantify Sinomenines ability to reduce anxiety as well as attenuate addictive behavior. Further research could also focus on gaining a more definitive understanding of Sinomenine's mechanism of action. Past research indicates that Sinomenine binds to opioid receptors and induces cAMP increase through receptor activation. However, since there are many types of g-protein coupled receptors which can activate adenylyl cyclase, further research may pertain to the effects of receptor antagonism on other g-protein receptors and how it affects adenylyl cyclase and CREB mediated transcription. Additionally, CREB has multiple kinases which can phosphorylate it such as Ca^{2+}/calmodulin-dependent protein kinase (CAMK). Further research may investigate the roles of NMDA receptors and calcium signaling in various addictions in direct comparison to the role of opioid receptors. What makes opioid receptors particularly relevant to other addictions is that their activation is implicated in both cAMP/PKA/CREB which upregulates TH and by direct modulation of dopaminergic neurons. However, inhibition of NMDA receptors could prevent CREB mediated transcription through regulation of CAMKII and LTP as well. Since both receptors have multiple pathways implicated in the development of addiction, a comparison of the effects of inhibition of each receptor may lead to greater understanding of the synergy of these pathways and how a combination of medications may be most effective in treating the development of addiction. Circling back to the present study, future research may focus on the effects of Sinomenine on alcohol and nicotine induced behaviors in more complex model organisms such as mice or humans which have established opioid reward in behavioral assays.

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