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NUCLEUS ACCUMBENS AND ETHANOL CONSUMPTION**

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Honors Thesis

EFFECTS OF CATHARANTHINE ON DOPAMINE RELEASE IN THE NUCLEUS
ACCUMBENS AND ETHANOL CONSUMPTION

By
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Submitted to Brigham Young University in partial fulfillment of graduation requirements
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ABSTRACT

EFFECTS OF CATHARANTHINE ON DOPAMINE RELEASE IN THE NUCLEUS ACCUMBENS AND ETHANOL CONSUMPTION

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Bachelor of Science

This thesis discusses the history of catharanthine and related compounds, and their potential anti-addictive properties. Current research is exploring possible mechanisms of these properties. Past studies have found catharanthine has effects on neurons that project to the mesocorticolimbic system, an area implicated in addiction. We have seen that catharanthine decreases evoked dopamine (DA) release but increases basal DA release. This is the first study to investigate catharanthine's effect on DA transmission *in vivo*. Using microdialysis, we determined the effect of catharanthine on DA in the nucleus accumbens of the striatum. This study determines the effect of different doses of catharanthine, kinetics of catharanthine, and the effect of catharanthine and ethanol injections. We also used the drink in the dark behavioral method to determine if catharanthine decreased drinking behavior in mice.

ACKNOWLEDGEMENTS

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REVIEW OF CRITICAL LITERATURE

Impact of Alcohol Addiction on Society

The most prevalent substance use disorder is alcohol addiction (Degenhardt et al., 2018). In 2019, the National Institute of Health reported that 14.1 million adults in America suffered from alcohol use disorder (AUD). Those who suffer will continue to use alcohol (also called ethanol or EtOH) despite its physical, social, and emotional adverse consequences (NIH, 2020). As of 2015, there were over 90,000 alcohol related deaths a year in the United States, including an average of 29 years of life lost per each death (Esser et al., 2020). Alcohol abuse is the third leading cause of preventable death in the United States. It is estimated that alcohol abuse costs the United States of America over 250 billion dollars annually (CDC, 2015). AUD doesn't only cause problems for the individual but has adverse effects on a person's family and social circle. Children of alcoholics have higher rates of certain cognitive disorders and behavioral disorders (Rothenberg et al., 2017). Unstable home environments for children of alcoholics lead to many of these deficits (Park & Schepp, 2015). Acts of domestic violence and crime are often associated with alcohol use (Quigley & Leonard, 2000).

Alcohol withdrawal symptoms include anxiety, headaches, nausea, sweating, delirium, and seizures (Muncie et al., 2013). If chronically abusing alcohol, these make it dangerous to abruptly quit drinking. Withdrawal symptoms are psychologically and physiologically painful, making it hard to detox and further recover from AUD. A major goal of research is to find better treatments for AUD.

There are currently three FDA approved medications for treating AUD. Antabuse inhibits the enzyme that metabolizes acetaldehyde, a byproduct of alcohol metabolism. Excess acetaldehyde leads to adverse symptoms like dizziness, nausea, and an increased heartrate. The idea behind Antabuse is that if drinking causes these unpleasurable symptoms, people will not drink. For the same reason, the drug compliance for those prescribed Antabuse is low (Hardt, 1992). Naltrexone is a mu opioid receptor antagonist, blocking the euphoria associated with drinking (Sudakin, 2016). Acamprosate is prescribed to reduce craving in AUD patients who have already detoxed from alcohol (Mason, 2006). None of these treatments acts as a “cure” for AUD. Each has its merits and its problems, which is why a goal for researchers is to find better pharmacological treatments to help addicts.

Dopamine's Role in Reward and Addiction

The mesocorticolimbic system is often called the reward circuit because of its integral role in motivation and pleasure. It is naturally an important brain system, as it is involved in vital behaviors like eating and drinking (Agmo et al., 1995). Our current understanding of this system focuses on dopamine (DA) neurons from the ventral tegmental area (VTA) projecting to the nucleus accumbens (NAc). The circuitry is much more complicated than these two areas. This system gets inputs from many brain areas and has many outputs. However, the current dogma of reward and addiction states that increased DA release in the NAc signals pleasure (Gardner, 2011). Addictive drugs will take over this circuitry. Drugs of abuse can increase DA to 10 times the levels associated with natural rewards. Therefore, in this model of addiction drugs will be intensely rewarding and lead to perseveration and addiction (Kalivas & Volkow, 2005).

Dopamine is not only implicated in the pleasurable effects of alcohol and other drugs, but also in the craving and withdrawal symptoms. While drugs will initially raise DA levels, when the drug is gone, DA will fall to a level below the pre-drug baseline level. The current theory states that this depletion of DA leads to the “craving” of a drug and need to take a drug again to achieve higher dopamine levels (Koop, 2003). When it comes to alcohol, this lowest point of DA is when the urge to drink is the strongest. Indeed, chronic alcohol abusers have lower levels of baseline DA than non-addicts (Volkow & Wang, 2007).

Catharanthine and Related Compounds

Ibogaine is a natural compound that comes from the root of the *Tabernanthe Iboga* tree. Ibogaine may decrease craving and withdrawal symptoms (Maisonneuve & Glick, 2003). Initial evidence for this in humans was anecdotal, with recreational ibogaine users noting they didn’t desire other drugs they were addicted to (Lotsof, 2001). Some animal studies have shown ibogaine may decrease self-administration of EtOH (Rezvani et al., 1995), morphine (Glick, 2003), and cocaine (Cappendijk, 1993). Despite some promising research, the side effects of ibogaine make it a less than ideal treatment for addiction. In many patients ibogaine has induced tremors, cardiac arrhythmias, and nausea. Ibogaine is also hallucinogenic and has led to weeks of mania in some patients (Litjens, 2015).

Ibogaine belongs to a class of compounds called iboga alkaloids. Other compounds in this family include coronaridine, 18-methoxycoronaridine (18-MC), and catharanthine. Another name for these compounds is coronaridine congeners. These compounds are promising research targets for addiction treatments with fewer side

effects (Maisonneuve & Glick, 2003). Ibogaine activates a variety of nicotinic acetylcholine receptor (nAChR) subtypes and possibly NMDA receptors as well (Glick et al., 2002). 18-MC and catharanthine bind more specifically to nAChRs than ibogaine does with no NMDA activity. This is likely why they have fewer side effects. In fact, catharanthine binds to $\alpha 3\beta 4$ subtype nAChRs with much more specificity than any other receptor type, where ibogaine also binds to $\alpha 4\beta 2$ receptors (Arias, 2017). Catharanthine also activates GABA_A receptors, inducing a mild sedative effect (Arias et al., 2020).

Medial Habenula NACHRs and Anti-Addictive Properties

$\alpha 3\beta 4$ receptors are mostly found in the medial habenula (MHb) (Grady et al., 2009), as opposed to $\alpha 4\beta 2$ receptors which are in many more brain areas. The medial habenula has already been considered for its possible role in reward systems. The habenula interacts with dopamine neurons to signal negative reward (Matsumoto & Hikosaka, 2007). Many genes associated with addiction are expressed in the MHb (Velasquez et al., 2014).

Inhibition of habenular $\alpha 3\beta 4$ nAChRs have been implicated in the anti-addictive effects of coronaridine congeners. Activity of nAChRs with $\alpha 3$ and $\beta 4$ subunits have been associated with the rewarding properties of nicotine (McCallum et al., 2012). Tonic activity of these same neurons happens during nicotine withdrawal as well (Gorlich et al., 2013). Antagonism of these receptors and decreased habenular ACh current reduce self-administration of morphine, methamphetamine (Glick et al., 2002) and nicotine (Toll et al., 2012). Knockout mice with no $\beta 4$ subunits have reduced nicotine withdrawal symptoms (Salas et al., 2004).

Anatomically, MHb axons project to the VTA (Sutherland, 1982) through the fasciculus retroflexus (Nishikawa et al., 1986). When administered with no other treatment, nicotine increases DA in the NAc. When 18-MC is injected directly in the MHb, nicotine administration does not increase accumbal DA (McCallum et al., 2012). Injection of $\alpha 3\beta 4$ inhibitors into the MHb decreases drug self-administration, but injection of these into the VTA does not (Glick et al., 2011). This shows that even when there is no direct pharmacological effect on the mesocorticolimbic system, inhibition of habenular nAChR activity somehow modulates neurotransmission in the reward pathway. However, we do not know the mechanism through which this modulation happens.

Rationale and Hypotheses

While the exact anti-addictive mechanism of coronaridine congeners is still unclear, their nAChR inhibition is likely the starting point. We know that a decrease of $\alpha 3\beta 4$ activity and ACh current is associated with decreased drug intake. Decreased habenular nAChR activation can block the dopamine increase, withdrawal symptoms, and rewarding effects involved with nicotine (McCallum et al., 2012). We also know the MHb synapses on the VTA (Sutherland, 1982). This evidence combined suggests that coronaridine congeners and other $\alpha 3\beta 4$ antagonists inhibit MHb activation, which may decrease neurotransmission to the VTA from this area. This would mean less activation of VTA, and therefore less dopamine release on the NAc. More work is required to determine if this idea is correct, and the exact receptors involved in the process.

Catharanthine inhibits habenular $\alpha 3\beta 4$ receptors with high affinity and decreases ACh current (Arias, 2017). Since many studies have shown that inhibition of $\alpha 3\beta 4$ NACHRs and decreased ACh current can decrease self-administration of many drugs, so

catharanthine it has the potential to decrease alcohol self-administration. In fact, much of the current research uses ibogaine or 18-MC to inhibit NACHRs and often low levels of many $\alpha 3\beta 4$ blockers have to be combined (Toll et al., 2012). Catharanthine has higher specificity for habenular $\alpha 3\beta 4$ s than other coronaridine congeners, so it could be a better target for an anti-addictive compound (Arias, 2017).

There are currently no published studies about catharanthine's effects on DA transmission. We understand catharanthine's actions on habenular receptors and theorize that this inhibition further inhibits dopamine release through MHb and VTA connections. Thus, we decided to start looking at DA systems directly to see if this idea holds true. As discussed, dopamine is an important signal for learning and reward. Our lab has performed some preliminary work on catharanthine and dopamine, but until this thesis there has been no *in vivo* work. Understanding catharanthine's effect on dopaminergic systems in a living rodent model can help us better understand its possible anti-addictive effects. In addition to catharanthine's effects alone, it will be interesting to determine if catharanthine could block the DA increase expected with drugs of abuse.

It is also interesting to note that the sedative properties of catharanthine may also factor into its use for alcohol withdrawal. Although not FDA approved for this purpose, it is common for physicians to prescribe benzodiazepines and other sedative drugs to alleviate the anxiety associated with alcohol withdrawal (Muncie et al., 2013). Benzodiazepines have some dependence liability (Licata & Rowlett, 2008), but catharanthine's sedative effects utilize a benzodiazepine-independent mechanism (Arias et al., 2020). While this GABAergic mechanism is not the focus of this thesis, it is a factor to consider in interpreting behavioral results. Thus, we hypothesize that

catharanthine will modulate DA transmission similar to EtOH. Catharanthine may target the same neural substrates as EtOH, serve to substitute for EtOH effects, and similar to benzodiazepines, ameliorate withdrawal from chronic EtOH without the side effects of benzodiazepines.

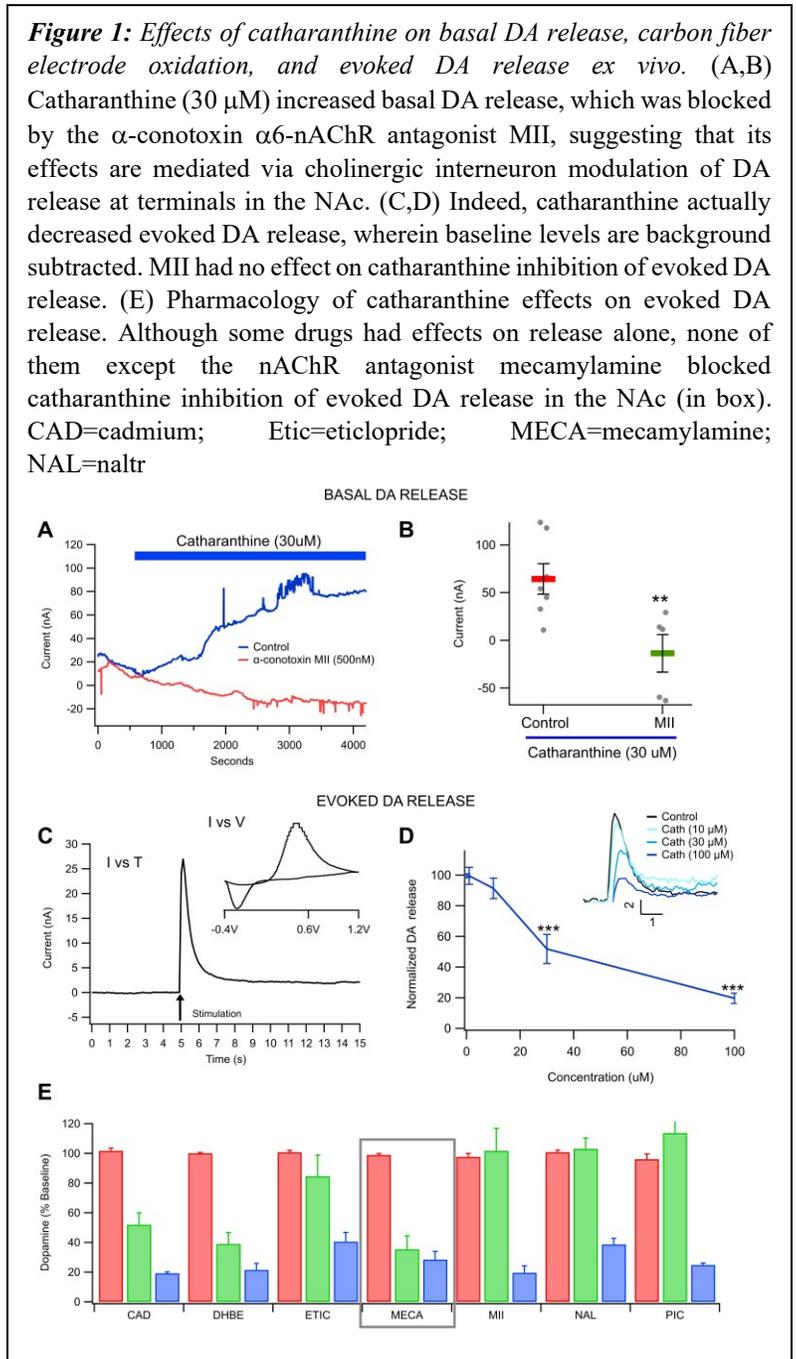
The inhibitory effect of catharanthine on habenular $\alpha 3\beta 4$ nAChRs could be important when it comes to alcohol withdrawal. Since $\alpha 3\beta 4$ nAChRs seem to be required for withdrawal of some drugs (Gorlich et al., 2013) and $\alpha 3\beta 4$ knockout mice have reduced withdrawal (Salas et al., 2004)—catharanthine could help alleviate withdrawal symptoms with its cholinergic mechanisms. Knowing catharanthine decreases cholinergic activity in the MHB, an area associated with negative reward, means it could also inhibit negative reward involved with withdrawal.

PRELIMINARY WORK

Catharanthine

Preliminary studies in our lab have shown that catharanthine enhances basal DA release but reduces evoked DA release *ex vivo*. **Figure 1** shows the effects of 30 μM catharanthine on basal and evoked DA release. Catharanthine enhances basal (**Fig. 1 A, B**) and reduces (**Fig. 1 C, D**) evoked DA release *ex vivo* in a dose dependent manner This was accomplished using fast scan cyclic voltammetry in the NAc of drug-naïve mice. However, catharanthine oxidizes at the same voltage as DA. However, this is a confound in the basal DA release studies, but not in evoked DA release as the evoked values come from subtracting from baseline values. This makes the data on basal DA release hard to interpret. We sought to determine the mechanism underlying catharanthine inhibition of

evoked DA release. As catharanthine effects appear similar to EtOH, we investigated diverse pharmacological tools to dissect catharanthine effects similar to what we have done with EtOH. We tested cadmium (CAD), NACHR blocker DHBE, the D2R blocker eticlopride (ETIC), mecamylamine (MECA), the non- $\alpha 7$ nAChR blocker, the $\alpha 6$ conotoxin MII, the mu-opioid receptor antagonist naltrexone (NAL), or the GABAR blocker picrotoxin (PIC) affected catharanthine inhibition of evoked DA release in the NAc (**Fig. 1D**). None of these well-known and relevant drugs affected catharanthine inhibition of evoked DA release but MECA, suggesting that nAChRs are involved in catharanthine effects on DA release similar to what has been shown on behavioral measures by other researchers. Given the confound of direct catharanthine effects on voltammetric recordings, it is important to evaluate its effects *in vivo* with methods other than voltammetry to establish the physiological relevance and justification for studying its effects



on EtOH consumption, especially since catharanthine oxidizes at similar potentials as DA. In addition, it is hypothesized that catharanthine's enhancement of DA release will reduce the rewarding properties of EtOH in naïve mice and the adaptive properties of EtOH dependent mice.

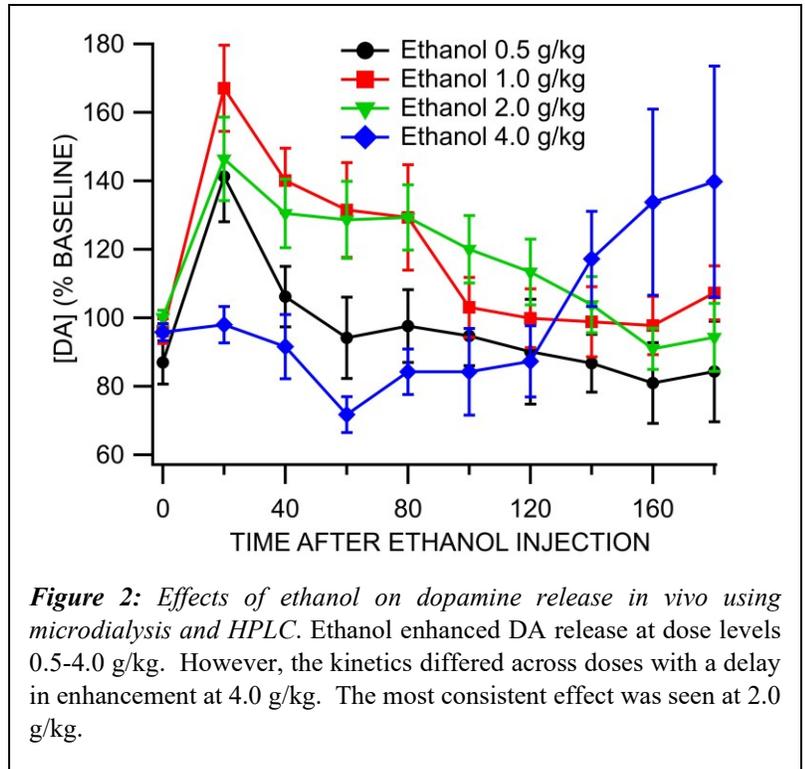
Ethanol

Previous lab work has determined that 2.0 g/kg EtOH maximally enhances DA in the core of the nucleus accumbens. **Figure 2** shows the effect of ethanol on DA release at ethanol doses.

METHODS

Microdialysis

Male VGAT-Cre/GAD67 mice were anesthetized using isoflurane. In a non-survival surgery, a hole was drilled to the NAc (+2.0 AP, -9 ML, -3.6 DV from bregma) and a microdialysis probe (MD-2211 1mm Membrane Probe, BASi Instruments, West Lafayette, IN, USA) was inserted and held in place. During the experiment, artificial cerebrospinal fluid (aCSF) flowed at a rate of 2 μ L/min. aCSF went through the probe and through the membrane, so dialysate samples contained neurotransmitters released in



the NAc. Dialysate samples were collected every 20 minutes. All catharanthine doses were dissolved in a vehicle of 95% saline (9% NaCl) solution and 5% Tween 80.

Catharanthine Dose Response

Samples were collected as a baseline for 40 minutes. A 5 mg/kg dose was injected at time zero and dialysate samples were collected for another 40 minutes. After 40 minutes, the 10 mg/kg dose was injected. The 20 and 50 mg/kg doses were also collected for 40 minutes after the previous dose.

Catharanthine Time Course

Samples were collected as a baseline for 60 minutes. A 10 mg/kg dose was injected at time zero and dialysate samples were collected for two hours post-injection.

Catharanthine + EtOH experiments

In the catharanthine + ethanol experiments, baseline samples were collected for 60 minutes. A 10 mg/kg dose of catharanthine was injected at time zero. 20 minutes later, 2.0 g/kg ethanol was injected. Samples were collected for another 100 minutes post EtOH injection.

HPLC

Samples were evaluated using high pressure liquid chromatography with electrochemical detection (HPLC-ECD). Analysis of DA was done using an Ultimate 3000 system (Thermo Fischer Scientific, Waltham, MA, USA) connected to a Coulochem III electrochemical detector (Thermo Scientific, Waltham, MA, USA) with an Acclaim RSLC Polar Advantage II Column to perform separations and the

Chromeleon computer program. The mobile phase consisted of 150 mM sodium dihydrogen phosphate, 4.76 mM citric acid, 3 mM sodium dodecyl sulfate, 50 μ M EDTA, 15% HPLC grade acetonitrile, 10% HPLC grade methanol, and 75% HPLC grade water. Mobile phase was adjusted to pH 5.6 using NaOH. This mobile phase recipe comes from Thermo Scientific's "Chromatography for Neuroscience Applications Notebook" for Monoamines and Metabolites."

Drink in the Dark

Twelve adult VGAT-Cre/GAD 67 were used for this procedure. Drink in the dark (DID) experiments took place during the mice's dark cycle, 3 hours after lights turned off. During the DID procedure, mice were caged individually. In the time in between drinking, mice were housed with one other cage mate in the same experimental group as them. At the beginning of the procedure, bottles of 16% ethanol were put in the cage. On days 1-3, mice were allowed to drink for two hours. On day 4, mice drank for 4 hours to better simulate binge drinking and a max amount of ethanol drank. During week 2, mice were injected with either catharanthine in vehicle or vehicle only. There were six mice in each experimental group. Catharanthine injections were 10 mg/kg, and vehicle injections were an equal volume per body weight ratio. Injections were done intraperitoneally 20 minutes before the drinking protocol. On days 5-7, mice drank for two hours and on day 8 mice drank for 4 hours. Bottles were weighed before and after. Differences were measured in grams. Three bottles were also placed in empty cages and measured to control for the bottles leaking. The average of leak control was subtracted from the amount drank in all calculations.

RESULTS

Catharanthine injected on its own increased basal DA. The initial dose curve micro dialysis experiments (n=9; **Figure 3**) showed that the 5 mg/kg, 10 mg/kg, 20 mg/kg, and 50 mg/kg doses all enhance DA release in the NAc *in vivo* as recorded with microdialysis/HPLC. The maximal effect was around 10 mg/kg, increasing basal DA to around 250%

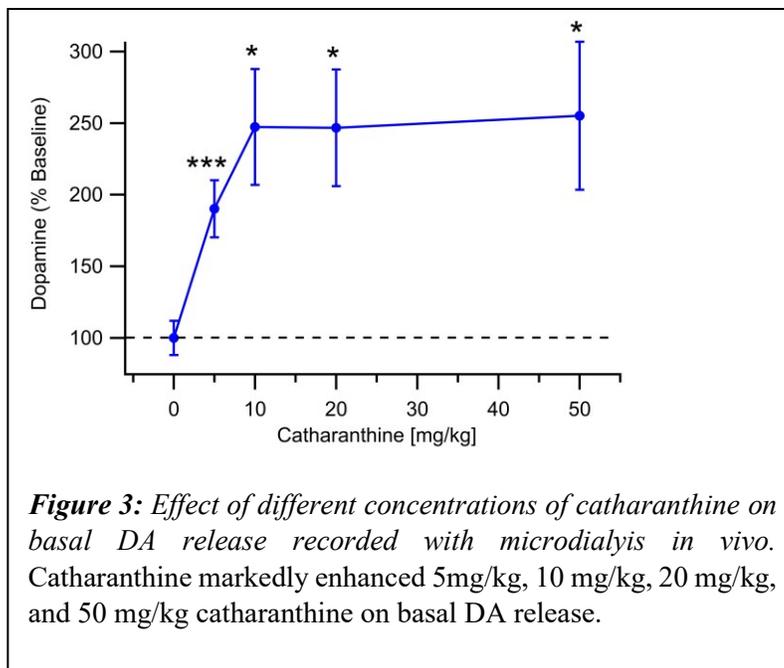


Figure 3: Effect of different concentrations of catharanthine on basal DA release recorded with microdialysis *in vivo*. Catharanthine markedly enhanced 5mg/kg, 10 mg/kg, 20 mg/kg, and 50 mg/kg catharanthine on basal DA release.

of baseline. This agrees with previous voltametric readings showing catharanthine increases basal DA. Based on this data, we decided to continue experiments using the 10 mg/kg dose. Since the 10, 20, and 50 mg/kg doses showed similar effects, using the 10 mg/kg was desirable to reduce the injection volume used in mice.

Further kinetics experiments (n=3) showed that catharanthine's enhancement of basal DA occurred 20 minutes post-injection and recovered to baseline in 20 minutes (data not shown). Thus, further microdialysis and behavior experiments involve catharanthine treatment 20 minutes before ethanol treatment.

In the next experiments, 10 mg/kg catharanthine was injected. Twenty minutes later, a 2 g/kg dose of ethanol was injected. Ethanol is normally expected to increase basal dopamine. Although preliminary (n=4), it appears that catharanthine inhibits the typical enhancement of DA release by EtOH (**Figure 4**).

We performed DID experiments that aimed to see if catharanthine would decrease drinking behavior in mice. Mice were separated into two groups of six mice each. Three hours into the dark cycle they were allowed to drink EtOH. Mice drank for a week previous to any injections, and for week two they were injected with catharanthine or vehicle twenty minutes before drinking. The results of this experiment were inconclusive.

Catharanthine decreased week 2 drinking in some mice, but not in others (**Figure 5**). Vehicle also decreased drinking in some mice, so it is possible some effects were due to timing and previous ethanol exposure.

CONCLUSION

This *in vivo* work, along with previous *ex vivo* work, shows that catharanthine increases basal dopamine in the nucleus accumbens. This effect on its own shows that catharanthine is psychoactive in some way and has some effect on the reward pathway. It is

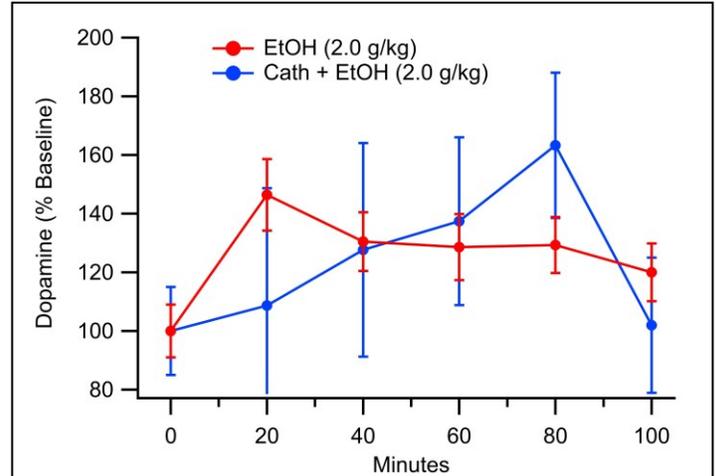


Figure 4: Catharanthine may reduce ethanol effects on DA release *in vivo*. Ethanol (2.0 g/kg) moderately enhances DA release in 20 min (n=7) with some recover over 1.5 hrs. Administration of Catharanthine (10 mg/kg) 20 min before EtOH reduced the ability of EtOH to enhance DA release (n=4).

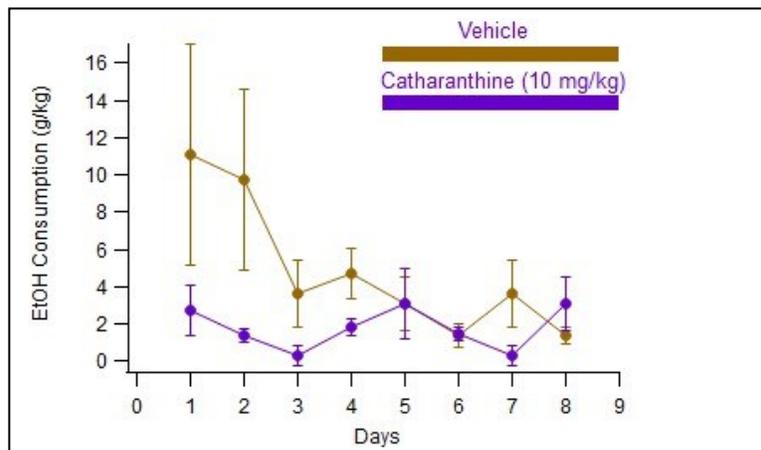


Figure 5: Effects of 10 mg/kg catharanthine on EtOH consumption in the DID procedure. Perhaps due to EtOH leak from the bottle, some mice consumed large quantities of EtOH the first week. Regardless, although drinking stabilized somewhat, there was no significant difference between vehicle and catharanthine treatment.

important to note that depending on the size of this effect in relation to other drugs or rewards, catharanthine may or may not be pleasurable itself. That is a topic of future study for our lab.

While a DA increase is expected in response to alcohol, catharanthine can block that. This is an important find in finding a possible mechanism for catharanthine's anti-addictive properties. Catharanthine and EtOH are interacting in some way, but we do not currently know how. In future research, we hope to use blockers for specific receptors *in vivo* to study the mechanisms catharanthine may use to modulate DA transmission. Specifically, we want to see how catharanthine's modulation of $\alpha 3\beta 4$ nAChRs modulates dopamine transmission. This could also help us better understand catharanthine's effect when combined with EtOH.

Our DID data didn't tell us much about catharanthine's effect on drinking behavior. However, this may be due to an error in experiment design. Our DID conditions likely did not simulate alcohol dependence like we wanted it to. Our mice drank freely but were not ethanol dependent. It takes 2-3 cycles of DID to produce dependence, shown by progressively enhanced consumption of ethanol. Since we are studying catharanthine as a possible treatment for patients going through withdrawal, this specific experiment probably can't say much about catharanthine's efficiency. So, while this data was useful to see if catharanthine did do anything to baseline drinking levels, the fact it didn't doesn't necessarily mark the end of catharanthine DID studies. The next step will be to make mice dependent through chronic EtOH injections or EtOH chambers and then see if catharanthine effects drinking in a dependent group. These experiments cannot be included in this thesis due to time constraints.

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