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Benjamin M. Williams
Brigham Young University

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Master of Science

Scott C. Steffensen, Chair
Jordan Yorgason
Jeff Edwards

Neuroscience Center

Brigham Young University

ABSTRACT

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Benjamin M. Williams
Neuroscience Center, BYU
Master of Science

Catharanthine is derived from the *Catharanthus roseus* plant and is an analog to ibogaine, a drug that reduces opioid and alcohol withdrawal symptoms and decreases drug self-administration in both animals and humans. Catharanthine has promise to be an alternative pharmacological treatment for addiction without the adverse side effects associated with ibogaine. The objective of this study was to evaluate catharanthine's effects on dopamine (DA) transmission in the mesolimbic DA system as well as determine its effects on both ethanol withdrawal induced anxiety and drug-seeking behaviors in mice. We hypothesized that catharanthine would inhibit evoked DA release in the nucleus accumbens (NAc) while also reducing anxiety and drug seeking behaviors in mice. We found that superfusion of catharanthine (1-100 μM) to mouse brain slices significantly inhibits evoked DA release in the NAc of the striatum in a dose dependent manner, while also slowing DA reuptake through inhibition of the dopamine transporter (DAT), measured using fast-scan cyclic voltammetry (FSCV). We also found that intraperitoneal administration of catharanthine in live mice significantly increases extracellular DA, measured via microdialysis with electrochemical detection. Catharanthine inhibition of evoked DA release was significantly reduced by the non-selective nAChR antagonist mecamylamine, the α4 nAChR antagonist dihydro-β-erythroidine hydrobromide (DhβE) and the α6 nAChR antagonist α-conotoxin MII, suggesting that catharanthine inhibits α4 and α6 nAChRs in the NAc. Iontophoresis and in-vivo data indicates that catharanthine slows DA reuptake and increases extracellular DA in the NAc through partial inhibition of DATs. Catharanthine also blocked increases in anxiety-like behavior during ethanol withdrawal in mice in the elevated plus maze. Lastly, preliminary data suggests that catharanthine increases both water and ethanol drinking in a 24-hour two-bottle choice drinking paradigm, which was contrary to our hypothesis.

Keywords: addiction, catharanthine, 18-methoxycoronaridine, dopamine, alcohol, withdrawal, anxiety, voltammetry, nucleus accumbens, nicotinic acetylcholine receptors, dopamine transporter

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INTRODUCTION

Impact of Drug Abuse

Excessive drug use negatively impacts the lives of millions of people around the world. Addiction to drugs of abuse, especially alcohol and nicotine, places a substantial financial burden on the U.S. economy. Alcohol use disorder (AUD) has been estimated to cost the US economy $249 billion per year (Sacks, Gonzales, Bouchery, Tomedi, & Brewer, 2015) with an estimated 140,000 deaths annually (CDC, 2022). Cigarette smoking, of which nicotine is the main addictive component, has been estimated to cost the US economy $169.3 billion per year (Xu, Bishop, Kennedy, Simpson, & Pechacek, 2015) with an estimated 480,000 deaths annually (General, 2014). Alcohol Use Disorder is detrimental to the physical and mental health of those who suffer, however AUD can be even more detrimental to family and others close to the individual (Ersche et al., 2020). For these reasons it is critical that we find an effective treatment for AUD and other drug use disorders.

Drugs of Abuse and the Mesolimbic Dopamine System

Dopamine is the canonical neurotransmitter implicated in movement, motivated behavior and reward learning. Current dogma maintains that DA neuron activation and release in the nigrostriatal DA system originating in the midbrain substantia nigra (SN) and projecting to the dorsal striatum underlies the initiation of movement, hence its involvement in Parkinson's disease, wherein dopamine (DA) neurons are depleted in the SN. The mesolimbic DA system originating in the midbrain ventral tegmental area (VTA) and projecting to the nucleus accumbens (Vuckovic et al., 2010) of the medial temporal lobe is thought to be involved in reward from natural behaviors such as feeding, drinking and sex (Koob & Volkow, 2010). The NAc is known as the "pleasure center" of the brain due to the dense DAergic projections it receives from the VTA. One theory

states that DA release in the NAc is a scalar index of reward, however it has become evident that DA release in the NAc is not only involved with reward, but also involved with the expectation of reward, reward prediction error coding, learning to avoid aversive stimuli and coping with stressful events (Berridge & Kringelbach, 2008; Cui et al., 2020; Kramar, Castillo-Diaz, Gigante, Medina, & Barbano, 2021; Schultz, 2016). Dysregulation of this mesolimbic system is a hallmark of drugs of abuse and has been implicated in the habit-forming actions of several addictive drugs of abuse (Wise, 2004). Within this pathway, an increase of DA in the NAc is implicated in substance addictions. The level of DA release by some drugs of abuse can be 10 times that produced by naturally rewarding behaviors such as eating, drinking, and sex. However, the onslaught of DA release is transient and often results in adaptations including progressive, compensatory lowering of baseline DA levels during withdrawal from chronic drug abuse.

The main cell types in the NAc are γ-aminobutyric acid (GABA)-ergic medium spiny neurons (MSNs) expressing either DA D1 or D2 receptors (D1R or D2R) and cholinergic interneurons (CINs). D1-MSNs form a positive feedback loop in a direct pathway back to the VTA with VTA GABA neurons increasing DA output, while D2-MSNs form a negative feedback loop in an indirect pathway with GABA neurons in the globus pallidus (GP), which connect to glutamate neurons in the subthalamic nuclei (STN), which excite GABA neurons in the VTA, inhibiting DA release in the NAc (Macpherson, Morita, & Hikida, 2014). VTA GABA neurons perform an important role in inhibiting VTA DA neurons and are an important target of dopaminergic modulation. VTA DA neurons also form a diffuse modulatory system with multiple brain regions, including the dorsal hippocampus, pre-frontal cortex (PFC), anterior insular cortex, basolateral amygdala (BLA), ventral striatum (or NAc) and the anterior cingulate cortex (aCC) (Koob & Volkow, 2010). This modulatory system is heavily involved with reward and learning.

Overview of Coronaridine Congeners Ibogaine, Catharanthine and 18-Methoxycoronaridine

Ibogaine is one of several terpene indole alkaloids compounds that are found in the root bark of the African shrub *Tabernanthe iboga*. Ibogaine is a member of a small group of drugs known as coronaridine congeners, including but not limited to its active metabolite noribogaine, coronaridine, catharanthine, 18-methoxycoronaridine (18-MC), 18-hydroxycoronaridine and 18-methylaminocoronaridine, which have recently gathered interest for their anti-addictive properties. Ibogaine has been found to act on a wide variety of targets, including voltage-gated sodium channels, kappa-opioid receptors, mu-opioid receptors, sigma receptors, NMDA glutamate receptors, monoamine transporters, metabotropic 5-HT_2 and ionotropic 5-HT_3 receptors, nicotinic acetylcholine receptors (nAChRs) and muscarinic g-protein coupled receptors (mGPCRs) (Codd, 1995; Deecher et al., 1992; Sershen, Hashim, Harsing, & Lajtha, 1992; Sershen, Hashim, & Lajtha, 1997; Sweetnam et al., 1995). Ibogaine reduces opioid and ethanol withdrawal symptoms and decreases drug self-administration in both animals and humans (Brown & Alper, 2018; Maisonneuve & Glick, 2003), however ibogaine has been found to increase heart arrhythmias, induces body tremors, loss of cerebellar purkinje cells, has hallucinogenic properties and has even lead to sudden deaths (Grogan, Gerona, Snow, & Kao, 2019; Knuijver et al., 2021; Koenig & Hilber, 2015). For this reason, ibogaine has been classified as a schedule I substance by the DEA in the United States and for the most part, has been prohibited as an addiction treatment. Outside the US, ibogaine has found moderate success in treating addiction (Barber, Gardner, Savic, & Carter, 2020; Knuijver et al., 2021), however alternative pharmacological treatments without negative side effects are needed.

18-methoxycoronaridine, a synthetic derivative of ibogaine, is an α3β4 nAChR antagonist (Arias, Jin, Feuerbach, & Drenan, 2017; Arias, Targowska-Duda, Feuerbach, & Jozwiak, 2015) and has been gathering interest over the past 20 years of research for its anti-addictive properties. 18-MC has been found to reduce self-administration of nicotine, cocaine, morphine and ethanol (Maisonneuve & Glick, 2003; Rezvani et al., 2016; Rezvani et al., 1997) and recently has been found to decrease neuropathic pain (Arias, Tae, et al., 2020). 18-MC was also found to decrease extracellular dopamine in the NAc (Glick, Kuehne, Maisonneuve, Bandarage, & Molinari, 1996).

Catharanthine is also an ibogaine analog and can be derived from the African shrubs *Tabernaemontana divaricate* and *Catharanthus roseus.* To date, no research has investigated the effect of catharanthine on drug seeking behaviors or on DA transmission. Catharanthine is known to inhibit nAChRs in the brain, specifically α3β4 and α9α10 nAChRs with higher potencies compared to that for the α4β2 subtype (Arias et al., 2017; Arias, Tae, et al., 2020; Arias et al., 2015). Catharanthine and 18-MC have been recently found to also inhibit voltage-gated N-type calcium channels ($Ca_V2.2$), which in conjunction with α9α10 nAChR inhibition, is implicated in the antinociceptive activity of these congeners (Arias, Tae, et al., 2020). Recent studies also showed that catharanthine and 18-MC potentiate $GABA_A$ receptors, and this activity has been

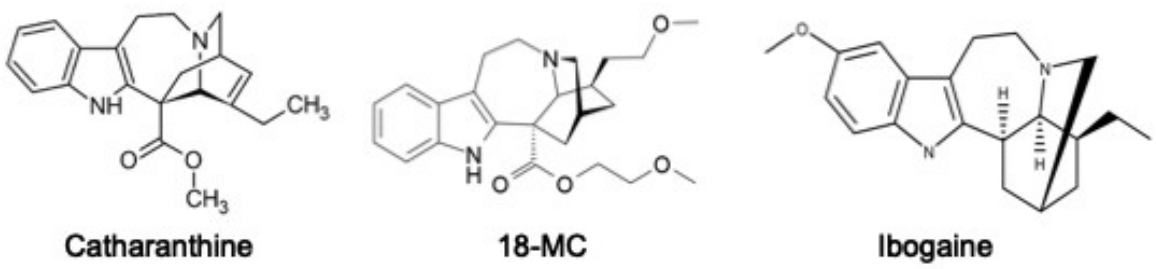

Figure 1. Molecular structure of coronaridine congeners catharanthine, 18-methoxycoronaridine (18-MC) and ibogaine.

associated with the observed sedative activity in mice elicited by catharanthine at high doses (63 mg/kg) (Arias, Do Rego, et al., 2020).

Role of Nicotinic Acetylcholine Receptors in Modulating Dopamine Release

Nicotinic acetylcholine receptors are pentameric ligand-gated ion channels widely expressed on a variety of cells types throughout the brain. Nicotinic receptors are classified as either heteromeric, expressing α, β or other subunits (e.g., α4β2 nAChRs) or homomeric receptors expressing only one kind of subunit (e.g., α7 nAChRs). α4β2, α6 and α7 nAChRs are expressed in the VTA (Klink, de Kerchove d'Exaerde, Zoli, & Changeux, 2001) and nAChRs containing both α4 and α6 in particular have been shown to mediate the reward effects of ethanol (EtOH) (Liu, Hendrickson, et al., 2013; Liu, Zhao-Shea, McIntosh, & Tapper, 2013). Striatal CINs fire spontaneously at 2-10 Hz both in-vivo and in brain slice (Goldberg & Wilson, 2005; Reynolds, Hyland, & Wickens, 2004; Wilson, Chang, & Kitai, 1990). CINs are important in DA modulation through local ACh release onto DA pre-synaptic terminals in the NAc, which are known to express α6β2β3, α6α4β2β3 and α3β2 nAChRs (Exley & Cragg, 2008). Activation of nAChRs allow calcium to flow through the membrane, depolarizing the pre-synaptic terminal, which removes Mg^{2+} ions blocking the NMDA ion channel, causing more calcium to flow into the terminal (Dajas-Bailador & Wonnacott, 2004; Dani, 2001). The extra calcium binds to synaptic proteins initiating vesicle fusion and subsequent neurotransmitter release (Hu et al., 2002; Miklavc & Frick, 2011). However, nAChRs quickly desensitize, leading to short bursts of striatal DA release (Cuevas & Berg, 1998; Grady, Wageman, Patzlaff, & Marks, 2012; Pidoplichko et al., 2004). Optogenetic activation of striatal CINs causes DA release in the NAc through activation of nAChRs expressed

on dopamine pre-synaptic terminals, which supports the role of nAChRs and CINs in dopamine release (Brimblecombe et al., 2018).

Interactions Between Ethanol and Nicotine: Role of nAChRs

It is known that ethanol elevates dopamine release in-vivo in the NAc via inhibition of VTA GABA neurons (Lof, Ericson, Stomberg, & Soderpalm, 2007; Ludlow et al., 2009). However, high doses of ethanol have been shown to inhibit DA release when applied to brain slices by activating $GABA_A$ and glycine receptors (Yorgason, Ferris, Steffensen, & Jones, 2014; Yorgason, Rose, McIntosh, Ferris, & Jones, 2015). Interestingly, EtOH has also been shown to potentiate nAChRs, particularly α3β4 nAChRs (Abburi & McDaid, 2021). Mecamylamine, a nonspecific nAChR antagonist, reduces self-administration of ethanol in mice, suggesting that nAChRs as an important target for ethanol pharmacology (Bhutada et al., 2012; Ericson, Blomqvist, Engel, & Soderpalm, 1998; Ford et al., 2009).

Multiple brain regions also regulate VTA DA release to the NAc, such as the habenula and the laterodorsal tegmental nucleus (LDTg). Activation of glutamatergic neurons in the LDTg results in increased DA release in the NAc via activation of nAChRs and glutamate receptors on VTA DA neurons (Forster & Blaha, 2000). The habenula is a small brain structure located posterior to the thalamus and adjacent to the third ventricle, subdivided into the medial habenula (MHb), which modulates ACh transmission, and lateral habenula (LHb), which modulates norepinephrine and serotonin transmission. The habenula is critical in modulating reward through exciting GABAergic neurons in the rostromedial tegmental nucleus (RMTg), which inhibit VTA GABA neurons, thereby disinhibiting VTA DA neurons (Velasquez, Molfese, & Salas, 2014). Local administration of 18-MC into the MHb has been shown to decrease morphine self-administration in mice, through acting on α3β4 nAChRs. However, local administration of 18-MC

into the VTA did not affect self-administration of morphine (Glick, Ramirez, Livi, & Maisonneuve, 2006). This is likely due to the lack of α3β4 nAChRs expressed in the VTA. It is unknown what the effect would be of local administration of 18-MC or catharanthine into the nucleus accumbens.

Rationale and Hypothesis

It is well established that coronaridine congeners such as ibogaine and 18-methoxycoronaridine induce anti-addictive effects, particularly in reducing nicotine and ethanol self-administration in animals. No research to date has investigated catharanthine, an ibogaine analog, in reducing drug self-administration or on dopamine dynamics. We hypothesized that catharanthine will induce anti-addictive effects through two mechanisms: elevating extracellular dopamine, thereby alleviating drug craving, and inhibiting dopamine release in the NAc, which would reduce the rewarding effects of drugs. Additionally, we hypothesized that administration of catharanthine in-vivo will inhibit EtOH self-administration in a 24-hour 2 bottle choice paradigm and alleviate anxiety during EtOH withdrawal in EtOH dependent mice.

METHODS

Brain tissue preparation

C57BL/6J mice were anesthetized with isoflurane (Patterson Veterinary, Devens, MA) and were sacrificed by decapitation. Brains were rapidly removed and transferred into ice-cold, pre-oxygenated (95% O2/5% CO2) artificial cerebral spinal fluid (ACSF) consisting of (in mM): NaCl (126), KCl (2.5), NaH2PO4 (1.2), CaCl2 (2.4), MgCl2 (1.2), NaHCO3 (25), glucose (11), pH adjusted to 7.4. Tissues were sectioned into 220 μm-thick coronal slices containing the striatum with a vibratome tissue slicer (Leica VT1000S, Vashaw Scientific, Norcross, GA). Brain slices are placed in a submersion recording chamber (Warner Instruments, Hamden, CT, USA) that was modified to prevent leakage (i.e., static flow system) and bubbled with 95% O2/5% CO2 gas to maintain pH at 7.2–7.4 and perfused at 1 ml/min at 36 °C with oxygenated ACSF.

Carbon Fiber Electrodes and Fast Scan Cyclic Voltammetry

For fast scan cyclic voltammetry (FSCV) recordings, carbon fiber electrodes were prepared by inserting a 7.0 μm diameter carbon fiber into borosilicate glass capillary tubing (1.2 mm outer diameter; A-M Systems, Sequim, WA, USA) under negative pressure and subsequently pulled on a vertical pipette puller (Narishige, East Meadow, NY, USA). The carbon fiber electrode (CFE) was then cut under microscopic control with 30-70 μm of bare fiber protruding from the end of the glass micropipette. The CFE was backfilled with 3 M KCl. Voltammetric recordings of dopamine signals were performed and analyzed using Demon Voltammetry and Analysis Software (Demon Voltammetry and Analysis; Yorgason, Espana, & Jones, 2011). The carbon fiber electrode (7 μm X ~150 μm) potential was linearly scanned as a triangular waveform from -0.4 to 1.2 V and back to -0.4 V at a scan rate of 400 V/s. Cyclic voltammograms were recorded at the carbon fiber electrode every 100 msec by means of a potentiostat (Dagan Corporation, Minneapolis, MN).

Dopamine release was evoked every 2 min through a bipolar stimulating electrode. Fig. 2 demonstrates that dopamine becomes oxidizes into dopamine-o-quinone at 0.6 V on the initial voltage ramp and then reduces back to dopamine at -0.2 V upon the downward voltage ramp. Single pulse baseline dopamine signals were collected (0.5 msec, 350 μA) until signals were stable across 4-5 collections. Catharanthine was then dissolved into ACSF and applied on the slice via bath application.

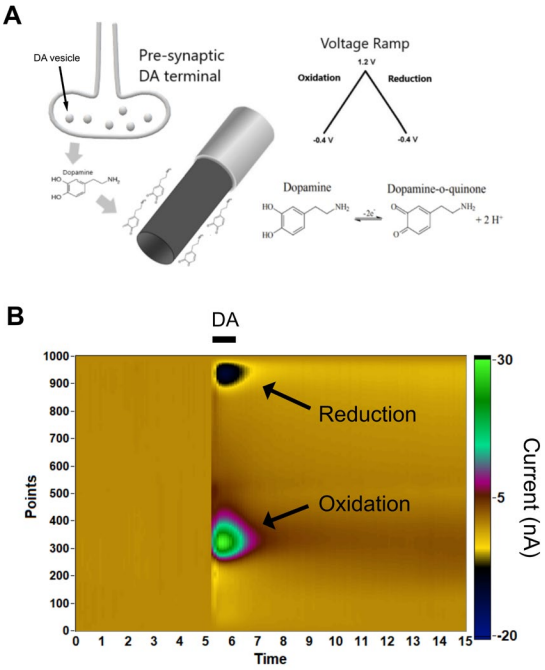

Figure 2. Analysis of dopamine via Fast Scan Cyclic Voltammetry (FSCV). (A) Dopaminergic pre-synaptic terminals release dopamine (DA), which is subsequently converted to DA-o-quinone and reduced back to DA upon exposure to a triangular waveform that is applied through a carbon fiber electrode. The magnitude of the oxidation/reduction is measured by a respective change in current. **(B)** The y-axis is a point system ranging from 0 to 1000 representing the voltage ramp (-0.4V to 1.2V to -0.4V) is plotted against time on the x-axis with color coded changes in current on the z-axis shown here with the DA oxidation peak at 5 seconds, 300 points (0.6V).

In frequency response experiments, each drug application including the control was first stabilized with single pulse recordings to stabilize the drug effect. Following the stabilization period, a protocol was run to test the effect of multiple pulses stimulating at different frequencies. The program was written in the Demon voltammetry program to have 3 single pulse baseline recordings, followed by five more recordings, each at 5 rapid pulses at the frequencies 10, 20, 40, 80 and 200 Hz.

Iontophoresis

Iontophoresis is a similar technique to FSCV in that it used CFEs inserted into a mouse brain slice to measure DA kinetics, except DA is exogenously applied through a second electrode

instead of electrically evoking a physiological dopamine response from the brain slice. Iontophoresis specifically measures the reuptake of exogenously applied DA into the slice through DA transporters (DATs). Application of DA saturates the DATs, allowing us to fit the curve to the Michaelis-Menten equations to calculate K_m and V_{max}.

Patch clamp electrophysiology

Horizontal brain slices (250–300 mm thick) were obtained from transgenic ChAT mice viral injected with channel rhodopsin 2 (ChR2) inserted into the membrane of striatal cholinergic interneurons (CINs) using a vibratome slicer. Slices were maintained at 36 degrees Celsius in oxygenated ACSF and visualized with infrared interference contrast microscopy. A glass pipette was pulled with a micropipette puller to have a sharp tip and is filled with 150 mM NaCl. The pipette is placed close to a neuron in the slice while applying positive air pressure through the pipette until it is resting directly on the membrane of the neuron. Then the positive air pressure is released, and a seal is formed between the pipette and the neuron. In our experiments, we aimed to obtain a "loose patch" between 10-50 MΩ between the membrane and the pipette to enable us to record the firing rate of the cell. We compared the difference in firing rate in CINs between pre and post drug administration.

Oocyte Electrophysiology

Protocols (No. 17-07020) for obtaining oocytes from *Xenopus laevis* frogs were approved by the University of Utah's Institutional Animal Care and Use Committee. Frogs were purchased from Xenopus1 (Dexter, MI, USA) and maintained by university personnel in an AAALAC accredited facility. Oocytes were obtained from frogs anesthetized with 0.4 % wt/vol Tricaine-S

(Thermo Fisher Scientific, Waltham, MA, USA) and were sacrificed after removal of the ovarian lobes.

Methods for the preparation of cRNA constructs for expression of nAChRs in Xenopus laevis oocytes have been previously described (Hone et al., 2018). Clones for human β2, β3, β4 and the rat α6/α3 construct were provided by J. Garrett (Cognetix, Salt Lake City, UT, USA). The human α6/α3 construct was provided by J.M. Lindstrom (University of Pennsylvania, Philadelphia, PA, USA), and rat β2, β3, and β4 clones were provided by C. Luetje (University of Miami, Miami, FL, USA). The α6/α3 constructs were used because injection of oocytes with cRNAs encoding human or rat α6 subunits results in few or no functional receptors when co-expressed with β2 and β3 subunits (Dowell et al., 2003; Kuryatov, Olale, Cooper, Choi, & Lindstrom, 2000). Furthermore, functional human α6β4 nAChRs also fail to express in oocytes (Hone et al., 2021). For the purposes of this work, α6/α3 refers to the chimeric expression construct used here for functional studies of α6/α3β2β3 and α6/α3β4 nAChRs in oocytes.

Stage IV-V oocytes were injected with equal ratios cRNAs encoding cloned human or rat nAChR subunits and subjected to two-electrode voltage-clamp electrophysiology 3-5 days after injection. The concentrations of acetylcholine (ACh) used were 100 μM for α6/α3β2β3 and 300 μM α6/α3β4 nAChRs. ACh was applied at 60 s intervals for one second. For the assessment of (+)-catharanthine activity, oocytes were continuously perfused with frog saline (control solution) consisting of 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1.0 MgCl2 and buffered to pH 7.4 with 5 mM HEPES and NaOH. Bovine serum albumin 0.1% (wt/vol) was added to all solutions to prevent non-specific adsorption of the compound to the tubing and glassware. The oocyte membranes were clamped at a holding potential of -70 mV and stimulated with ACh for 1 s once every 60 s, until a

stable baseline-response was observed. The saline was then switched to one containing the antagonist and the ACh responses monitored for changes in amplitude. (+)-Catharanthine sulfate was prepared as 10 mM stock solutions in distilled water and added to perfusion solution to obtain concentrations in the 0.1-100 μM range. Each concentration was perfused until a steady-state level of inhibition of the ACh-gated currents was observed.

Microdialysis

Using a surgical drill, a small hole was created in the skull of a mouse at the coordinates 1.5mm anterior of bregma, 0.9 mm lateral of midline to give access to the NAc. A microdialysis probe (MD-2211 1mm Membrane Probe, BASi Instruments) was lowered 4.2 mm into the NAc. The probe was used to flow artificial cerebrospinal fluid (aCSF) at a rate of 1-2 uL/min. The dialysate samples were collected every 20 min for two hours after injection. The samples were analyzed using a Thermo Fischer Ultimate 3000 High Performance Liquid Chromatography (HPLC) instrument with detection by a Coulochem III electrochemical detector. This technique determined how much DA is in each sample in order to monitor its levels over time.

Elevated Plus Maze

The elevated plus maze (EPM) paradigm is used to measure anxiety related behaviors. The EPM apparatus consists of a "+" shaped maze elevated above the floor with two oppositely positioned enclosed arms with high walls, two oppositely positioned open arms with no walls and a center area between the four arms. Each mouse is placed into the center of the maze and then allowed to roam freely for 5 min while being timed. The time that a mouse spends in the open arms of the maze corresponds with a lack of anxiety. An increase in time spent in the open arm from pre-treatment to post-treatment indicates an anxiolytic effect elicited by the drug under study.

Marble burying

Mice in the natural environment often bury their food or other objects when stressed. The marble burying test takes advantage of this instinctive behavior to measure anxiety-like behavior in mice. Each mouse is placed in a container filled half-way with corncob bedding with a set number of marbles placed in a grid inside. After 30 min, the number of marbles that were more than three-quarters buried were counted. We compared the number of marbles buried between mice that received saline (control) and catharanthine injections.

24-hour two-bottle choice drinking

Mice were given continuous access to 2 water bottles placed on either side of a cage. After a 1-week habituation period, mice were given 15% EtOH in one bottle and water in the other. Bottle weights were measured multiple times a day to quantify amount drank in grams. Each day, the bottle placement was switched to reduce the possibility of conditioned-place preference. Mice were subsequently exposed to EtOH vapors in a chronic intermittent exposure (Martinez-Pinilla et al., 2020) model of 4 days on, 3 days off. Drinking was measured again following 4-5 weeks of exposure to EtOH vapor.

Statistical analysis

Dopamine release was analyzed in Demon Voltammetry software (Yorgason et al., 2011) and measured at peak oxidation currents. The software performs a running subtraction of capacitative currents to account for changes in extracellular DA levels and to reduce noise. Post-subtracted data is then compared across time against calibrated DA cyclic voltammograms, from which it calculates an r^2 value to measure signal clarity. Statistics were performed in R studio and figure creation was done in IGOR Pro (Wavemetrics, Lake Oswego, OR). Single ANOVAs were used to compare concentration specific differences between drug applications with Tukey HSD

correction method for post-hoc analysis. Linear regression models were fit on catharanthine/ 18-MC dose response curves to measure correlation between DA release and concentration of coronaridine congeners.

RESULTS

Effect of Coronaridine Congeners on Dopamine Release and Dopamine Kinetics

Fast scan cyclic voltammetry (FSCV), which is the technique used here to determine the effects of catharanthine on dopamine (DA) release and kinetics, is an electrochemical technique capable of quantitatively measuring DA release through depolarizing nearby DA terminals via a simulating electrode. Further analysis of the time and velocity of the downward side of the DA curve can reveal the level of DA reuptake through dopamine transporters (DATs). It is known that catharanthine inhibits nicotinic acetylcholine receptors (nAChRs), so we hypothesized that catharanthine would inhibit evoked DA release. Catharanthine is already known to inhibit both the serotonin transporter (SERT) and the DAT, which would elevate baseline DA levels, however due to FSCV employing background subtraction, any effect catharanthine has on basal levels of DA should be subtracted out in the DA peak. We also know that 18-MC has the opposite effect of reducing basal DA levels making it unlikely that 18-MC similarly inhibits the DAT. Additionally, catharanthine is known to inhibit α3 and α9α10 nAChRs subunits, therefore, since α3 nAChRs subunits are expressed on DA terminals in the NAc, catharanthine should inhibit DA release, which is what we observed (Fig. 3A, B). A color plot representation of scanning for dopamine release over a range of voltages (y-axis) over time (x-axis) with corresponding increases in current (z-axis) with subsequent inhibition by catharanthine is shown (Fig. 3B).

We found that both catharanthine base and catharanthine sulfate significantly inhibit DA release in the NAc in a concentration dependent manner with half maximal inhibitory concentrations (IC50s) of 30 and 20 μM respectively (Fig. 3C). Catharanthine base significantly inhibits DA release at 30 and 100 μM in an ANOVA model with Tukey adjustment (overall: F =

35.91, p = 1.914e-10, 30 μM: 95% confidence intervals (CI) [-67.2, -26.9], $p < 0.0001$; 100 μM: 95% CI [-107.0, -57.5], $p << 0.0001$, Fig. 3C). Catharanthine sulfate, which has additional sulfate groups added to improve solubility, significantly inhibits DA at 10, 20 and 30 μM (overall: F = 32.54, p = 6.099e-10, 10 μM: 95% CI [-53.4, -2.3], p = 0.0267, 20 μM: 95% CI [-74.7, -23.6], p = 0.0000506; 30 μM: 95% CI [-108.3, -59.6], $p << 0.0001$, Fig. 3C). There are no results with catharanthine sulfate at 100 μM due near complete inhibition of the DA signal at 30 μM. 18-MC also significantly inhibits evoked DA release in the NAc, but only to 79.6% of the baseline at 100 μM (F = 3.830, p = 0.00658, Fig. 3C). Fitting a linear regression to our model with an adjusted r-squared of 0.4204, we see that catharanthine base and catharanthine sulfate inhibit DA release significantly more than 18-MC across all doses (t = -2.578 and -4.630, p = 0.0114 and 1.08e-05 respectively, Fig. 3C). In this model, we also see that concentration significantly predicts DA release across the 3 different drugs (t = -8.298, p = 4.59e-13, Fig. 3C). Adjusting our regression model to compare against catharanthine base, we see that catharanthine sulfate inhibits DA release significantly more than catharanthine base across all doses (t-value = -2.019, p = 0.0461, Fig. 3C). Comparing the three coronaridine congeners at 30 μM, we see that catharanthine base and catharanthine sulfate significantly inhibit DA release more than 18-MC (F = 5.94 and 13.01, p = 0.0288 and 0.0036 respectively, Fig. 3D). Catharanthine base inhibits DA release more than 18-MC at 100 μM (F = 15.19, p = 0.00297, Fig. 3E).

Analyzing the DA curve, we can determine the effects of catharanthine on DA reuptake using FSCV. Catharanthine base significantly slowed the downward side of the DA curve at 30 and 100 μM, increasing the time it takes for DA to return into the DA pre-synaptic terminals in an

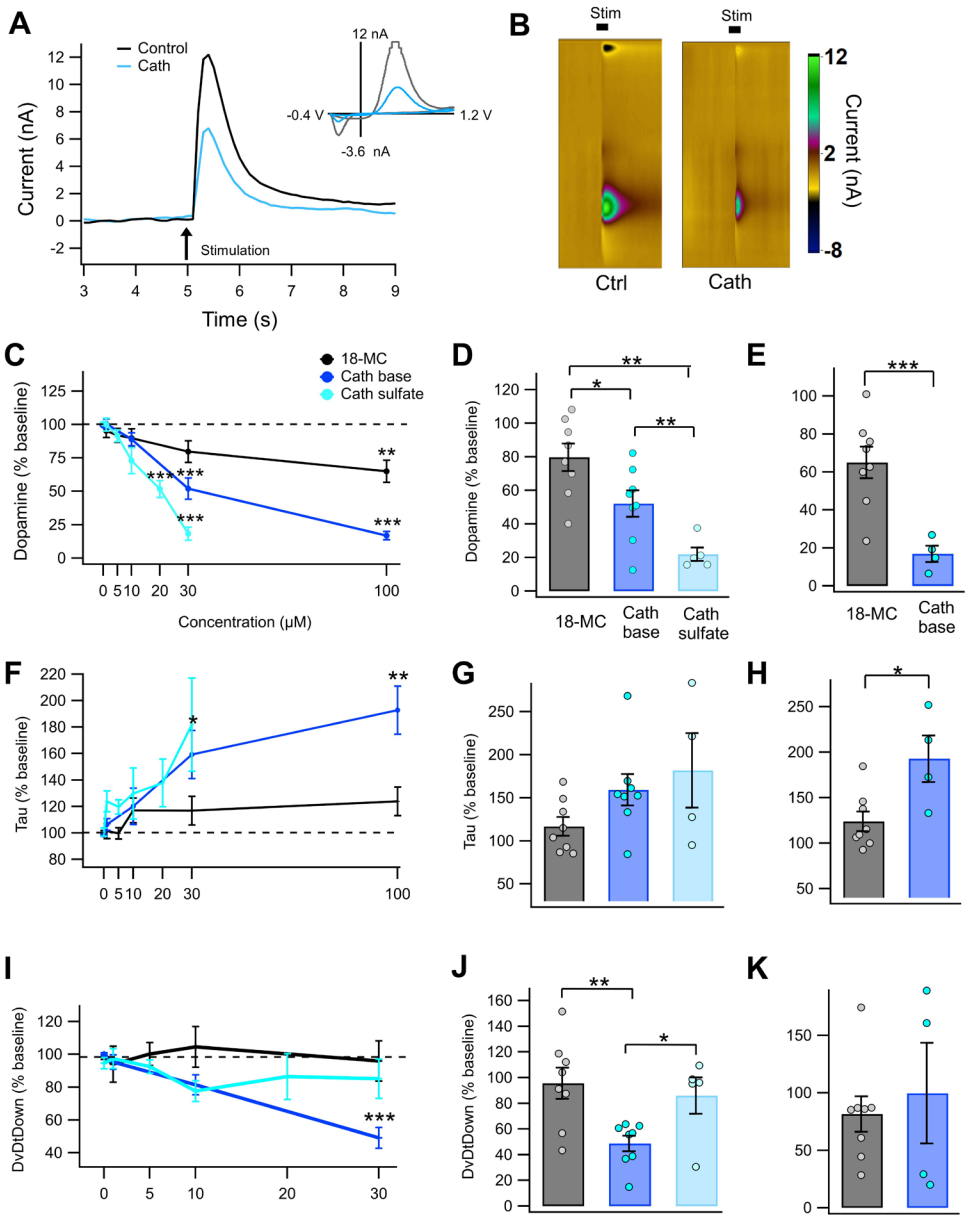

Figure 3. The effects of coronaridine congeners 18-methoxycoronaridine (18-MC), catharanthine base and catharanthine sulfate on evoked DA release and reuptake. (A) Example current trace (I/T) demonstrating DA release with overlaid effect of catharanthine base (30 μM). (B) Corresponding 3-dimensional color plots (V/T over I) demonstrating DA signal and effect of catharanthine base (30 μM). (C, D, E) Comparison of 18-MC, catharanthine base (cath base) and catharanthine sulfate (cath sulfate) on DA peak height. (D) In depth comparison at 30 μM. (E) In depth comparison at 100 μM. (F, G, H) Comparison of 18-MC, cath base and cath sulfate on DA reuptake, measured by Tau. (G, H) similar to (D, E). (I, J, K) Comparison of 18-MC, cath base and cath sulfate on downward velocity (DvDtDown), a second measure of reuptake. 100uM DvDtDown data not included due to inaccuracies of this variable becoming more pronounced with smaller signals. (J, K) similar to (D, E). Asterisks *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

ANOVA (overall: F = 6.7266, p = 0.000683; 30 μM: t = 3.401, p = 0.002105; 100 μM: t = 4.3, p = 0.000199, Fig. 3F). Catharanthine sulfate also significantly slows DA reuptake, increasing tau at 30 μM (t = 2.726, p = 0.0123, Fig. 3F). 18-MC only significantly slows DA reuptake at 100 μM (t = 2.061, p = 0.0462, Fig. 3F). There were no significant differences in tau between catharanthine base, catharanthine sulfate or 18-MC at 30 μM (Fig. 3G). Catharanthine base significantly increased tau compared to 18-MC at 100 μM, indicating that catharanthine has a higher affinity for the DAT than 18-MC (F = 8.79, p = 0.0142, Fig. 3H). Fitting a linear regression model to our data with adjusted r2 = 0.1959, catharanthine base and catharanthine sulfate inhibit DA reuptake, significantly increasing tau more than 18-MC (F = 2.674 and 3.086, p = 0.00875 and 0.00262 respectively, Fig. 3F). Adjusting our regression model to compare against catharanthine base, we see that catharanthine sulfate does not significantly inhibit DA reuptake more than catharanthine base (F = 0.537, p = 0.592, Fig. 3F).

Catharanthine base significantly slowed the downward velocity (DvDtDown) of the DA curve at 30 μM, but not at 100 μM, due to the sensitivity of this variable to fluctuations in the current traces, which are present at increased inhibition of DA at higher doses of catharanthine (Fig. 3I). We observed a significant difference in DvDtDown between catharanthine base and 18-MC, further supporting the idea that catharanthine base has greater affinity for the DAT than 18-MC (F = 11.44, p = 0.00447, Fig. 3J). Additionally, catharanthine base slows DvDtDown more than catharanthine sulfate significantly more at 30 μM (F = 7.107, p = 0.022, Fig. 3J). Oddly, catharanthine sulfate did not slow DvDtDown significantly across the concentrations. There was no significant difference between catharanthine base and 18-MC at 100 μM (Fig. 3K). Fitting a

linear regression model to our data, we did not observe any significant difference between catharanthine base, catharanthine sulfate or 18-MC across concentrations (Fig. 3I).

Pharmacology of catharanthine

In isolating the mechanism of action for catharanthine induced inhibition of DA release, we tested a variety of receptor antagonists in the presence of catharanthine in slice. We first tested nAChR antagonists α-conotoxin MII, dihydro-β-erythroidine (DHβE) and mecamylamine, which are α6, α4 and a general nAChR antagonists, respectively. We observed that DHβE 300 nM and 1 μM significantly inhibited DA release on their own to 38.9% and 46.2% of the baseline with standard error 7.73 and 11.17 respectively ($F = 59.5$ and 13.08, $p = 0.000249$ and 0.00681 respectively). Mecamylamine 2 μM and 10 μM also significantly inhibited DA release to 44.9% and 35.5% of the baseline with standard error 7.97 and 9.06 respectively ($F = 47.59$ and 51.83, $p = 1.65e\text{-}05$ and 0.000364). MII (500 nM) did not significantly inhibit DA release on its own.

Catharanthine sulfate (20 μM) induced inhibition of dopamine (DA) release is completely blocked by α6 and α4 nAChR antagonists MII and DHβE respectively and partially blocked by general nAChR antagonist mecamylamine in a concentration dependent manner (Fig. 4A, B). Example current traces demonstrate no change in peak height following bath administration of catharanthine sulfate (20 μM) (Fig. 4A). Catharanthine still inhibits DA release in the presence of 300 nM DHβE ($F = 21.51$, $p = 0.00355$), but not in the presence of 1 μM DHβE ($F = 2.273$, $p = 0.17$, Fig. 4B). Catharanthine also still inhibits DA release in the presence of 2 μM mecamylamine ($F = 38.64$, $p = 4.48e\text{-}05$), but it does not with 10 μM mecamylamine ($F = 2.917$, $p = 0.139$, Fig.

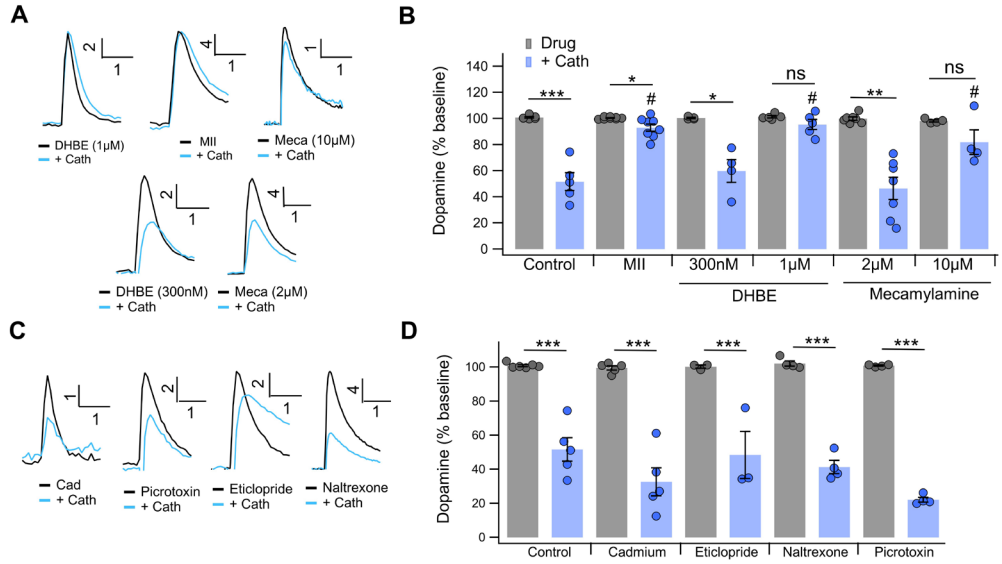

Figure 4. Mechanism of action for catharanthine sulfate in inhibition of DA release in the NAc. (A) Example current traces of nicotinic acetylcholine receptor (nAChR) antagonists α-conotoxin MII, DHβE and mecamylamine (Meca) with and without catharanthine sulfate (20 μM), normalized to the addition of the nAChR antagonist. (B) Effect of various nAChR antagonists on blocking catharanthine induced inhibition of DA release in the NAc. (C) Example current traces of receptor antagonists for voltage-gated calcium channels (cadmium), $GABA_A$ receptors (picrotoxin), DA D2 receptors (eticlopride) and delta opioid receptors (naltrexone). with catharanthine sulfate (20 μM). (D) Effect of other receptor antagonists on blocking catharanthine induced inhibition of DA release in the NAc. Asterisks * and ** indicate $p < 0.05$ and $p < 0.01$ respectively, ns = no significance. # indicates statistical significance to catharanthine sulfate control group.

4C). Our results also indicate that catharanthine is blocked in the presence of 500 nM a-conotoxin MII with catharanthine normalized to administration of MII, inhibiting DA release to 93% of the baseline with standard error of 2.73, however this effect is significant in a one-way ANOVA ($F = 7.045$, $p = 0.0189$, Fig. 4C). Catharanthine sulfate (20 μM) inhibits DA release significantly more than catharanthine sulfate in the presence of DHβE 1 μM, mecamylamine 10 μM and MII (500 nM) in a two-way ANOVA with Tukey adjustment (95% CIs [21.7, 65.8], [6.92, 53.6] and [21.5, 61.2], $p = 0.0001342$, 0.0087405 and 0.0000755, Fig. 4B).

To determine whether catharanthine acts on other receptors previously reported as targets for ibogaine, we tested catharanthine in the presence of cadmium, picrotoxin, eticlopride and naltrexone, which are antagonists for voltage-gated calcium channels, $GABA_A$ receptors, DA D2

receptors (D2Rs) and delta opioid receptors (DORs) respectively. Cadmium significantly inhibited DA release on its own to 50.8 ± 7.58 % of the baseline ($F_{1,6}$ = 38.58, p = 0.000804), however the other antagonists picrotoxin, eticlopride and naltrexone did not significantly affect DA release on their own ($F_{1,6}$ = 0.747, p = 0.421; $F_{1,4}$ = 1.032, p = 0.367; $F_{1,4}$ = 0.173, p = 0.695 respectively). Catharanthine sulfate (20 µM) did significantly inhibit DA release in the presence of cadmium, picrotoxin, eticlopride and naltrexone ($F_{1,6}$ = 44.64, p = 0.000545; $F_{1,6}$ = 3266, p = 1.93e-09; $F_{1,4}$ = 14, p = 0.0201; $F_{1,4}$ = 1228, p = 3.96e-06; respectively, Fig. 4D).

Catharanthine slows dopamine reuptake in a concentration dependent manner

Following vesicular release of DA in the brain, DA is funneled back into the pre-synaptic terminals through DATs. Using FSCV, evoked DA signals can transiently increase or decrease over time, changing the amount of DA reuptake observed, thus making it less efficient in measuring DA reuptake. Iontophoresis can more directly measure DA reuptake by externally applying a fixed amount of DA to the slice and recording the change in current as DA is funneled into the pre-synaptic terminals. Using iontophoresis, we can evaluate DA reuptake and thereby measure the activity of the DAT. We hypothesize that due to catharanthine's affinity for inhibiting the DAT, catharanthine will slow reuptake of iontophoretically applied DA in a concentration dependent manner. Following external application of DA, we observe a sharp increase in current with subsequent

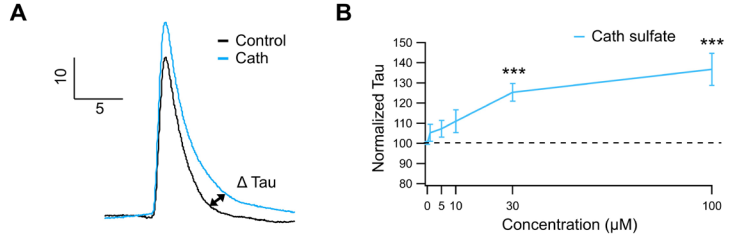

Figure 5. Catharanthine inhibits DA reuptake through inhibition of the DAT. (A) Example current trace demonstrating effect of iontophoretically applying DA to a brain slice before and after exposure with catharanthine base (30 µM). DA reuptake through DA transporters (DAT) is measured through the downward side of the current trace. Catharanthine base (30 µM) slows DA reuptake through blocking the DAT. (B) DA reuptake is slowed in a concentration dependent manner via catharanthine. Asterisks *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

slowing of the downward slope of the curve with 30 μM catharanthine sulfate (Fig. 5A). We found that catharanthine significantly slowed DA reuptake, increasing tau across all doses in a linear regression model ($F_{1, 31}$ = 27.84, p = 9.71e-06, Fig. 5B). In a two-way ANOVA with Tukey adjustment, catharanthine sulfate increases tau at 30 and 100 μM (95% CIs [0.066, 0.44] and [0.13, 0.51], p = 0.00379 and 0.000212, Fig. 5B).

Catharanthine inhibits cholinergic interneuron excitability in the nucleus accumbens core

Next, we investigated whether catharanthine would have any effect on the firing rate of striatal cholinergic interneurons (CINs). It is known that CINs modulate DA levels through local acetylcholine release onto pre-synaptic DA terminals expressing nAChRs. Thus, we hypothesized that catharanthine would inhibit DA release through inhibition of striatal CINs. To confirm this hypothesis, we patched onto CINs in the NAc in a cell-attached voltage-clamp configuration with a "loose seal" from 10-50 MΩ to enable recording of firing rate of the neuron. Bath application of catharanthine sulfate in increasing concentrations demonstrated inhibition of CIN firing rate (Fig. 6A). Fitting a linear model to our curve with an adjusted r^2 = 0.6914, catharanthine sulfate significantly inhibited CIN firing rate ($F_{1, 25}$ = 59.26, p = 4.705E-08, Fig. 6B). In a two-way ANOVA, we found that catharanthine sulfate specifically inhibits CIN firing rate at 10, 20, 30 and 100 μM (t = -2.70, -2.38, -4.85 and -7.74, p = 0.0137, 0.0273, 9.62e-05 and 1.91e-07 respectively, Fig. 6B).

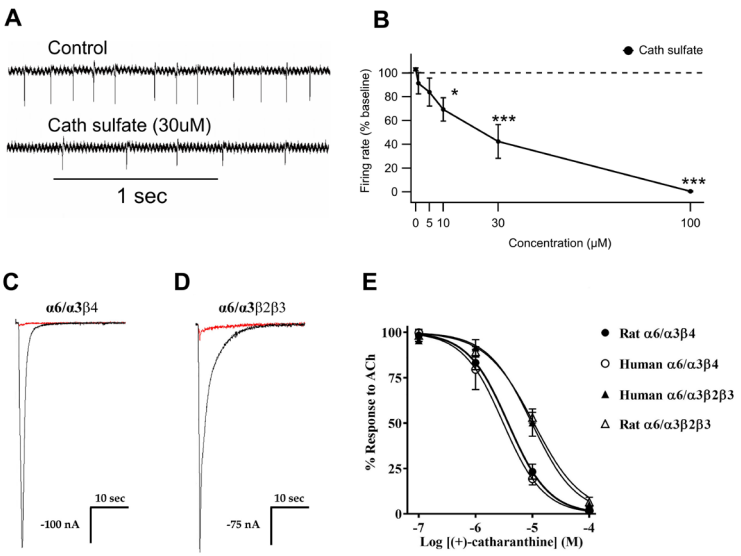

Figure 6. Catharanthine inhibits striatal cholinergic interneurons (A,B) Firing rate recordings were obtained via patch clamp electrophysiology on cholinergic interneurons (CINs) in the nucleus accumbens. (A) Example recordings with catharanthine sulfate (30 μM) (B) Dose response curve for catharanthine sulfate. (C,D,E) . Effect of (+)-catharanthine on human and rat α6/α3β4 and α6/α3β2β3 nAChRs expressed in *Xenopus* oocytes. (C,D) ACh-evoked responses of α6/α3β2β3 or α6/α3β4 receptors were recorded at 100 μM and 300 μM ACh, respectively, using two-electrode voltage-clamp at a holding potential of -70 mV. (C,D) Examples of current traces from oocytes expressing human α6/α3β4 (C) or rat α6/α3β2β3 (D) nAChRs, and the inhibition produced by 100 μM (+)-catharanthine (red traces). (c) Concentration-response curves for (+)-catharanthine (mean ± SD; n = 4 independemt determinations) at rat (●) and human (○) α6/α3β4 nAChRs and rat (Δ) and human (▲) α6/α3β2β3 nAChRs, gave IC_{50} values of 3.7 ± 0.5 μM and 10.6 ± 1.4 (rat) and 3.1 ± 0.6 μM and 9.8 ± 1.7 μM (human), respectively. Asterisks *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

To determine the specificity of catharanthine in inhibiting nAChR with α6 and α3 subunits, we used cell cultures of oocytes specially derived to express α6/α3β4 and α6/α3β2β3 nAChRs. We then used 2 electrode voltage-clamp with a holding potential of -70mV and bath applied 100 μM acetylcholine (ACh) to stimulate the nAChRs. We subsequently bath applied 100 μM catharanthine, which completely blocked the ACh induced currents (Fig. 6E).

Effect of catharanthine on in-vivo dopamine dynamics

Although catharanthine had fairly robust effects on evoked DA release in the slice preparation ex-vivo, we wanted to determine if it was active in-vivo. We know that catharanthine has specificity for the DAT, thus we hypothesized that catharanthine would increase extracellular DA levels. In contrast, 18-MC is known to decrease extracellular DA levels, however the mechanism is unknown. We injected catharanthine intraperitoneally (IP) into an anesthetized mouse and collected DA samples via microdialysis every 20 min. Catharanthine base significantly

elevated extracellular DA levels in a dose dependent manner in a linear regression model ($F_{1,33} = 8.09$, $p = 0.00758$, Fig. 7B). In a two-way ANOVA with Tukey HSD adjustment, catharanthine base significantly elevates extracellular DA at 5, 10, 20 and 50 mg/kg (95% CIs [43.7, 336.6], [100.9, 393.7], [100.2, 393.2] and [108.6, 401.5], $p = 0.00604$, 0.000283, 0.000292, 0.000185 respectively, Fig. 7B).

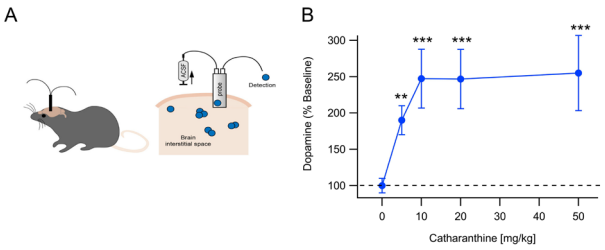

Figure 7. Catharanthine elevates extracellular DA levels in the NAc. (A) Illustration demonstrating in-vivo measurements of DA via microdialysis. DA is measured in the samples via electrochemical detection via HPLC. (B) Following increasing doses of IP catharanthine injections, DA samples are taken and measured at each concentration. Catharanthine increases extracellular DA content in the nucleus accumbens (NAc). Asterisks *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

Effects of catharanthine on self-administration of ethanol in a 2-bottle choice paradigm

To determine whether catharanthine possesses anti-addictive properties, we tested EtOH dependent mice divided into 2 groups, vehicle and catharanthine on EtOH drinking in a 24-hour two-bottle choice paradigm. Due to catharanthine's biphasic effects in elevating extracellular DA levels and inhibiting evoked DA release in the NAc, we hypothesized that catharanthine would both reduce drug craving and blunt the rewarding effects of EtOH, which would result in reduced drinking in EtOH-dependent mice.

Following a 1-week period of habituation in the cages, we measured the mice's baseline EtOH drinking behavior over the course of one week by placing one bottle with 16% EtOH and another bottle with water in the cages. We measured the bottle weights several times a day to quantify the amount of EtOH consumed. We injected half of the mice with catharanthine base (10 mg/kg) and the other half with saline. Next, we exposed the mice to chronic EtOH vapors in an

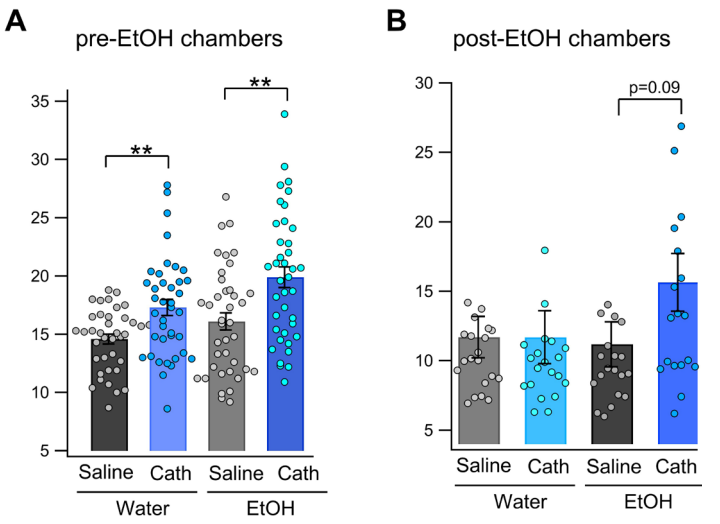

Figure 8. Effect of catharanthine on voluntary EtOH drinking. (A) Prior to being exposed to ethanol (EtOH) vapor chambers, mice were tested in the 24-hour two bottle choice (TBC) drinking paradigm for one week. Catharanthine (10mg/kg) or saline IP injections were given once daily. (B) After 5 weeks of exposure in EtOH vapor chambers we repeated the TBC procedure. Results were inconclusive. Asterisks *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively.

EtOH vapor chamber system with 16 hours of EtOH vapor per day. Following a period of 5-weeks of exposure to intermittent EtOH vapor, we tested EtOH drinking again using the same procedure used initially. Catharanthine base was again IP injected into half of the mice once per day. Surprisingly, we found that catharanthine increased both water and EtOH drinking prior to EtOH chambers ($F_{1,76} = 13.03$, $p = 0.000548$; $F_{1,76} = 12.23$, $p = 0.000788$, Fig. 8A). There were no statistical differences between groups after vapor chambers (Fig. 8B).

Catharanthine alleviates ethanol induced anxiety during withdrawal

Elevated plus maze. It is well known that withdrawal following heavy ethanol use can be one of the most stressful experiences that humans or animals can undergo. It has also been well established that ethanol use modulates the mesolimbic DA system, thus it has been theorized that the intense craving experienced during withdrawal results from reduced DA release in the NAc. Since catharanthine elevates extracellular DA levels, we theorized that catharanthine would reduce drug craving during EtOH withdrawal, thereby also reducing anxiety. In an elevated plus maze (EPM) paradigm, increased time spent in the open arm of the maze is correlated with reduced anxiety. Mice will typically spend more time in the closed arms of the maze where they feel more

protected, especially when they are stressed. We found that increased EtOH exposure significantly decreased time spent in the open arm in both saline and catharanthine exposed mice (saline: $F_{4, 10} = 8.67$, $p = 0.00274$; catharanthine: $F_{4, 10} = 9.17$, $p = 0.00221$, Fig. 9A). Interestingly, mice exposed to catharanthine significantly spent more time in the open arm of the maze compared to saline

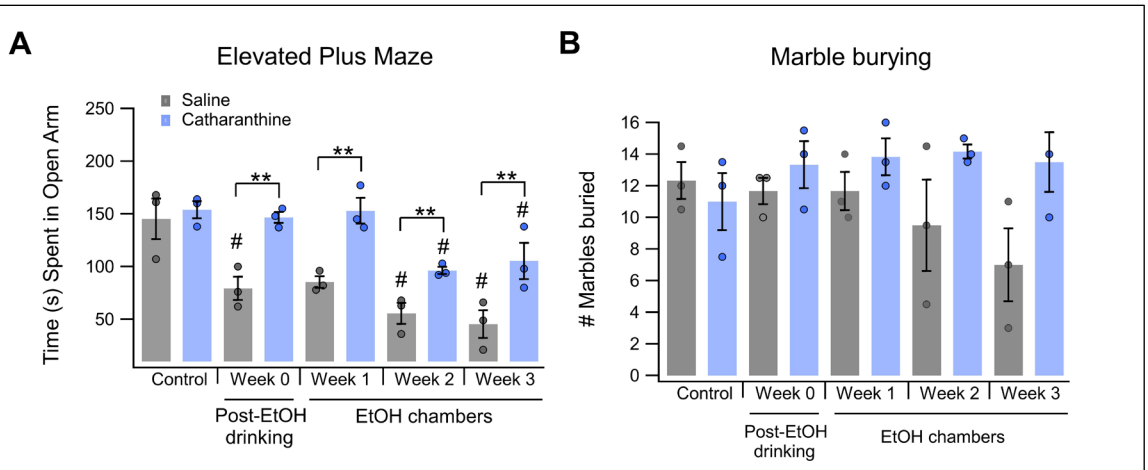

Figure 9. Catharanthine alleviates alcohol withdrawal induced anxiety. (A, B) Mice were tested on the elevated plus maze and marble burying procedures before exposure to alcohol and catharanthine (Control), after exposure to 1 week of EtOH drinking with once daily catharanthine sulfate (10 mg/kg) IP injections (Week 0) and once per week during a chronic intermittent exposure (CIE) model to alcohol in alcohol vapor chambers. No catharanthine injections were given during time spent in EtOH vapor chambers. Asterisks *, ** and *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. # indicates $p < 0.05$ for that groups respective control.

mice at all time points following exposure to EtOH (week 0: $F_{1,4} = 30.1$, $p = 0.00537$; week 1: $F_{1,4} = 25.5$, $p = 0.0072$; week 2: $F_{1,4} = 15.0$, $p = 0.0179$; week 3: $F_{1,4} = 7.72$, $p = 0.0498$; Fig. 9A). In a Tukey HSD adjustment, we found that mice exposed to catharanthine at weeks 2 and 3 in the EtOH vapor chambers spent significantly less time in the open arm compared to before the vapor chambers, but not at week 1 in the vapor chamber (week 1: 95% CIs [-52.8, 39.4], $p = 0.988$; week 2: 95% CIs [-109.4, -17.2], $p = 0.00763$; week 3: 95% CIs [-100.4, -8.22], $p = 0.0201$, Fig. 9A). Additionally, we found that mice exposed to saline significantly decreased the amount of time they spent in the open arm compared to control at time points week 0, week 2 and at week 3, but not at week 1 (week 0: 95% CIs [-109.1, -2.19], $p = 0.0405$; week 1: 95% CIs [-103.2, 3.81], $p = 0.0719$; week 2: 95% CIs [-132.8, -25.8], $p = 0.00449$; week 3: [-143.1, -36.2], $p = 0.0018$, Fig. 9A).

Marble burying. Marble burying is a behavioral paradigm that corresponds with natural behaviors exhibited by mice in the wild. Mice naturally bury their food or other objects when stressed, thus we can take advantage of this instinctive behavior to measure anxiety in the laboratory. Increases in the number of marbles buried is correlated with increases in anxiety. Oddly, we saw no significant differences between groups in the marble burying behavioral test (Fig. 9B), however saline mice tended to decrease the number of marbles buried with more time in the EtOH vapor chambers while catharanthine mice slightly increased the number of marbles they buried. The comparison at week 3 between saline and catharanthine groups is suggestive of a difference, but is not significant ($F_{1,4} = 4.74$, $p = 0.0951$, Fig. 9B). The lack of significant results in marble burying could be due to the low sample size in our data.

DISCUSSION

Catharanthine modulates dopamine release and reuptake in the nucleus accumbens

It has been well established that DA release in the nucleus accumbens (NAc) is involved in learning and potentially mediating the rewarding properties of drugs of abuse. We have shown in this study that catharanthine modulates DA release and reuptake in the NAc in the μM range, indicating that catharanthine may potentially influence the hedonic properties of drugs of abuse (Figs. 3-5). Our data suggests that catharanthine modulates DA through a biphasic mechanism inhibiting vesicular DA release while also slowing DA reuptake through inhibition of the DAT. Interestingly, we found that catharanthine sulfate inhibits DA release significantly more than catharanthine base, which may be due to catharanthine sulfate's increase aqueous solubility (Fig. 3). Next, we tested 18-methoxycoronaridine (18-MC) on DA release, a compound that is also a coronaridine congener and has been found to decrease self-administration of EtOH and nicotine in animals. We found that 18-MC weakly inhibits DA release in a concentration dependent manner, but significantly less than catharanthine in a linear regression (Fig. 3).

Next, we wanted to determine the mechanism of action for catharanthine's inhibition of DA release in the NAc. We found that catharanthine's inhibitory effects were significantly reduced by DHβE, an α4 nAChR antagonist, with α-conotoxin MII, a selective α6 nAChR antagonist and with mecamylamine, a general nAChR antagonist (Fig. 4). For this reason, we suggest that the mechanism for catharanthine's inhibition of DA release is via inhibition of nicotinic acetylcholine receptors (nAChRs) containing either α4 or α6 subunits. Others have shown that catharanthine also inhibits α3 nAChRs, which are also expressed on DA terminals and such are also likely to be involved in catharanthine induced inhibition of DA release. We should also consider that the drugs DHβE and MII may have weak affinity for other nAChR subunits, thus future experiments

investigating this effect are warranted. It is currently unknown whether catharanthine induced inhibition of DA release occurs via nAChRs expressed on DA pre-synaptic terminals or on nAChRs expressed on CINs, however it is likely a combination of both.

Catharanthine's second mechanism in modulating DA is through slowing DA reuptake through inhibition of DATs expressed on DA pre-synaptic terminals (Fig. 3), which we were able to confirm through iontophoresis experiments recording the reuptake of externally applied DA onto a brain slice (Fig. 5). Due to catharanthine's affinity for serotonin transporters (SERT) and for dopamine's slight affinity for SERTs, it is possible that part of this effect is mediated by inhibition of SERTs. It appears that catharanthine base may have a greater affinity for the DAT, due to catharanthine sulfate's lack of a significant effect on downward velocity (Fig. 3). Future experiments could include analysis of 18-MC and catharanthine base on DA reuptake via iontophoresis, effect of α3 nAChR antagonists on catharanthine induced inhibition of DA release, genetic knockouts of α4 and α6 nAChRs to confirm catharanthine's mechanism of action and analysis of the effect of catharanthine on isolated serotonin release via FSCV.

Effect of catharanthine on cholinergic interneuron and nicotinic acetylcholine receptor excitability

Following our analysis of catharanthine on DA dynamics, we decided to determine whether catharanthine has any effect on the firing rate of CINs in the NAc. CINs are important modulators of DA release through releasing acetylcholine onto pre-synaptic terminals expressing nAChRs. Using cell-attached mode patch clamp electrophysiology, we found that catharanthine inhibits CIN firing rate in a concentration dependent manner with an IC50 of 30 μM (Fig. 6). This supports our theory that catharanthine inhibits evoked DA release partially through inhibition of striatal CINs.

Another future experiment we would like to do is test catharanthine on CIN firing rate in the presence of EtOH. We have shown that EtOH increases CIN firing rate in slice through interaction with nAChRs, thus it would be interesting to see if EtOH blocks catharanthine induced inhibition of CIN firing rate. Lastly, we would also like to investigate the effect of 18-MC on CIN firing rate and compare it with catharanthine to help us understand the differences seen in inhibition of DA release.

Effects of catharanthine on dopamine dynamics in-vivo

To better understand the effects of catharanthine on DA dynamics in the brain, we decided to measure extracellular levels of DA in a live animal using microdialysis and high performance liquid chromatography (HPLC) combined with electrochemical detection. In-vivo measurements are important to make because it shows you what happens to the brain on a systemic level. It is likely that catharanthine modulates a large variety of different brain regions due to how widely nAChRs are expressed in the brain. We hypothesized that catharanthine would slightly elevate extracellular DA levels in the NAc due to its affinity for the DAT. We found that catharanthine elevated DA levels to nearly 200% of the baseline at 5 mg/kg and to 250% of the baseline at 10 mg/kg (Fig. 7). Increasing doses of catharanthine to 20 and 50 mg/kg did not result in any further increase in DA levels, which was unexpected due to our results in-vitro showing increasing concentrations of catharanthine resulted in increased slowing of the DAT. It is possible that catharanthine acts through an additional mechanism to elevate DA levels in the NAc, however we currently don't have any data to support that. Furthermore, we suspected that the increases in extracellular DA observed following IP administration of catharanthine were due to catharanthine oxidizing at a similar voltage as DA during the HPLC analysis, which would confound our results.

So we tested catharanthine on its own and found that it did not oxidize similarly to dopamine in our HPLC analysis.

Based on our data ex-vivo and in-vivo, we suggest that catharanthine elevates extracellular DA levels in the NAc in mice through inhibition of the DAT, although it is possible that there are nAChR effects influencing this phenomenon. Further experimentation is warranted to better understand this effect. We suggest that further experiments investigate the effect of DAT knock-outs on extracellular DA levels following administration of catharanthine. We hypothesize that knock outs of the DAT would block the elevations in DA we observe in the NAc. Additional experimentation could also investigate DA levels in different brain regions that don't express the DAT, such as the cerebellum.

Effect of catharanthine on mice behavior

Ethanol self-administration. To determine the suitability of catharanthine as a treatment for addiction, we tested it on both EtOH self-administration and EtOH withdrawal induced anxiety (Figs. 8, 9). Unfortunately, our self-administration data was inconclusive due to a poor experimental design (Fig. 8). We were expecting to see a large increase in EtOH drinking following a 5-week period in EtOH vapor chambers designed to induce dependence. Instead, the mice drank nearly the same amount of EtOH following the vapor chambers compared to before the vapor chambers. A potential reason for this lack of results could be that we did not use the chronic intermittent exposure (CIE) model which has been shown to increase EtOH dependence. The CIE model consists of 4 days with vapor on and 3 days with vapor off for the mice to repeatedly go through withdrawal and to better learn the rewarding effects of EtOH. Our experimental design consisted of exposing the mice to EtOH vapor for 16 hours from 10 AM to 2

AM every day. Additionally, we started doing injecting pyrazole, an alcohol dehydrogenation inhibitor, once every 3 days half way through the 5-week period in the vapor chambers to slow EtOH metabolism in the mice. Pyrazole injections have been shown to increase blood EtOH levels in mice and to increase dependence, so it is possible that we didn't do pyrazole injections for enough time. Lastly, we also did not wait for sufficient time following the end of the EtOH vapor chambers to induce withdrawal before collecting EtOH drinking data. Mice will binge drink to dependence after several days of feeling the negative effects of withdrawal. We propose to redo this drinking experiment with the above mentioned adjustments to better understand the effect of catharanthine on drug craving and drug use.

Ethanol withdrawal induced anxiety. Withdrawal from heavy EtOH use has a large variety of negative side effects in both humans and animals, among them being severe anxiety. A pharmaceutical treatment that reduces anxiety during EtOH withdrawal, thereby reducing the negative reinforcing effects of EtOH, could potentially reduce drug craving. We found that catharanthine reduces anxiety-like behaviors during EtOH withdrawal, specifically in the amount of time that they spend in the open arm of the elevated plus maze (Fig. 9). In these experiments, we tested mice before and during exposure to EtOH vapor chambers in a CIE model of 4 days of vapor with 3 days without vapor. We tested mice in both the elevated plus maze and marble burying paradigms once a week on the first day without vapor, 10 hours into withdrawal. Significant differences were noted between catharanthine and vehicle groups on each time point tested, indicating that catharanthine has an anxiolytic effect in mice. We also observed a significant decrease in the time the control group spent on the open arm, indicating that the withdrawal from the EtOH vapor chambers was anxiety inducing. It is known that there are residual effects from

testing mice in the EPM repeatedly, which may partly confound our results. However, due to the significant differences seen between the catharanthine and vehicle mice, this is not likely to overly influence the data.

There remains a lot of work to do in fully determining the effects catharanthine has on the brain and whether catharanthine is a suitable treatment for addiction. Due to catharanthine being a naturally occurring substance, it could potentially be acting on other receptors, further influencing behavior in unexpected ways. Future studies investigating catharanthine should determine its effects in different brain regions, different neurotransmitters in the brain and further characterize its effect on drug seeking behavior in rodents.

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