2021

REWARD-RELATED EFFECTS OF AMPHETAMINES ADMINISTERED BY ELECTRONIC CIGARETTE

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

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July 2021

Acknowledgements:

I would like to take this opportunity to thank my family, who have supported me relentlessly throughout my educational career and in my life. I would also like to thank Dr. Keith Shelton for granting me the opportunity to study in his lab and for guiding me through my master's and thesis work. Additionally, thank you Dr. Katherine Nicholson for your assistance and time in helping me carry out my lab research. I also would like to thank Dr. Krista Scoggins, the director of the Pharmacology department during the first portion of my master's degree, for assisting me in my journey and helping ensure I have fulfilled my requirements. Thank you to my committee members, Dr. Keith Shelton, Dr. Joseph Porter, Dr. Jennifer Wolstenholme, and Dr. David Walentiny for your continual feedback and for investing time and energy into my project.

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List of Abbreviations

Coc: Cocaine

d-Amph: d-Amphetamine

ICSS: Intracranial Self-Stimulation

IP: Intraperitoneal

Meth: Methamphetamine

PG: Propylene Glycol

SC: Subcutaneous

THC: Tetrahydrocannabinol

VG: Vegetable Glycerol

Abstract

REWARD-RELATED EFFECTS OF METHAMPHETAMINE ADMINISTERED BY ELECTRONIC CIGARETTE

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While e-cigarette vaporizers are primarily used for the delivery of nicotine, they can also be used to administer a wide range of other substances of abuse including stimulants such as d-amphetamine and methamphetamine. Methamphetamine and prescription amphetamines are widely abused throughout the United States and across the world leading us to believe there may be abuse potential with vaping methamphetamine as vaping becomes increasingly popular. In the present study, methamphetamine and amphetamine vapor were assessed using vapor capture methods. The vapor samples were then analyzed using liquid chromatography mass spectroscopy (LC-MS). Three experiments were carried out to examine the abuse potential of vaping methamphetamine. The first experiment was a locomotor assay, to determine if behaviorally relevant doses of methamphetamine were achieved under the chosen test conditions. The other two experiments were intracranial self-stimulation and a self-administration assay. Vapor analysis using LC-MS showed that the altered variables including voltage, flow rate, and puff time influenced the amount of drug vaporized and, in turn, would be likely to impact drug exposure. The locomotor assay revealed dose-dependent changes in total distance traveled with

methamphetamine given subcutaneously (at doses of 0.3, 1, and 1.7 mg/kg) as well as concentration-dependent changes in locomotion after vaporized methamphetamine exposure (at e-liquid concentrations of 25, 50, 100, and 200 mg/ml). Time spent in the zones along the wall was also assessed as a model of anxiety-like effects. Vehicle vapor as well as vaporized methamphetamine at higher e-liquid concentrations (100 and 200 mg/ml) produced anxiogenic-like effects based on time spent in the zones along the walls of the locomotor chamber. Methamphetamine vapor puffs did not serve as reinforcers in the self-administration assay. ICSS data indicated that d-amphetamine vapor did not facilitate performance but further experimentation should be done due to the small sample size and a number of other challenges encountered.

Introduction

BACKGROUND AND SIGNIFICANCE

Electronic Cigarettes (e-cigarettes, e-cigs, vapes) are electromechanical devices marketed as nicotine-delivery systems. The most widely used type of e-cigarettes heats and vaporizes a liquid commonly composed of varying proportions of propylene glycol, glycerol, and nicotine base and/or a nicotine salt. The popularity of e-cigarettes has been on the rise over the last decade. In 2015, 3.5% of U.S. adults were current e-cigarette users and by 2017, 5.6% of U.S. adults reported being either a current user or as having tried an e-cigarette (Rodu & Plurphanswat, 2018). Although the long-term health consequences of using these devices is still unknown, there is growing evidence that e-cigarettes may have a unique set of health consequences compared to combustible tobacco products (Eltorai et al., 2019; Rom et al., 2015); (Callahan-Lyon, 2014). While nicotine remains the primary drug used in e-cigarettes, the technology itself is adaptable to any of a number of other drugs with high abuse liability including psychomotor stimulants such as amphetamine, methamphetamine, and methylphenidate.

The use of electronic cigarettes as a delivery mechanism for abused stimulants is potentially alarming. In 2018, approximately 1.9 million people over the age of 12 years old reported using methamphetamine within the past year, corresponding to a 0.7 percent of the total United States population (NSDUH, 2018). In addition, roughly 2.1% of adults in the U.S. reported misusing amphetamine-containing medications, like Adderall and Vyvanse, and methylphenidate-containing medications, like Ritalin (Compton et al., 2018). This corresponds

to roughly 5.1 million adults abusing stimulant medications in 2018. Furthermore, over six million adolescents have been diagnosed with ADHD and in 2016, 62% of those adolescents diagnosed with ADHD were reported to be receiving pharmacotherapy, including stimulant medications like d-amphetamine and methylphenidate (Danielson et al., 2018). Given that over 300,000 people aged 12-25 are estimated to have used methamphetamine within the past year and millions report abusing other psychostimulants like amphetamine and methylphenidate (Jones & Salzman, 2020; NSDUH, 2018; Ritchie & Roser, 2019) coupled with the rising popularity of vaping among teenagers and young adults, there is the possibility of the emergence of vaping as a novel psychomotor stimulant delivery system. Given the higher e-cigarette use among adolescents, experimentation with their medications may be likely (Danielson et al., 2018).

Some anecdotal evidence exists that points to illicit drugs such as methamphetamine already being used in vaporizers with varying degrees of self-reported success. Such reports emphasize the ability to conceal the use of illicit substance in public places and being able to better regulate the rewarding effects of the drug (Greenhill, 2018; bluelight, 2014, https://www.bluelight.org/xf/threads/vaping-meth-2014.731118/). Many of the user reports emphasize the importance of using a sufficiently high concentration of drug in the e-liquid in order to get the desired effects. For instance, one user reported that when using a high concentration of methamphetamine dissolved in e-liquid, the effects were different compared to traditional smoking and the high was described as heavy-headed (bluelight, 2014, https://www.bluelight.org/xf/threads/vaping-meth-2014.731118/).

Unfortunately, at this time relatively little is known regarding the abuse liability or the pharmacological effects of amphetamines administered by vapor delivery. The available

literature will be discussed is subsequent sections of this paper but it is first necessary to briefly discuss the evolution of e-cigarettes into effective drug delivery devices.

ELECTRONIC CIGARETTES

Electronic cigarettes (e-cigarettes) have become commonplace as an alternative to smoked tobacco. E-cigarettes deliver nicotine in the form of a fine aerosol, which is commonly mislabeled as being a vapor, hence the slang term for e-cigarette use is "vaping". They are handheld devices, often small enough to fit in one's pocket and be taken anywhere like a pack of cigarettes. Common to all vaporizer-type e-cigarettes is a battery-heated resistance wire coil, much like that in an incandescent lightbulb or toaster. The coil is wrapped in an absorbent material such as cotton. Activation of the coil aerosolizes the e-liquid in the device, which is then inhaled. Increasing both wattage and voltage increases the amount of vapor produced. An e-cigarette's electrical resistance can be varied by the composition of the coil which is made from various alloys of stainless steel, nickel, or titanium. This also impacts the amount of vapor produced. There are several types of vaporizers, which have been categorized into four generations.

Figure 1. First, second, and third generation e-cigarette devices. A. Generic tobacco cigarette, B. First generation e-cigarette, C. Second generation e-cigarette with refillable tank, D. Third generation e-cigarette (also known as a box mod) with a larger refillable tank and the ability to manipulate voltage or wattage.

Stratton, k., Kwan, L., and Eaton, D. Public Health Consequences of E-Cigarettes (January 23, 2018), The National Academies Press, National Academies of Sciences, Engineering, and Medicine, doi/10.17226/24952, https://pubmed.ncbi.nlm.nih.gov/29894118/

First generation e-cigarettes referred to as a cig-a-like vaporizers, mimics a tobacco cigarette in appearance and feel. The heating coil voltage is low and fixed and the e-liquid cartridge in the unit is inaccessible to the user. Once the e-liquid has been depleted, the e-cigarettes is either disposed of as a whole or the fluid liquid reservoir is disposed of and replaced with a new, filled cartridge. The subsequent second generation vaporizers are referred to as clearomizers, which have a higher-volume tank that can be filled with e-liquid and allow the user to alter voltage. The atomizing unit is removable allowing the coil to be changed and the tank to be refilled with e-liquid chosen by the user. The third generation of e-cigarettes are referred to as "mods". Mods are far more sophisticated microprocessor-controlled e-cigarettes equipped with

powerful high-amperage batteries allowing manipulation of wattage and voltage. The greater ability to vary these parameters gives users more control over the amount of vapor produced and, concomitantly, the dose of drug administered. Third generation vaporizers can generally be classified as either variable-voltage (VV) vaporizers or variable-wattage (VW) vaporizers. Increasing wattage or voltage will both increase vapor output and likely the amount of drug delivered (Harvanko et al., 2018). The most recent evolution of e-cigarettes is the fourth generation pod-type vaporizers, such as Juuls, which utilize prefilled e-liquid pods. This generation contains a fixed voltage rechargeable battery but the design generally looks more akin to a USB drive than a traditional cigarette. Fourth generation devices are gaining rapid popularity due to being cheap, easy to use, and widely available. (Williams & Talbot, 2019). With several different types of vaporizers available for the delivery of nicotine and other substances of abuse, this raises questions over the advantages and disadvantages of vaping compared to traditional smoking.

TRADITIONAL SMOKING VERSUS VAPING

To the user of an illicit drug, vaping could provide a number of real or perceived advantages over traditional smoking or other more common routes of administration such as injection and insufflation. One of the most obvious advantages is covertness (Jenssen & Boykan, 2019). E-cigarettes vaporize drug rather than burn it, the exhaled vapor from an e-cigarette typically has the odor of the flavoring component of the liquid e-juice rather than a noticeable drug odor. Therefore, one could conceivably vape illicit drugs in public and it would be difficult

to differentiate illicit drug vapor from typical nicotine vapor. In addition to being not easily identified by law enforcement, there are also potential pharmacological advantages of vaping which may include enhancement of the intensity and speed of onset. Drugs delivered to the lungs are distributed by oxygenated arterial blood flow to highly perfused tissues such as the brain before being distributed to less well perfused tissues, potentially resulting in regionally specific tissue concentrations higher than that which would be the case following other routes of administration. As such, the effects of drug administered as vapors would be expected to be rapid. Further, in comparison to oral administration, a given dose would be expected to have greater bioavailability, since vaped drugs would avoid first pass metabolism. First pass metabolism is a phenomenon that occurs with some methods of administration where the concentration of certain drugs is substantially reduced before it reaches circulation (Herman & Santos, 2021). This is largely attributed to the metabolism of the drugs by the liver. This phenomenon is relevant because only certain routes of administration are susceptible to first pass metabolism. Inhaled drugs, such as the vaporized amphetamines being studied in the present project, avoid first pass metabolism. Therefore, not only are the effects of vaporized methamphetamine rapid due to rapid absorption through the lungs but also the drug has a high bioavailability with this route of administration. It has been long established that the speed of onset of effects of abuse drugs such as psychostimulants is directly correlated with the reinforcing effects, therefore, vaping may be a highly reinforcing method of administration (Kimmel et al., 2007; Balster & Schuster, 1973). However, at the present time, only a few studies have been conducted to explore the pharmacokinetics and pharmacodynamics of drugs other than nicotine administered as vapors and the extent to which this route of administration

may impact abuse-related effects of these drugs (Jaffe et al., 1989; Javadi-Paydar et al., 2019; Vendruscolo et al., 2018).

In contrast to other drugs of abuse, the effects of nicotine delivered by e-cigarettes has been studied in some detail. One notable difference between vaping and smoking is the pattern of drug delivery engaged in by traditional cigarette smokers versus vapers. Smokers typically smoke a cigarette in a single sitting while vapers inhale intermittently and may only take a single puff at a time. As a result, plasma nicotine levels of vapers have been shown to be more stable whereas the bolus dosing from cigarettes results in greater fluctuations (St.Helen et al., 2016). This difference in the pattern of intake, intermittent use versus bolus use in cigarette smoking and vaping respectively, suggests that further research into how plasma drug levels differ in vaping compared to that those of traditional smoking is important. The demographics of smokers and vapers are also dissimilar. For example, those who both smoke and use e-cigarettes, were significantly younger and more likely to be white, have more education, report a history of psychiatric co-morbidity, and smoke fewer cigarettes per day than traditional smokers. Because e-cigarette use is prevalent among a demographic in which substance abuse and tradition smoking is less common, it may therefore be the case that e-cigarette as a delivery mechanism result in new users of illicit drugs rather than simply resulting in existing users changing their preferred route of delivery (Piper et al., 2019).

While many factors associated with e-cigarette use are unique to humans, there are a number of aspects of the potential abuse liability and health consequences which might be addressed through the use of animal models. Pre-clinical research on e-cigarettes has increased over the past decade in direct response to their popularity. Several studies have attempted to produce replicable, reliable models of vaping in rodents. One of the earliest studies used sufentanil to assess a potential rodent vapor self-administration model (Jaffe et al., 1989). In this study, short bursts of an aerosol mist of sufentanil citrate generated by a nebulizer were delivered to rats in response to lever presses. The speed of acquisition of an operant response which produced drug effects was then examined. Rats given access to sufentanil vapor in overnight training sessions reached an average of one reinforcement per hour on a fixed ratio 5 schedule of reinforcement significantly sooner than did rats given access to water vapor. Responding maintained by sufentanil during 2 hour daily testing sessions was dose dependent. Substituting water vapor for each sufentanil concentration significantly reduced responding within 5-20 sessions.

Another more recent study purported to validate a rat model of the abuse-related effects of nicotine delivered by electronic nicotine delivery systems (ENDS) (Javadi-Paydar et al., 2019). Rats were implanted with radio telemetry devices for the reporting of temperature and activity and were exposed to vapor puffs under different conditions. Vapor inhalation conditions included propylene glycol (PG) vehicle, nicotine (1, 10, 30 mg/mL in the PG) and delta-9-THC (12.5, 25 mg/mL). The study reported that nicotine puff inhalation increased spontaneous locomotion and decreased body temperature of rats in a manner similar to that produced by

nicotine administered by other routes. Pretreatment with the nicotinic cholinergic receptor antagonist mecamylamine prevented the stimulant effects of nicotine vapor inhalation and attenuated the hypothermic response produced by nicotine. They also noted that combined inhalation of nicotine and THC resulted in apparently independent effects. Specifically, when combined together, nicotine and THC vapor produced additive effects on hypothermia whereas the effects on locomotor activity were antagonistic.

Another relevant study examined sufentanil delivered by a ENDS type device (Vendruscolo et al., 2018). Rats were trained in 2-hour daily sessions to perform an operant nose-poke response to receive 10 seconds of vaporized sufentanil delivery into a sealed exposure chamber. Rats were reported to concentration-dependently self-administer vaporized sufentanil. Rats exhibited a significant increase in responding for sufentanil when given naloxone, which was interpreted by the authors to indicate that naloxone was producing somatic signs of withdrawal and that a greater dose of sufentanil was required to alleviate the effects of withdrawal. The authors also noted that rats that were given long access periods but not short access to vaporized sufentanil escalated their drug intake over time. While this study clearly demonstrated that sufentanil vapor produced physiological effects the authors were not able demonstrate that sufentanil was self-administered at high rates than vehicle vapor, which is the hallmark of a drug serving as a reinforcer. Specifically, in the short access sufentanil vapor group, the rats performed on average about 5 nose pokes during the first hour of the session and approximately 10 nose pokes during a two hour session. The tests measuring response to just the cue and/or non-sufentanil vapor showed response rates of about 15-20 nose pokes per two hour session. Therefore, if anything, sufentanil was demonstrated to be suppressing operant responding compared to vehicle vapor.

Several laboratories, including our own, continue work toward developing a rodent model of vaping that parallels more traditional intravenous self-administration procedures. Collectively, these studies indicate that it is possible to study vaping in rodent models and drug-related behavior can be reliable assessed. However, at the present time there is insufficient data to convincingly show that e-cigarette delivered vapors are reinforcing in rodents and no studies have yet explored if other stimulant vapors such as amphetamines will produce abuse-related effects via electronic cigarettes. While the majority of the published and ongoing work is focused on self-administration, it is not the only potential paradigm to explore the reward related effects of drug vapor. Given the prevalence of amphetamine and psychostimulant abuse in the United States, developing alternative rodent models will be valuable in determining the abuse potential of stimulant vapors, especially if amphetamine vapor cannot be established as a reinforcer in rodents due to the technical challenges involved. One potential model which may have utility in this regard is the intracranial self-stimulation procedure.

INTRACRANIAL SELF-STIMULATION

In ICSS experiments, naïve subject, usually rats or mice undergo a stereotaxic surgical procedure in which an electrode is surgically implanted, most commonly, into the medial forebrain bundle (MFB), a region that is abundant in dopaminergic neurons and serves as a connection between the ventral tegmental area and nucleus accumbens, two key structures in what is widely referred to as the mesolimbic reward pathway (Kempadoo et al., 2013)(Figure 1). Following electrode implantation, subject are placed into an operant chamber and trained to

lever-press for electrical stimulation. Stimulation of the MFB is highly reinforcing as exhibited by the observation that rodents with accurate electrode placements typically quickly learn to lever-press, emitting hundreds of responses for brief pulse trains of electrical stimulation in each experimental test session (Olds, 1958; Goodall & Carey, 1975).

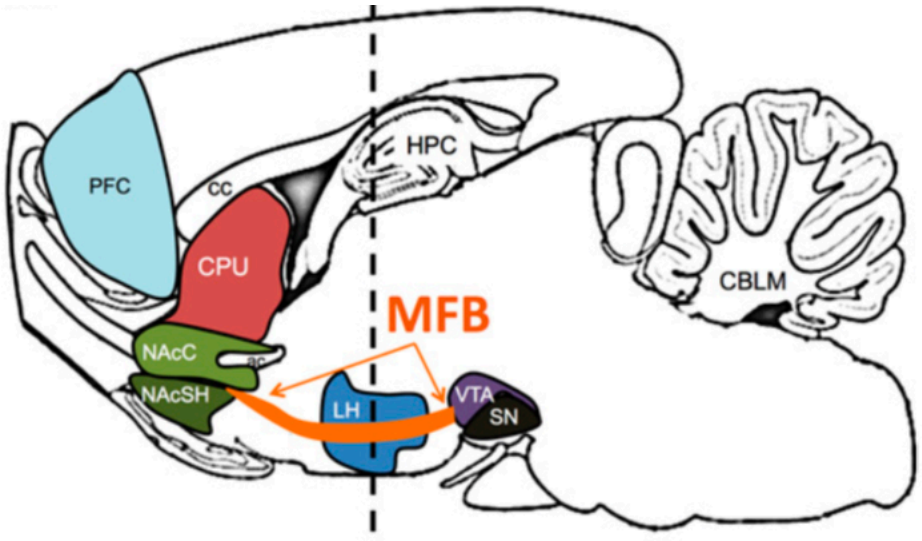

Figure 2. Coronal section showing the location of the medial forebrain bundle. The MFB (orange) connects the nucleus accumbens (green) to the ventral tegmental area (purple) and is commonly known as the reward pathway.

Negus, S. S., & Miller, L. L. (2014). Intracranial self-stimulation to evaluate abuse potential of drugs. *Pharmacological reviews*, *66*(3), 869–917. https://doi.org/10.1124/pr.112.007419

Intracranial self-stimulation has been widely utilized to assess the abuse-related effects of drugs and most drugs which are reinforcing in self-administration assays also produce positive results in ICSS assays (Negus & Miller, 2014). Abused drugs facilitate ICSS due to potentiation of the mesolimbic reward pathway. Briefly, stimulation of the MFB during ICSS is thought to activate "first stage" descending myelinated neurons originating in the lateral hypothalamus (LH) (Figure 2). Collateral branches of these descending neurons project to "second stage"

unmyelinated mesolimbic dopaminergic neurons in the ventral tegmental area (VTA). Activation of these mesolimbic neurons, mimicking the actions of drugs of abuse on this pathway, is the end result. Different classes of abuse drugs increase the function of the mesolimbic dopamine system through a variety of direct and indirect mechanisms. Psychostimulants such as amphetamine will stimulate the reward pathway by increasing dopamine release, which, in turn, increases the reinforcing effects of low ICSS stimulation intensities or low stimulation frequencies that, when given alone, are insufficient to support lever-pressing.

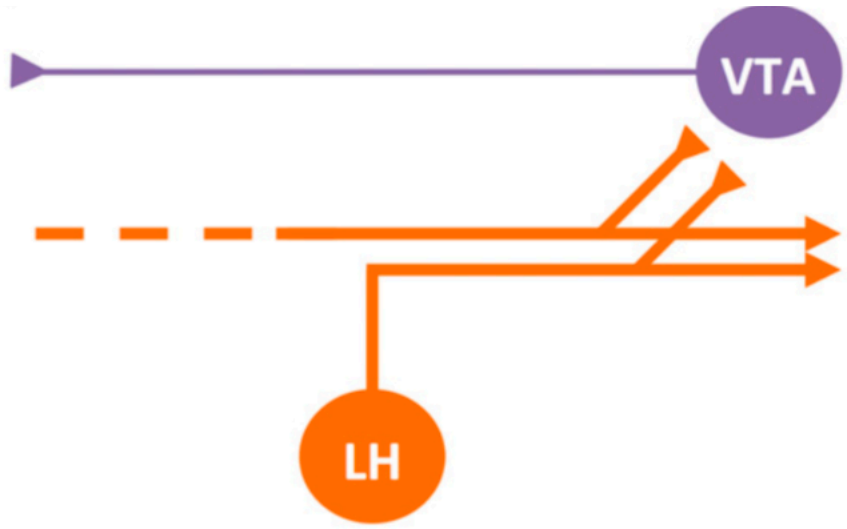

Figure 3. Diagram of neurons thought to contribute to ICSS. Stimulation of the MFB is thought to directly activate "first stage" descending myelinated neurons (orange) that originate in the lateral hypothalamus. These first stage neurons have collateral branches that project to and activate "second stage" unmyelinated mesolimbic dopaminergic neurons (purple) in the ventral tegmental area.

Negus, S. S., & Miller, L. L. (2014). Intracranial self-stimulation to evaluate abuse potential of drugs. *Pharmacological reviews*, *66*(3), 869–917. https://doi.org/10.1124/pr.112.007419

We have chosen to explore ICSS in addition to self-administration for several reasons. First, vapor itself, regardless of whether a drug is present or not may be aversive to rodents without extended training to habituate the subjects to the exposure. In addition, in ICSS procedures, the vaporized drug can be administered independent of the subjects' behavior and at the experimenter-chosen concentrations and durations, which allows an exploration of a wider variety of exposure and dosing conditions than would volitional self-administration. Furthermore, ICSS also proves advantageous over self-administration studies in that self-administration functions on the assumption that the drug is a reinforcer (Panlilio & Goldberg, 2007). When using a self-administration model, a drug must be reinforcing in order to be self-administered and produce significant rates of responding. However, in an ICSS model, a drug does not necessarily have to be reinforcing because the drug is administered by the experimenter where the rates of responding for stimulations are then assessed. In other words, in self-administration models, the self-administration of a drug is an operant response that is reinforced by the drug's effects. In ICSS, drugs that are not reinforcing or conditions that may be aversive, such as the inhalation of an unpleasant vapor with a drug in it, may still be assessed because the operant response is to brain stimulations, which is independent of drug administration. However, even though operant response to brain stimulations is independent of the drug administration, the drug's effects will still affect operant responding for brain stimulations and consequently, the behavioral and abuse-related effects can be assessed.

As with self-administration procedures, most ICSS studies utilize simple fixed-ratio schedules of reinforcement, usually FR1, meaning the animal receives one pulse-train of stimulation each time it operates the manipulandum (Neill et al., 2002). However, other schedules of ICSS-reinforced behavior have been used, such as fixed-interval schedule, where a

stimulation is delivered following a response after a designated amount of time (Hunt & Atrens, 1992; Elder et al., 2016), or progressive-ratio schedule, where the number of responses required to deliver a stimulation is progressively increased following each successfully completed ratio (Easterling et al., 2000; Tracy et al., 2014). Generally, the data generated from procedures utilizing differing schedules are consistent with one another (Tracy et al., 2014).

While a number of operant schedules of ICSS-reinforced behavior can be utilized, most studies examining the effects of drugs on ICSS most commonly use either the threshold or rate-frequency procedure. Rate-frequency methods allows for the creation of what is referred to as a rate-frequency curve, plotting the frequency of available stimulation on the Y axis and rate of operant responding on the X axis (Negus & Miller, 2014; O'Neill & Todtenkopf, 2010). Briefly, the frequency of available stimulation is progressively increased or decreased in discrete steps either within a single experimental session or over the course of repeated sessions. Frequency changes are ordered on a logarithmic scale with the lowest available frequency being 56 Hz and the highest being 158 Hz. As high frequencies result in greater neuronal activation, animals will respond at high rates at high frequencies and then responding will slow and eventually stop at low frequencies of stimulation. Rates of responding after vehicle pretreatment are then compared to rates of responding after injection of a test drug. Test drugs with abuse liability will result in lower frequencies stimulation maintaining responding than those which supported responding in the vehicle condition, due to the synergistic effect of drug-induced and electrically-driven mesolimbic activity. When examined across the entire frequency-response curve, a leftward shift can usually be detected when comparing frequency-response curves generated under control and drug conditions. In contrast, in threshold procedures the aim is to establish a minimum threshold current that will support operant responding by increasing and

decreasing the available current depending upon operant response rates (Marcus & Kornetsky, 1974; Elder et al., 2016). Drugs of abuse generally decrease the threshold that will support ICSS behavior.

INTRACRANIAL SELF-STIMULATION IN ASSESSING STIMULANT DRUG EFFECTS

There is fairly extensive literature on the effects of stimulants on intracranial self-stimulation, examining a variety of experimental questions. Earlier studies focused on the ISCSS-facilitating effects of stimulants. For example, one study showed that administration of amphetamine had a dose-dependent effect on ICSS reinforcement threshold with increasing doses of amphetamine progressively decreasing ICSS thresholds (Schaefer & Michael, 1988). Other studies have explored more challenging questions. For instance, a study by Lin and colleagues sought to assess changes in threshold for self-stimulation with variation in time between amphetamine injections based on the hypothesis that the amount of time between successive injections of psychostimulants plays a role in the development of neuroadaptive responses to these drugs (Lin et al., 2000). They found that threshold elevations associated with withdrawal from amphetamine diminished after repeated drug challenges at 5-day intervals. In contrast, daily injections of the same dose of amphetamine did not alter the acute threshold-lowering effect of the drug but resulted in progressive increments in thresholds at later time points. The effects of chronic drug treatment on ICSS have also been explored. In a study by Bauer et al., the impact of chronic amphetamine administration on cocaine-induced facilitation of ICSS was examined (Bauer et al., 2014). In this study, cocaine was shown to facilitate ICSS,

after which amphetamine was administered regularly for two weeks. Cocaine-induced facilitation of ICSS produced a sustained facilitation of the baseline-ICSS, upon which amphetamine had little additional impact. Other stimulants such a caffeine have also been shown to alter ICSS performance (Lazenka et al., 2015). However, when compared to amphetamine and cocaine, two psychostimulants with higher abuse potential, caffeine was found to produce an inverted U-shape dose effect curve but lower peak ICSS facilitation. The authors concluded that based on this data, caffeine demonstrated a lower abuse potential as well as potential aversive effects at higher doses.

The ICSS facilitating effects of amphetamines can also be demonstrated using procedures that do not involve lever-pressing behavior. For example, one study assessed the effects of nicotine and d-amphetamine on ICSS in a shuttle box test (Clarke & Kumar, 1984). Rats could turn on and off the stimulation by breaking photobeams on opposite sides of a shuttle box and the amount of time that the rats spent receiving stimulation was compared to a control baseline. When given 15 minutes prior to testing, d-amphetamine increased the time that the rats received stimulation and facilitated responding. Another study evaluated the motivational effects of methamphetamine by the runaway method using priming stimulation of intracranial self-stimulation behavior (Sagara et al., 2008). Methamphetamine or saline was administered intraperitoneally (IP) 30 minutes prior to testing and behavior was assessed relative to a baseline. Methamphetamine was found to increase running speed. This positive response was further increased with an increase in stimulation frequency. In summary, a number of studies using a wide variety of methods all demonstrated that amphetamine and methamphetamine administered by injection reliably facilitate ICSS under appropriate test conditions.

Although injected stimulants reliably facilitate ICSS, we are aware of only one prior study has used ICSS to examine the reward-related effects of a psychomotor stimulant delivered as a vapor (Nguyen et al., 2016). In this study, the authors explored the effect of vaporized methamphetamine and cathinones on reward threshold. Vapor delivery of methamphetamine lowered ICSS reward threshold in rats in dose-dependent manner. Reward threshold, or positive reinforcement threshold, as described previously is a minimum threshold current that maintains ICSS responding (Marcus & Kornetsky, 1974). Methamphetamine's lowering ICSS reward threshold would suggest that methamphetamine either increases the sensitivity to ICSS's reinforcing effects or increases the animals motor capabilities, allowing it to perform the operant behavior at a higher rate. It is worth noting that methamphetamine vapor in this study was administered 30 minutes prior to testing. Given the hypothesized rapid onset of drug effects when administered via vapor inhalation, it is conceivable that the peak effects of methamphetamine were considerably sooner than the 30 min timepoint examined. If such was the case, the result may not accurately represent the maximal effects of the methamphetamine vapor and therefore underestimated its potential potency as well as abuse liability. As previously noted, the immediacy of a drug effect is an important predictor of its reinforcing effects and it is unlikely that a human user of amphetamine vapor would find a 30 min delay of effects to be preferable to routes that provide more immediately CNS activity. As such, we believe that it is important to examine post-exposure time points more analogous to that which would be found in humans in order to determine the abuse liability of vaporized amphetamine. Furthermore, the prior study only examined methamphetamine and at the present time we are aware of no studies that focused on d-amphetamine, despite its widespread availability both as an illicit product as well as in medications widely used to treat ADHD.

Numerous studies have assessed methamphetamine's abuse potential with self-administration models in order to address a variety of questions. One study assessed the neurochemical consequences of methamphetamine self-administration in male and female rats and found that intravenous methamphetamine self-administration reduced striatal dopamine transporter in both sexes and elevated hippocampal brain-derived neurotrophic factor in males (Johansen & McFadden, 2017). Another study examining intravenous methamphetamine self-administration used a runway model where rats were allowed to traverse an alley where they would receive a dose of methamphetamine upon doing so (Akhiary et al., 2018). The results showed a U-shaped response with a dose of 0.5 mg/kg producing the strongest approach behavior which mimics the inverted U-shaped dose-response curve of most drugs that support self-administration in procedures utilizing lever-pressing operants. Furthermore, the rats showed no evidence of approach-avoidance behaviors that may be indicative of negative anxiogenic effects.

Methamphetamine administered as a vapor has also been examined, albeit far less frequently that intravenous methamphetamine in self-administration procedures. One such study gave mice access for one hour daily to a test chamber where nebulized methamphetamine was available (Juarez-Portilla et al., 2017). The mice could enter and exit the test chamber freely. Spending more time in the test chamber would expose them to more nebulized methamphetamine. Results showed that the mice would regulate their methamphetamine intake, administering for shorter periods if the concentration of methamphetamine vapor was increased. This study suggested that while reinforcing effects may be seen at some exposure concentrations,

aversive effects may be more prominent at other concentrations. Overall, the literature is overwhelming that rodents readily self-administer-methamphetamine when administered intravenously and there is some data also indicating that they may self-administer inhaled methamphetamine under certain conditions, although the data for the latter is much weaker and more sparse. It is therefore unclear, based on the current data if methamphetamine vapor has reinforcing effects and if they are as robust as those produced by intravenous methamphetamine.

LOCOMOTOR ASSAY

Although our primary focus is to determine if amphetamine vapor produces abuse-related effects, we also believe it is important to measure other behavioral outcomes to confirm that amphetamine vapors have other behavioral effects consistent with stimulants delivered by other routes of administration. Open field locomotor tests are a useful tool in assessing a variety of behavioral information ranging from ambulatory capabilities to the emotional state of the animal (Seibenhener & Wooten, 2015). In a typical locomotor assay, an experimental subject is placed into an open field apparatus in which they can move freely. Scoring of locomotion can be done by hand but is most commonly accomplished by a computerized system consisting of either photobeams or using a video tracking system. The locomotor activating effects of amphetamine and methamphetamine administered by injection have been examined in many assays (Nickolson, 1981; Mueller et al., 1989; Siviy et al., 2015). For instance, a study by Nickolson and colleagues found that amphetamine when given subcutaneously 30 min prior to testing at doses of 1 mg/kg and 2 mg/kg, stimulated most aspects of open-field behavior in rats, including

rearing, total distance traveled, and number of ambulation episodes (Nickolson, 1981). Therefore, we expect amphetamines given both subcutaneously and inhaled will stimulate locomotion in mice at moderate doses. A later study by Mueller showed that different doses of amphetamine given prior to a locomotor assay resulted in varying levels and types of stereotypy (Mueller et al., 1989). Briefly, lower doses of amphetamine increase locomotion and sniffing while at higher doses produce more focused stereotypy such as intense licking and biting a specific area of their environment. This indicates that locomotor activity assays result in dose-dependent behavioral outcomes. Doses of amphetamine that are above and below a specific dose will produce a sub-maximal locomotor response either due to insufficient CNS activity at low doses or due to the recruiting of responses such as stereotypy which competes with locomotion at high doses.

A few experiments have also been carried out that assessed inhaled methamphetamine's effects on locomotion. One study examined locomotion in mice after exposure to methamphetamine smoke delivered from a heated glass pipe, mimicking the common practice of methamphetamine smoking in humans (Meng et al., 1999). The ED_{50} for methamphetamine inhalation in this manner was found by assessing the change in activity at varying doses relative to baseline activity after breathing air. The ED_{50} for this type of exposure to inhaled methamphetamine was found to be 9.4 umol/kg. A more contemporary study exposed mice to either nebulized water or methamphetamine (Juarez-Portilla et al., 2017). Mice were placed in a nebulization chamber for 15 minutes daily and exposed to 1 mg/ml methamphetamine in water. Following administration, wheel running for the next three hours was examined. Results showed that methamphetamine at a concentration of 1 mg/ml produced significant increases in wheel running relative to nebulized water. Another study also exposed rats to vaporized

methamphetamine prior to a locomotor assay and found that the inhalation of methamphetamine significantly increased locomotion under their test conditions at methamphetamine concentrations of 25 and 100 mg/ml (Nguyen et al., 2016). We hypothesize that, under our test conditions, vaporized methamphetamine will generate similar dose-dependent effects on locomotor behavior as these other studies have shown even at relatively short periods of time after the cessation of exposure.

EVALUATING OTHER FACTORS INFLUENCING THE ABUSE-RELATED EFFECTS OF DRUG VAPORS

E-cigarette devices are commercial products and there are no standards of function or delivery characteristics across the dozens of available devices. As such, we believe that assessing the delivery characteristics of the actual vaporizer within the same study in which behavior is assessed is an important component of a comprehensive analysis. There are several variables related to the delivery system and E-liquid composition that may impact the abuse potential of drugs administered as vapor. For instance, e-liquid compositions vary but they usually contain both propylene glycol (PG) and glycerol. A recent study on particle size and e-liquid composition found that EC vapors composed of a 50:50 ratio of PG to vegetable glycerol (VG) produced the greatest amount of small particles, which allowed nicotine to achieve the greatest lung penetration (Mulder et al., 2019). A vehicle of pure VG also achieved a substantial, but slightly lower, amount of small particles and deep lung penetration. A vehicle comprised of

solely PG performed worse than either a combination of VG and PG or VG alone (Mulder et al., 2019).

Another potentially important variable is vaporizer output power which, as described previously, has increased greatly in newer generation e-cigarettes. Mulder and colleagues also examined the extent to which vaporizer output power impacted particle size, reporting that varying voltage played no role in particle size distribution. Because voltage and wattage are directly related, particle size should not be impacted by varying device power by altering wattage which is our intention. While higher voltages may not impact particle size, they do create a greater volume of vapor which we hypothesize will increase the amount of drug available for inhalation. Beyond the composition of the e-liquid and voltage, the influence of other factors such as wattage and puff time on drug delivery is still relatively unknown. Understanding the delivery characteristics of the vaporizer will be valuable information both to determine if behaviorally active doses of vaporized amphetamine can even be produced and may also provide some indication of whether vaping amphetamine is both practical and an appealing option for human users relative to other methods of administration.

OVERARCHING HYPOTHESIS

Our overarching hypothesis is that vaporized methamphetamine delivered by 3rd generation e-cigarettes has behavioral effects which may result in substantial abuse potential. We will test this hypothesis through two aims. The first aim will focus on the delivery characteristics of the device itself. The second will assess if vaporized amphetamines produce

behavioral effects consistent with other abused stimulants using an ICSS model, a self-administration model, and a locomotor assay.

Methods

Aim 1. Assess the methamphetamine vapor concentration produced by a 3rd generation e-cigarette.

As described in the introduction, there are a number of variables related to the function of an e-cigarette vaping system which may alter the total amount of drug to which a subject is exposed. We believe that puff duration, e-cigarette wattage, exposure chamber volume, and flow rate are all potentially important determinants of total maximum drug exposure (Farsalinos et al., 2013). However, it was impractical to explore each of these variables so we have focused only on those we hypothesized to be most critical.

In order to approximate how much drug a mouse might inhale during an exposure, theoretical calculations were performed to select what we hoped to be appropriate test conditions. First a vaporizer was filled with e-liquid and the atomizer was weighed. As the goals of the present study were not to address the relevance of particle size, we chose a constant 50:50 ratio of PG and VG in order achieve maximum penetration of an aerosolized drug into the lungs of the user (Mulder et al., 2019). To simulate the projected exposure parameters, the vaporizer was then activated 10 times for 6 seconds each at 18 watts and a flow rate of 1 liter/min. The vaporizer was then reweighed to determine the total amount of liquid vaporized. This process was repeated three times and the average weight that the vaporizer lost was

calculated to be 0.22 g. This number was then divided by the total number of seconds that the vaporizer is active in each session, in this case 60 seconds, in order to determine the total weight of vehicle vaporized per second. This was found to be 0.0037 g/s. Negligible displacement of the drug in the vehicle e-liquid was assumed such that the volume and amount vaporized did not change when we introduce the 50 mg/ml concentration of d-amphetamine into the equation. The value of 0.0037 g/s was then divided by the molecular weight of d-amphetamine, which is 171.67 g/mol, to express the value in moles delivered per second of e-cigarette activation, which was determined to be 0.000022 mol/s. The total volume of the vapor exposure chamber we used for behavioral studies was 2.07 liters. The amount of amphetamine vaporized per session was therefore divided by the chamber volume to determine the amount of drug present per 2.07 liters with the assumption that the vapor would be distributed evenly throughout the chamber. The d-amphetamine distribution for all ten puffs combined was found to be 0.00438 μmol/cm^3. Finally, the tidal volume and respiratory rate of the mouse was factored into the calculation in order to determine how much amphetamine the mouse might be expected to inhale. Multiplying tidal volume, which is 0.15 cm^3 for an adult mouse, by respiratory rate, which is roughly 181 breaths/min for the C57BL/6J strain we utilized, gave the volume inhaled per minute, which was found to be 27.15 cm^3/min. This was divided by 60 seconds to determine the volume inhaled per second and then was multiplied by the number of seconds the mouse would be exposed to the vapor. Then, this was multiplied by the amphetamine distribution in the chamber. This procedure should yield an approximation of the amount of amphetamine inhaled per puff of vapor. Under these conditions, for 10 individual 6 second puffs, a mouse would theoretically inhale 0.002575 μmol of d-amphetamine per puff. Multiplying this final number by 10 puffs in a session gave the total theoretical amphetamine inhalation, which totals 0.02575 μmol. For a 30 g

mouse, this equals a dose of 0.858 μmol/kg or 0.147 mg/kg. For reference, the ED50 for inhaled methamphetamine, based on a locomotor assay, was estimated to be 1.75 mg/kg, which is about 0.05 mg for a 30 g mouse (Meng et al., 1999). These calculations suggest that there could be a wide discrepancy between the amount of d-amphetamine which can be vaporized within a limited period of time and the dose required to produce changes in behavior. However, at least one study has demonstrated that methamphetamine vapor exposure using an e-cigarette-based system alters locomotor performance (Juarez-Portilla et al., 2017). Therefore, either the theoretical calculations are in some way missing some critical variable or the potency of methamphetamine delivered by e-cigarettes is greater than that noted by Meng.

Our calculations above were premised on the hypothesis that amphetamine would be vaporized efficiently and consistent with the rate at which vehicle was vaporized. This may or may not be an accurate assumption. It could be that amphetamine is not vaporized at the same rate as vehicle and that drug may accumulate in the unused liquid in the reservoir or that the converse is true and drug is preferentially vaporized from the e-liquid. Therefore, to determine if our theoretical calculations of vaporizer output based on a crude measure of vaporized vehicle weight were consistent with the amount of drug actually vaporized, we conducted a vapor vacuum trap experiment to collect amphetamine vapor under conditions similar to that used in our behavioral studies (Figure 4).

Vacuum traps capture vapor by condensing it into liquid and have been used previously to measure components of vaporized e-liquid (Peace et al., 2018). To conduct this study, a Smoant Cyclon 218 W Box Mod e-cigarette fitted with a Innokin iSubV vaporizer reservoir and a 0.5 ohm iSubV stainless steel coil was connected to 6.35 mm diameter tubing, which was fed into two 125 ml flasks and a 250 ml flask connected in series by additional lengths of 6.35 mm

tubing. Each of the 125 ml flasks contained 100 ml of deionized and the 250 ml flask contained 200 ml of deionized water. In each flask, the intake tubing was connected to a section of glass dip tube that extended below the water line of the flask. A piece of glass wool was placed in the tubing between the first and second flasks to prevent residual vapor from escaping the system. To draw e-cigarette vapor into the apparatus, negative pressure was applied to the distal end of the tubing using a Gast 1/8 HP diaphragm vacuum pump regulated by a rotometer. After the vacuum had been established, the vaporizer was turned on for a specified period of time to simulate a puff. Once the vaporizer was switched off and vapor generation ceased, the pump was shut off, the hose disconnected from the pump, and the hoses on both ends of the three flasks clamped. The vapor was then allowed to condense for approximately 5 minutes until no vapor was visible in the flasks. The flasks were then shaken, and decanted. The empty flasks were then washed with a 100 ml of DI water. The total collected volume of all the flasks and the wash water were combined for a total of 500 ml. The glass wool was allowed to soak in the water for several minutes. Finally, the collected water was shaken to mix it thoroughly and a micropipette used to collect samples for later analysis by liquid chromatography-mass spectroscopy (as described later).

Puff time is thought to affect drug delivery (DeVito & Krishnan-Sarin, 2018) and a previous study reported that variation in puff time, described as puff topography, may influence the amount of exposure to nicotine. Our procedure was to conduct a number of tests using the vapor-trap apparatus to determine how changes in puff duration and wattage impact the amount of amphetamine being vaporized. Our initial baseline condition was a puff time of 6 seconds, a wattage of 18 W, and a flow rate of 1 L/min. After five trials were carried out under these

conditions, the coil was changed, puff time was reduced to 5 seconds and then subsequently to 3 seconds.

After examining puff time, we sought to determine the effect of vaporizer wattage on total vaporized amphetamine yield. E-cigarette coil wattage has been shown to influence the total amount of nicotine vaporized in e-cigarettes (DeVito & Krishnan-Sarin, 2018) and is likely to also be relevant with regard to d-amphetamine. To test how wattage impacts the amount of amphetamine vaporized, we measured the amount of amphetamine in the vapor when the wattage was increased from 18 W to 36 W using a fixed 6 second puff duration and 1 liter/min flow rate. Three trials were carried out under these conditions.

A previous study has also shown the amount of nicotine present in vapor to also be correlated with the flow rate (DeVito & Krishnan-Sarin, 2018). Based on a published study, flow rates up to around 2.3 L/min should be possible in our vapor trap apparatus because the total volume of the trap is large enough to contain the vapor generated (Peace et al., 2018). However, the pump we used to produce vapor for the ICSS and locomotor studies achieved a maximum output of 1.25 L/min. To maintain comparable conditions, we altered flow rate while keeping other conditions constant at 18 W and a puff time of 6 seconds. Three trials were carried out at a flow rate of 1.25 L/min.

Two final tests were also performed with the wet vapor trap. The first was to provide comparison drug blank solutions in which one 6 second puff of vehicle vapor was collected at a vaporizer setting of 18 W. Three trials were carried out under these conditions. Lastly, a test in which 10 puffs of d-amphetamine vapor at 18 W with a 6 second puff duration, rather than a single puff, was generated and captured in the same vapor trap sample. The purpose of this was to determine whether the amount of d-amphetamine captured per puff was independent of the

number of puffs that are generated from a single vaporizer tank or if repeated puffs results in a change in the amount of drug generated in each puff.

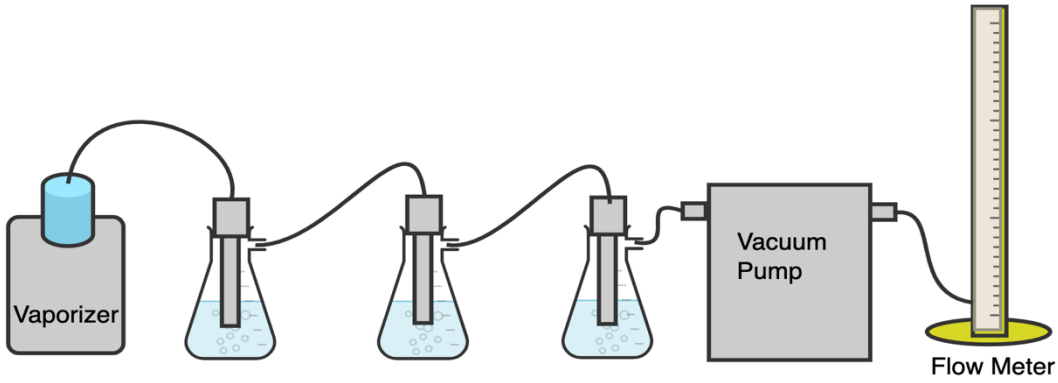

Figure 4. Diagram depicting the setup of the wet-trap experiment to capture the vapor. Three flasks, partially filled with DI water, are connected in series by tubing with the vaporizer attached at one end and a vacuum pump and flowmeter attached at the other end. The vacuum pump applies a negative pressure and flow through the system, quantifiable with the flowmeter, which pulls the vapor into the traps to be captured.

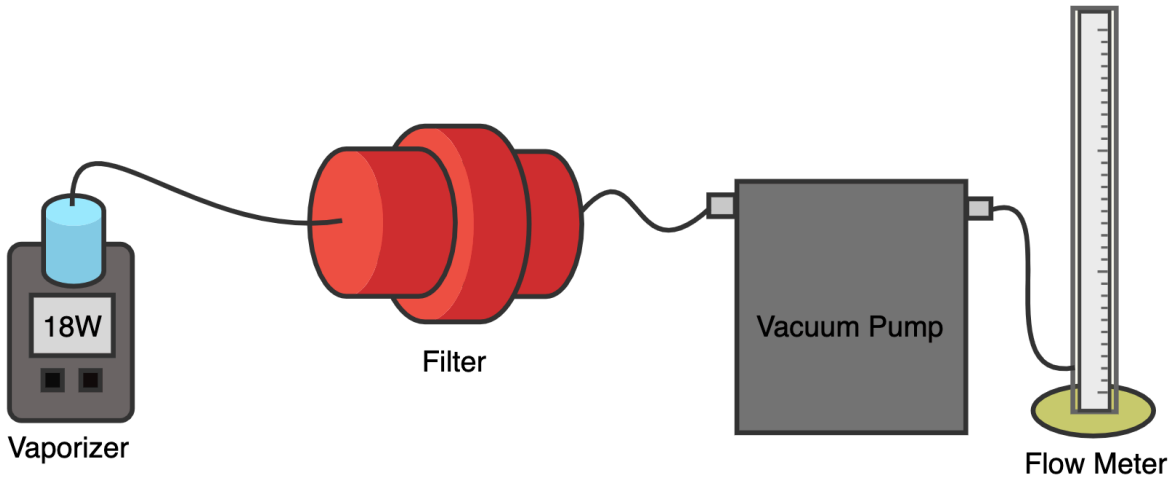

Figure 5. Diagram depicting the setup of the dry filter experiment to capture the vapor. A compartment that houses a dry particulate filter is connected by tubing in series with the vaporizer on one side and a vacuum pump and flowmeter on the other. The vacuum pump applies a negative pressure and flow through the system, quantifiable with the flowmeter, which pulls the vapor though the dry filter where it is captured.

A dry particulate filter experiment (shown in figure 5) was also carried out as an alternative methodology to the wet vapor trap apparatus. In these experiments, methamphetamine was utilized rather than d-amphetamine as it has much greater solubility in e-liquid. We hypothesized that this would increase the likelihood of obtaining behaviorally relevant doses in our behavioral studies. Dry particulate filters rely on the pore size of the filter being small enough to effectively capture aerosol particles passing through them. In this experiment, the Smoant Cyclon 218W Box Mod e-cigarette fitted with a Innokin iSubV vaporizer reservoir and a 0.5 ohm iSubV stainless steel coil was connected in series with the filter (Borgwaldt Korber Solutions, Hamburg, Germany), a vacuum pump, and a flow meter using 6.35 mm just as it was in the water trap experiment. The filter was contained within a 2-

piece 3D printed housing with hose barbs on either end and sealed to be airtight. The same vacuum pump and flow meter used in the water vapor trap experiment were also utilized in this experiment.

The first set of conditions tested with the dry particulate filter experiment was a single puff of 100 mg/ml methamphetamine vapor with a wattage of 18 W, a flow rate of 1 L/min, and a puff time of 6 s. The vapor vacuum pump was first turned on to create a negative pressure through the system and then the vaporizer activated for a single 6 s puff. Once the vapor had been captured in the filter, the vacuum pump was turned off. The filter housing was then removed and flushed with 10 ml of wash solution consisting of 80% methanol/20% deionized water followed by a second syringe of air to expel the majority of the liquid remaining in the housing. The wash solution was collected in a 20 ml conical tube. The housing was then disassembled and the remaining liquid decanted into the conical tube. The filter membrane was then removed from the housing, immersed in the wash solution and the conical tube capped. Three trials were carried out under this set of conditions.

A second series of three independent vapor captures were also collected in which the same conditions were used, except that five, 6 s puffs at a vaporizer wattage of 18 W were captured with each filter. The vacuum pump ran continuously between each of the five puffs with a 30 s interval between each puff. Two quality control samples were also prepared. In the first, 10 ul of 1 mg/ml methamphetamine dissolved in 50% vegetable glycerol/50% propylene glycol was pipetted direct onto a filter membrane. The filter was then placed directly in a conical tube containing 10 ml of 80% methanol/20% deionized water. The second quality control contained 10 µl of 1 mg/ml methamphetamine solution pipetted directly into 10 ml of 80:20 methanol to water solution without any filter. After the samples were prepared the tubes were

placed on a mechanical rocker for 24 hours to equilibrate the solutions. Following equilibration, 1 ml aliquots were collected in triplicate from each conical tube and subjected to analysis by the Pharmacology and Toxicology Analytical Analysis Core Lab.

Both liquid chromatography-mass spectroscopy (LC-MS) and gas chromatography-mass spectrometry (GC-MS) have been shown to be a reliable method to analyze different components of a vapor, including drugs, alkaloids, solvents, and flavors (Peace et al., 2018; Trehy et al., 2011; Hadwiger et al., 2010; Pellegrino et al., 2012). We utilized LC-MS as that instrumentation was available within the Pharmacology and Toxicology department. E-liquid samples were analyzed for amphetamine or methamphetamine (Cerilliant, Round Rock, Texas) using amphetamine-d8 or methamphetamine-d8 (Cerilliant, Round Rock, TX) as the internal standard (ISTD) respectively. Freshly prepared seven-point calibration curves in the appropriate matrix with a range of 10 to 1000 ng/mL amphetamine, a drug-free control (negative control) containing only ISTD and a double negative control that contains neither amphetamine nor ISTD were analyzed with each batch of samples. Samples and controls were kept at -20° C until analyzed. E-liquid samples were diluted into the calibration range with dilutions of 1:10, 1:100 or 1:1000. In brief, 300 µL of 0.1 N hydrochloric acid (HCl), 100 ng/mL of internal standard, and the buffered calibrator, controls or sample were added to pre-conditioned OFXQ narrow-bore 1 cc, 5 mg columns (Tecan US, Inc, Morrisville, NC). Columns were then washed with 800 µL 0.1 N HCl, deionized water, and 50:50 methanol:water and allowed to dry for 10 minutes. Columns were then eluted with 400 µL 80:18:2 Ethyl Acetate:methanol:Ammonium Hydroxide (NH4OH). 20 µL 1% HCl in methanol evaporated to dryness. Extracts were reconstituted with 45 µL of 25:75 methanol:water transferred to auto-sampler vials. Analysis was performed on a Shimadzu LCMS-8050 Ultra-high-pressure liquid chromatography-tandem mass spectrometry (UPLC-

MS/MS) system (Shimadzu, Kyoto, Japan). Chromatographic separation was performed on an Agilent Poroshell 120 EC-C18 column 2.1 x 50 mm x 2.7 μm (Agilent Technologies, Santa Clara, CA). Samples were analyzed using positive electrospray ionization (±ESI) in multiple reaction monitoring (MRM) mode. Mobile phase consisted of 5 mM ammonium formate + 0.1% formic acid in H2O (MPA) and 5 mM ammonium formate + 0.1% formic acid in methanol (MPB), utilizing a gradient from 2 – 98% MPB. A linear regression of the peak area of ratios of the quantification and the ISTDs transition ion were used to construct the calibration curves.

Aim 2. Characterize the abuse potential of vaporized amphetamines by assessing their effects on ICSS-reinforced behavior, self-administration, and locomotion.

Intracranial self-stimulation

Male and female C57BL/J mice underwent surgery to implant stimulating electrodes into their right medial forebrain bundle. Briefly, anesthesia was induced by 3% isoflurane at a flow rate of 2 L/min. The mice were also administered 7.5mg/kg of morphine subcutaneously for both intraoperative and postoperative analgesia. The mice were then placed in the myneurolab.com stereotaxic device in a flat-skull position and anesthesia was continued at between 1.5 and 2% isoflurane, titrated to effect. The incision site on the scalp was shaved and swabbed with betadine and ethanol. An approximately 1 cm incision was then made in the scalp using a scalpel blade. A small hole was drilled in the top of the skull using a Dremel moto tool model 395 fitted with a stainless steel dental burr at coordinates -1.1 mm anterior/posterior and -1.1 mm medial/lateral for male mice and -1.0 mm anterior/posterior and -1.0 mm medial/lateral

for female mice relative to bregma. A stainless steel 2-channel twisted electrodes, supplied by PlasticOne (Roanoke, VA), was fitted into an electrode holder and lowered at the same coordinates to a depth of -4.8 mm dorsal/ventral for males and -4.7 mm for females. The electrodes was secured by three 000-120 x 3/32 stainless steel screws placed in the skull around the electrode and a pedestal created by Filtek Supreme Flowable Restorative dental cement which was deposited around the screws and electrodes. The dental cement was cured with UV light from a UV dental curing lamp and the mice were removed from the stereotaxis for recovery on a heating pad. The mice were given 3 mg/kg of carprofen subcutaneously post-surgery and then the same dose once/daily for the following two days. They were weighed and observed each day during the three-day post-operation recovery period.

The mice were allowed at least 5 days of recovery and then they began pretraining in daily 50 min sessions to facilitate subsequent ICSS reinforced responding. During pretraining each FR1 response resulted in 10 s of access to a 0.01 ml dipper containing a solution of 25% nonfat powdered milk, 25% cane sugar and 50% water, by volume. Once the mice had been adequately trained to respond for milk, they were moved to ICSS training.

During daily (M-F) ICSS training, each mouse was placed into an operant chamber fitted with a single channel electrical commutator and connection tether which was connected to a Med-Associates model 152 programmable ICSS stimulator. Operant sessions were controlled by a Med-Associates interface and PC computer. During initial training the stimulation intensity provided to each mouse was adjusted to achieve appropriate levels of responding, generally rates of between 100 and 300 lever presses in each 50 minute training session. The mice were first trained in a 50 minute protocol under a fixed-ratio 1 (FR1) schedule of reinforcement. Each response resulted in a 500 ms train of alternating current stimulation at a frequency of 158 Hz.

When response rates were sufficiently robust, typically when surpassing 5 lever presses per minute, test sessions were progressively shortened over repeated days to 10 minutes. Mice were then transitioned to a 50 minute program divided into three 10 minute ICSS response components separated by two 10 minute timeout periods. Once the mice had been trained to respond under the multiple component schedule, they were transition to the test program which shares a similar format as the previous interval program except that the frequency of the stimulations decreased across each minute during the 10 minute active ICSS component. Frequencies decreased in the following order: 158, 141, 126, 112, 100, 89, 79, 71, 63, and 56.

When the mice were responding at satisfactory rates under the test program, drug testing began. Drugs were administered intraperitoneally (IP) 15 minutes prior to the beginning of the session. A minimum of two training days in which no drugs were administered were carried out in between each drug test day. Test days were identical to training days. In order to be testable, a mouse's response rates must have been deemed stable both across components and across days. Stability was defined as according to two criteria. First, the mouse's response rate for each component must have been within 30% of the average response rate for all components. Second, the mouse's response rates across two consecutive training days must have been within 30% of the mean of the two training days. If the mouse satisfied these criteria, it was tested the following day.

In ICSS procedures, cocaine is one of the most robust facilitators of performance (Fish et al., 2010; Negus & Miller, 2014). As such we first established conditions in which cocaine robustly facilitates performance as our anecdotal experience has shown that if at least one dose of cocaine (1-17 mg/kg) fails to facilitate ICSS it is unlikely that other drugs will be effective. If responses to intraperitoneal injections were inadequate at a given dose, alternative cocaine doses

were tried or doses were repeated after altering stimulation intensity. Those mice that showed reliable facilitation by cocaine were tested on IP d-amphetamine in order to establish at least one dose in which injected d-amphetamine also reliably facilitates ICSS performance. D-amphetamine doses of 0.3-17 mg/kg were tested based on existing published data (Goodall & Carey, 1975; Schaefer & Michael, 1988).

After drug-responses to both injected cocaine and d-amphetamine had been established, mice began testing using vaporized d-amphetamine. An acrylic anesthesia induction chamber (9 in x 3.75 in x 3.75 in) was used. Subjects were placed individually in chamber for a period of 5 minutes. Test condition included exposure to 10, 6 s vapor puffs of the 50% propylene glycol and 50% glycerol vehicle, ten 6 s puffs of d-amphetamine at concentrations up to the solubility limit of 50 mg/ml, and a control condition in which the mice were placed into the chamber for 5 min but received no vapor exposure. Immediately after exposure to vapor was completed, the mice were placed directly into the operant chambers and tested in the 3 component ICSS procedure.

Self-administration

Self-administration models are a widely accepted and commonly used method of assessing the abuse potential of psychoactive substances. This study aimed to assess the reinforcing effects of methamphetamine vapor puffs. A total of 15 experimentally-naïve adult male Sprague Dawley rats obtained from Charles River Laboratories were used as subjects. Subjects were singly housed under a 12/12 hr reversed light/dark cycle (testing in the dark phase) in a temperature and humidity-controlled vivarium in microisolator cages on wood chip bedding. Laboratory rodent chow and water were available ad libitum except during experimental

sessions. Experiments were conducted in six 9 inch by 9.5 inch modified operant chambers that had been fitted with a nose-poke actuated vapor puff delivery/liquid dipper apparatus on the center of the front wall of the chamber. The apparatus consisted of a custom-designed 3D printed 45 mm X 45 mm ABS plastic enclosure with an internal 3 W yellow LED stimulus light. The aperture was connected to a curved ABS plastic exhaust chimney that terminated in a 30x30 mm muffin fan. All six chambers were housed in a floor to ceiling walk-in fume hood.

Vapor was generated by pressurizing a modified e-cigarette reservoir tank using a 12 V diaphragm air pump. During operation the air pump was adjusted to generate a flow rate of 1 L/min. The exhaust fan was likewise adjusted manually maintain the vapor generated by the e-cigarette entirely within the aperture. The vaporizer and tank containing the e-liquid were connected to the aperture via 6.35 mm diameter tubing. A photobeam was located inside the puff delivery aperture, which would be broken by the nose of the rat when a nosepoke was performed. An electronic package was designed in-house to allow control of the air pump and exhaust fan speed as well as interface the components to a Med-associates control system and PC computer. An electrically-operated liquid dipper was positioned under the vapor delivery aperture. The dipper allowed for a 0.01 ml dipper cup to be elevated into the aperture according to programming parameters to provide liquid reinforcers.

First rats were trained to perform a nose poke for a sweetened powdered milk solution composed of 25% powdered nonfat milk, 25% sugar, and 50% water by volume. In each 30 min (M-F) training session, the subject received 4 s of access to a dipper containing milk contingent upon placing their head in the vapor delivery aperture and breaking the internal photobeam sensor. After reliable responding for the milk dippers had been achieved, vapor was introduced. The vaporizers used in this experiment were Smok R200 200W TC Box Mods. When the rat

performed a nose poke for the milk dipper the system initiated a vapor puff of 4 seconds in duration. After the completion of the puff the milk dipper was elevated into the aperture for 4 seconds. One second after the puff began, the exhaust fan was activated and remained on until 1 second after the completion of the puff. Initially, vehicle vapor (50% propylene glycol/50% vegetable glycerol) containing no drug puffs were paired with milk dippers. This was carried out at a low initial wattage and across repeated training sessions, the wattage was then increased across a series of sessions until the highest wattage that did not suppress milk dippers was achieved. Under our set of conditions, this was 18 W. Following determination of the maximally tolerated wattage, methamphetamine was faded into the e-liquid starting with a concentration of 0.05mg/ml. Methamphetamine e-liquid concentrations were increased over sessions until response rates were depressed relative to drug-free vehicle. Once this concentration was determined, the methamphetamine concentration for each subject was then decreased to the next lower concentration until stable responding was achieved with at least 10 dippers+puffs per session. This concentration was then maintained while milk was gradually diluted with water over successive sessions until dipper presentations contained only water. At the conclusion of the study, we also tested an extinction condition in which nose pokes had no scheduled consequences.

Locomotor Assay

Locomotor assays are a conventional method of assessing the behavioral effects of psychoactive substances such as stimulants and depressants (Fernandes et al., 1996; Berquist & Fantegrossi, 2018; Gatch et al., 2019). This study sought to examine the effects of vaporized methamphetamine on locomotor activity in 4 male and 6 female C57BL/6J mice. Mice were

obtained from Jackson Laboratories (Bar Harbor, Maine). Subjects were single housed on a 12/12 hr light/dark cycle in a temperature and humidity-controlled vivarium in microisolater cages on wood chip bedding. Mice were on a free-feed diet allowing them constant access to standard laboratory rodent chow and water. They were weighed weekly to monitor weight and health. Mice were exposed to intraperitoneally administered or vaporized amphetamine as well as intraperitoneally administered cocaine prior to the locomotor assay. However, administration of these drugs was halted for several weeks prior to the locomotor assay. Clear acrylic bins measuring 11.75 in X 11.75 in X 9.5 in placed in the center of a larger acrylic rat open field locomotor chambers were used as test spaces. A black and white analog video camera was suspended 96 cm above each bin. Locomotor data was recorded using Anymaze video tracking software (Stoelting, Wood Dale, IL). The room was kept brightly illuminated with sufficient lighting to provide contrast between the subject and the chamber floor. The overhead lights in the room were kept on but the locomotor chambers were placed out of direct light such that contrast between the bins and the mice could be achieved but a glare would not be present on the bottom of the plastic bins.

The mice first underwent six habituation sessions. On habituation day 1, the mouse was placed into the locomotor chamber and locomotion was recorded for 30 min. On the following 5 days, the mouse was habituated again in the same manner. After the six habituation sessions, the mouse began treatment condition tests. Saline was administered subcutaneously 15 min prior to the start of the first test session as a control to assess changes in locomotor activity that could be attributed to the injection itself. After the injection, the mouse was placed back into its homecage until the 15 minutes pretreatment period had elapsed and then placed into the locomotor chamber for a 30 min test session. The same test procedure was repeated with

methamphetamine. Doses of 3, 10, and 17 mg/kg of methamphetamine were administered subcutaneously, given 15 minutes prior to the start of the session (Gentry et al., 2004). To prevent the induction of locomotor sensitization, test sessions with methamphetamine pretreatment were separated by two days, one in which they were not placed in the locomotor chambers at all and one in which they were placed in the locomotor chamber for 30 minutes but they were not exposed to any drug prior to the session. Only after these two days were they allowed to be administered methamphetamine again.

After completing the subcutaneous (SC) methamphetamine dose-effect curve, the subject moved to testing following vapor exposure. Vapor exposures were performed using custom manufactured apparatus consisting of a 3rd generation e-cigarette, air pump and control box. Briefly, a Smoant Cylon 218 W e-cigarette was fitted with a Innokin iSub V tank and an Innokin iSub SS BVC coil. The vaporizer tank was modified such that it could be pressured by a 12 V diaphragm air pump. Pressurization resulted in vapor being emitted from the e-cigarette and directed into an acrylic anesthesia induction chamber measuring 9 inches long by 3.75 inches wide and 3.75 inches tall with a volume of 2.07 liters. The interior of the anesthesia chamber was divided into 4 equal sections by wire bar separators. The operation parameters of the e-cigarette and the activation of the air pump were controlled by an Arduino microprocessor and electromechanical relays.

Prior to each test session, the e-cigarette tank was loaded with vehicle or methamphetamine-containing e-liquid (50:50 ratio of propylene glycol to glycerol). When ready to test a subject on vapor, the mice were placed in the vape box and the Arduino box was turned on, which activated the vaporizer and administered ten 6 second puffs. The interpuff interval was 30 seconds and the flow rate was set at 1 L/min. After the vapor had been administered, the

mice were removed from the vape box and immediately placed in the locomotor chamber and a 30 min locomotor test session began. Test were carried out with vehicle vapor as well as 25, 50, 100 and 200 mg/ml methamphetamine. Between each test session two days in which no test sessions were conducted were interspersed to prevent the occurrence of locomotor sensitization.

All locomotor data was saved to Anymaze for later analysis. Analysis parameters included distance traveled, number of line crossings, number of freezing episodes, time spent in zones along the wall, and total time mobile. For all analysis the Anymaze software placed a point on the center mass of the mouse and tracked locomotion based on the movement of this single point. Total distance traveled is simply defined as the total distance that the point, and consequently the mouse, moves during the 30 minute session. The number of line crossings is defined as the number of times the animal's center point moved from one area of the apparatus map to another. In our locomotor assay, the field was divided into a 3x3 grid with 9 zones, each zone measuring roughly 3.85 x 3.85 in. Therefore, when the center point of the animal crossed a line as it passed into a different zone, the software registered this as one line crossing. Anymaze defines the number of freezing episodes as the number of times the animal stopped moving for a predetermined amount of time during the test. Every time the mouse began to freeze, a counter was incremented and the counter's total value at the end of the session is recorded as the total number of freezing episodes. Total time in the wall zone was total session duration – time in the center zone. Therefore, total time along the periphery or in the wall zones was considered total time in the 8 outer zones which did not include time in the one center zone.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism V9 for Macintosh. Locomotor data following SC methamphetamine and methamphetamine vapor exposure were assessed independently by two-way (dose/concentration x time) mixed model analysis of variance (ANOVA) followed by Fisher post-hoc tests when main effects or interactions were statistically significant at the $P< 0.05$ level. When the effect of time segment was not significant, the session totals were collapsed and reanalyzed by one-way ANOVA, followed by Fisher post-hoc tests.

Results

Vapor Analysis

The initial set of samples assessing d-amphetamine e-cigarette aerosol concentration was conducted using the wet vapor trap procedure. First, one puff of d-amphetamine was captured under standard conditions (wattage: 18 W; puff time: 6 s; flow rate: 1 L/min). Under this condition, the water traps collected an average of 0.098 mg of d-amphetamine per puff with a range of 0.049 to 0.1355 mg across replicates (Table 1). When wattage was increased to 36 W while retaining a 6 s puff duration and 1 L/min flow rate, the amount of d-amphetamine captured increased to a mean amount of 0.46 mg of d-amphetamine with a range of 0.366 to 0.6155 mg across replicates (Table 2). Under condition in which the flow rate increased to 1.25 L/min while puff time remained at 6 s and wattage was again 18 W, the water traps captured a mean of 0.34 mg of d-amphetamine with a range of 0.1275 to 0.6742 mg across replicates (Table 3).

Lastly, altered puff time was assessed by decreasing the puff time to 3 s and to 5 s while wattage remained at 18 W and flow rate at 1 L/min. The mean amount of d-amphetamine captured at puff times of 3 s and 5 s was 0.03 mg and 0.05 mg respectively (Table 4). When vehicle vapor was tested in the vapor trap experiment under test conditions, an average of 0.0113 mg of d-amphetamine was present in the water trap fluid (Table 5). Lastly, we assessed the extent to which increasing the number of puffs increased d-amphetamine capture. When 10 puffs of 50 mg/ml d-amphetamine was captured at a flow rate of 1 L/min and 18 W an average of 84.3 mg of d-amphetamine was captured (Table 6).

Standard Conditions (Puff Time=6 s; Wattage=18 W; Flow Rate=1 L/min)	
Trial	Total d-amphetamine captured (mg)
Trial 1	0.1225
Trial 2	0.0855
Trial 3	0.0965
Trial 4	0.1355
Trial 5	0.049
Mean	0.0978
Standard Error	0.015

Table 1. D-amphetamine captured with the wet vapor trap experiment under standard conditions. One 6 s puff was captured in the water traps under standard conditions expressed above. Five trials were carried out and the mean and standard error were calculated.

Altered Wattage (Puff Time=6 s; Wattage=36 W; Flow Rate=1 L/min)	
Trial	Total d-amphetamine captured (mg)
Trial 1	0.366
Trial 2	0.6155
Trial 3	0.386
Mean	0.4558
Standard Error	0.08

Table 2. D-amphetamine captured with the wet vapor trap experiment at altered wattage. One 6 s puff was captured in the water traps after wattage was increased from 18 W under standard conditions to 36 W. Three trials were carried out and the mean and standard error were calculated.

Altered Flow Rate (Puff Time=6 s; Wattage=18 W; Flow Rate=1.25 L/min)	
Trial	Total d-amphetamine captured (mg)
Trial 1	0.222
Trial 2	0.6742
Trial 3	0.1275
Mean	0.3412
Standard Error	0.169

Table 3. D-amphetamine captured with the wet vapor trap experiment at altered flow rate. One 6 s puff was captured in the water traps after flow rate was increased from 1 L/min under standard conditions to 1.25 L/min. Three trials were carried out and the mean and standard error were calculated.

Altered Puff Time (Puff time=3 s or 5 s; Wattage=18 W; Flow Rate= 1 L/min)	
Trial	Total d-amphetamine captured (mg)
Trial 1 (3 s)	0.038
Trial 2 (3 s)	0.0225
Trial 1 (5 s)	0.048
Trial 2 (5 s)	0.052
Mean (3 s)	0.0303
Standard Error (3 s)	0.008
Mean (5 s)	0.05
Standard Error (5 s)	0.002

Table 4. D-amphetamine captured with the wet vapor trap experiment at altered puff time. Puff time was decreased to 3 s and one puff was delivered. Two trials were carried out. Puff time was then changed to 5 s and the same procedure was carried out.

Vehicle Only	
Trial	Total d-amphetamine captured (mg)
Trial 1	0.014
Trial 2	0.0125
Trial 3	0.0075
Mean	0.0113
Standard Error	0.002

Table 5. D-amphetamine captured with the wet vapor trap experiment with only vehicle vapor. One 6 s puff was captured in the water traps. No drug was present in the e-liquid. A small, non-behaviorally relevant amount of amphetamine was captured under these conditions.

10 Puff (Puff time=6 s; Wattage=16 W; Flow Rate= 1 L/min)	
Trial	Total d-amphetamine captured (mg)
Trial 1	250.7
Trial 2	1.192
Trial 3	0.897
Mean	84.263
Standard Error	83.22

Table 6. D-amphetamine captured with the wet vapor trap experiment with 10 puffs. 10 puffs of vapor were carried out under standard conditions allowing vapor to settle in the flasks between each puff.

The wet vapor trap methodology proved to be inconsistent. Therefore, a new dry filter trap experiment was also carried out to assess if it would prove more accurate and reliable. For this experiment, methamphetamine aerosol was measured rather than d-amphetamine aerosol. First, one puff of 100 mg/ml methamphetamine vapor was captured under standard conditions of 18 W vaporizer power, 1 L/min flow rate and 6 s puff time. Under these conditions, the dry particulate filter experiment captured an average of 0.1829 (+/- 0.0441) mg/ml methamphetamine with a range of 0.0098 to 0.2898 mg across replicates (Table 7). Five puffs of methamphetamine vapor under the same conditions captured an average of 2.6402 (+/- 0.6576) mg of methamphetamine with a range of 1.0310 to 4.7697 mg across replicates. As a quality control for the vapor trap samples, 10 µl of 1 mg/ml methamphetamine e-liquid deposited directly into 10 ml of 80/20 methanol/DI water, LC-MS quantified a total of 0.0054 mg of methamphetamine in the solution. When 10 µl of 1 mg/ml e-liquid was placed directly on a filter

and then the filter was placed into a solution of 80/20 methanol/DI water, LC-MS quantified a total of 0.0145 mg of methamphetamine in the solution.

1 Puff (Puff Time=6 s; Wattage=18 W; Flow Rate=1 L/min)	
Trial	Total Methamphetamine Captured (mg)
Trial 1	0.2898
Trial 2	0.0098
Trial 3	0.2491
Mean	0.1829
Standard Error	0.0441
5 Puffs (Puff Time=6 s; Wattage=18 W; Flow Rate=1 L/min)	
Trial	Total Methamphetamine Captured (mg)
Trial 1	1.0310
Trial 2	4.7697
Trial 3	2.1200
Mean	2.6402
Standard Error	0.6576
10 µl of 1 mg/ml meth in 10 ml of 80/20	
Trial	Total Methamphetamine Captured (mg)
Trial 1	0.0054
Meth 10 µl of 1 mg/ml liquid in 10 ml of 80/20 on filter	
Trial	Total Methamphetamine Captured (mg)
Trial 1	0.0145

Table 7. Methamphetamine captured with dry-particulate filter experiment under various conditions. Five puffs of vapor produced a non-linear increase in the amount of

methamphetamine captured relative to one puff. The 10 µl of 1 mg/ml meth in 10 ml of 80/20 conditions were carried out in order to ensure the assay was working properly. 10 µl of 1 mg/ml meth e-liquid dissolved in 10 ml of 80/20 methanol/DI water and the same conditions but with e-liquid placed on a filter before being dissolved in the methanol/DI water solution was expected to show roughly 0.01 mg of methamphetamine in the solution.

ICSS experiments

After surgical preparation of 25 subjects and initial training to respond for ICSS it was necessary to suspend training for a period of several months due to COVID-19 restrictions. Upon return from this hiatus, a significant percentage of subjects had either lost their electrode implant headpieces, could not be retrained successfully to criteria performance, or failed to complete all the test conditions. As such, the data presented for ICSS are based on 4 subjects and presented individually given not enough animals were available to generate meaningful statistical power in a group analysis. Furthermore, data was collapsed across the three components.

Initially, the effects of cocaine on ICSS were examined to determine optimal conditions for assessing the effects of methamphetamine on ICSS performance. Data are presented as mean number of ICSS reinforcers earned across all three components in each of the 10 frequencies. Cocaine was administered intraperitoneally at doses of 10 mg/kg or 17 mg/kg 15 minutes prior to starting the ICSS session. Cocaine showed an increase in ICSS responding in all four subjects at either one or both doses of cocaine tested (Figure 6). Cocaine given at a dose of 17 mg/kg

appeared to have the most pronounced effects showing greater increases in ICSS responding across frequencies in three of the four subjects compared to a dose of 10 mg/kg, which resulted in more robust ICSS facilitation in one subject than did 17 mg/mg cocaine.

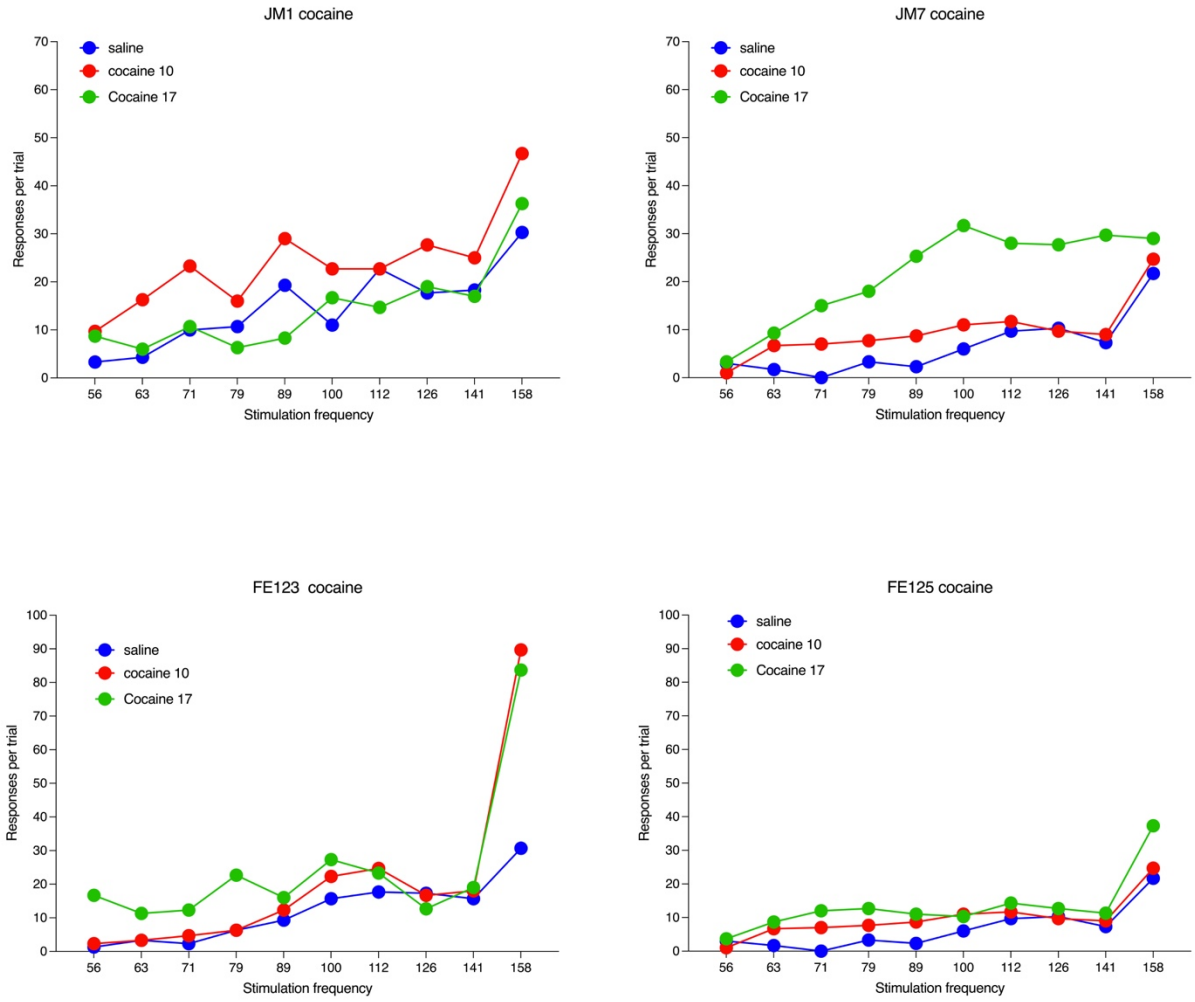

Figure 6a-d. Individual Data on ICSS Rates of Responding After Intraperitoneal Cocaine Administration. Cocaine was administered intraperitoneally at doses of 10 (red) and 17 (green) mg/kg, given 15 min prior to the start of the ICSS session. Rates of ICSS responding for each mouse were recorded and graphed as a function of the stimulation frequency and were compared to IP saline (blue).

After cocaine was demonstrated to facilitate ICSS responding in our test subjects, IP d-amphetamine at doses of 1.7 mg/kg and 3 mg/kg were administered 15 minutes prior to the session and data was examined. D-amphetamine administered intraperitoneally at a dose of 3 mg/kg showed an increase in ICSS responding relative to saline in two of the subjects, FE125 and JM7, while it showed a biphasic effect another subject, FE123 (Figure 7). In this subject, there was a general decrease in ICSS responding after being administered 3 mg/kg of d-amphetamine intraperitoneally but at the highest frequency, there was an increase in ICSS responding. One subject, JM1, showed possibly frequency-dependent changes in ICSS responding after 1.7 mg/kg d-amphetamine but changes appear to be minor. JM1 was not tested with 3 mg/kg d-amphetamine to determine if a higher dose would have facilitated ICSS performance.

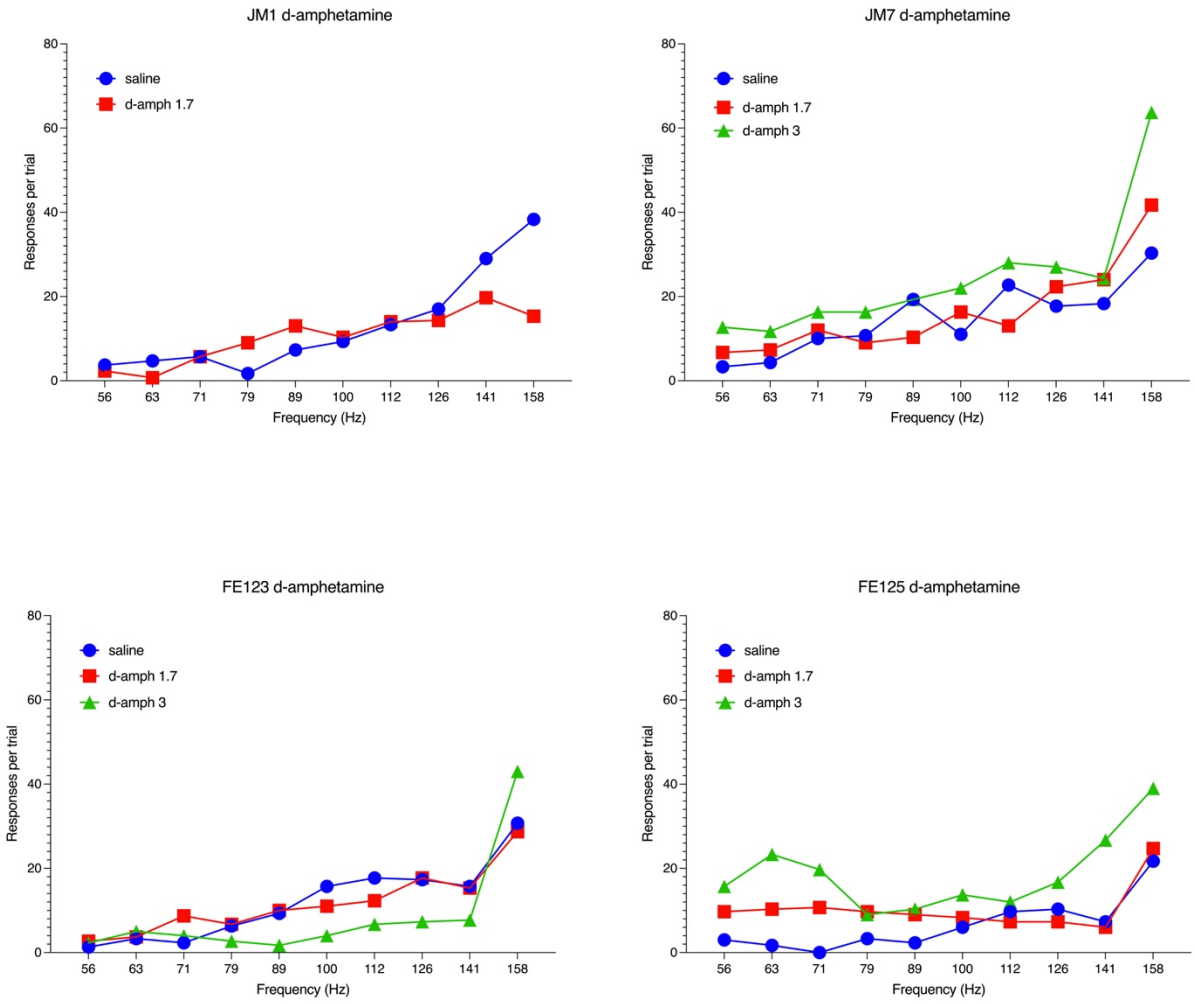

Figure 7a-d. Individual Data on ICSS Rates of Responding After Intraperitoneal D-amphetamine Administration. D-amphetamine was administered intraperitoneally at doses of 1.7 (red) and 3 (green) mg/kg, given 15 min prior to the start of the ICSS session. Rates of ICSS responding for each mouse were recorded and graphed as a function of the stimulation frequency and were compared to IP saline (blue).

Ten, 6 second puffs of 50 mg/ml d-amphetamine vapor was assessed in 4 subjects. In subject JM1 and FE123, d-amphetamine vapor exposure appeared to have little impact across either frequency curve. In mouse JM7, d-amphetamine vapor slightly increased responding at

intermediate frequencies of 71-100 Hz. Only FE125 appeared to show a consistent elevation in ICSS responding following d-amphetamine vapor exposure, with all 10 frequencies resulting in greater numbers of stimulations earned compared to the control condition.

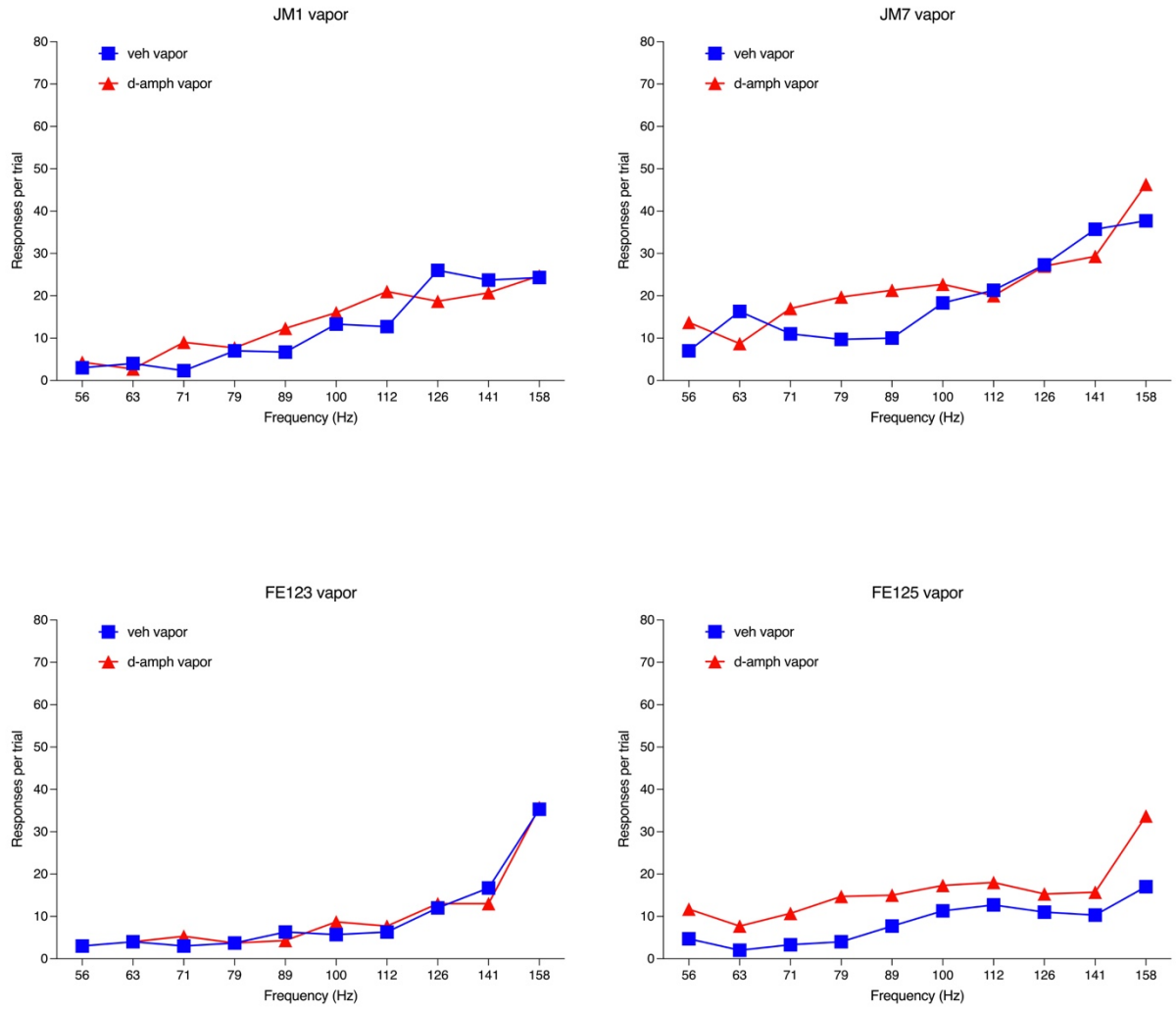

Figure 8a-d. Individual Data on ICSS Rates of Responding After Vaporized D-amphetamine Administration. D-amphetamine vapor was administered at an e-liquid concentration of 50 mg/ml (red), given immediately prior to the start of the ICSS session. Rates of ICSS responding for each mouse were recorded and graphed as a function of the stimulation frequency and were compared to vehicle vapor with no drug in it (blue). One or both doses of cocaine facilitated ICSS responding in all four subjects.

Self-Administration

Figure 9 shows the number of nose-pokes per session for milk dippers + vapor puffs as the methamphetamine concentration was increased from 1 mg/ml to 25 mg/ml. There was a d-amphetamine concentration-dependent reduction in number of nose-pokes per session as the concentration of methamphetamine was increased. Methamphetamine e-liquid concentrations at the lower end of the spectrum around 1 to 3 mg/ml produced response rates about four times greater than at the highest methamphetamine e-liquid concentrations of 25 mg/ml. Like nose-pokes, completed exposures resulting in milk-dipper presentation also decreased as methamphetamine concentration increased (Figure 10). At the lowest methamphetamine concentration of 1 mg/ml, the subjects received a mean of approximately 87 dippers in the 30 min test session. This declined to approximately 21 dippers at the highest methamphetamine exposure concentration of 25 mg/ml.

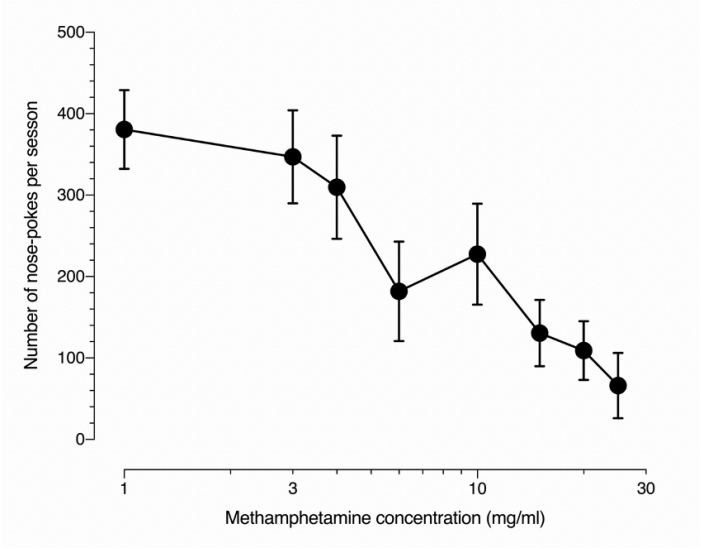

Figure 9. Number of nose-pokes per session at varying methamphetamine concentrations. The concentration of methamphetamine in the e-liquid was increased from 1 to 25 mg/ml while milk

concentration and other conditions were kept constant. Rates of self-administration (number of nose pokes) were recorded as methamphetamine e-liquid concentration was increased.

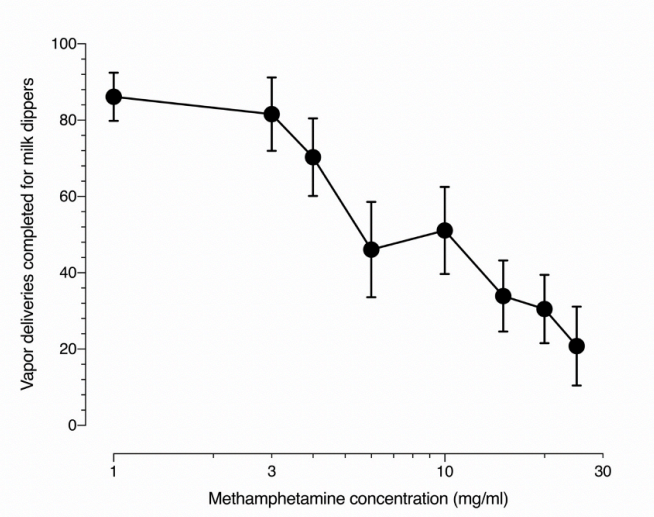

Figure 10. Number of vapor deliveries completed for milk dippers at varying methamphetamine concentrations. The concentration of methamphetamine in the e-liquid was increased from 1 to 25 mg/ml while milk concentration and other conditions were kept constant. Rates of self-administration (number of vapor deliveries) were recorded as methamphetamine e-liquid concentration was increased.

The results of reducing the concentration of milk concurrently presented with methamphetamine vapor puffs at the highest concentration each of 5 subjects would tolerate without a suppression of responding is show in figure 11. In the 100% milk + vapor condition the number of dippers+puffs earned was high, ranging from 42-79 in individual subjects (Figure 11, symbols plotted on top of each bar). A dilution of the milk with water to 50% milk produced a pronounced decrease in the number of dippers+puffs with a bimodal distribution emerging in which two subjects maintained high rates and 3 subjects exhibited a dramatic decrease in reinforcers earned. A further dilution of the milk to 25% of the starting concentration produced

even greater reductions in responding where only one subject continued to maintain a high number of dippers+puffs. When milk was completely replaced with water and behavior was reinforced only by methamphetamine, vapor puffs responding almost completely ceased, generating levels of puffs comparable to the extinction condition in which nose-pokes had no scheduled consequence.

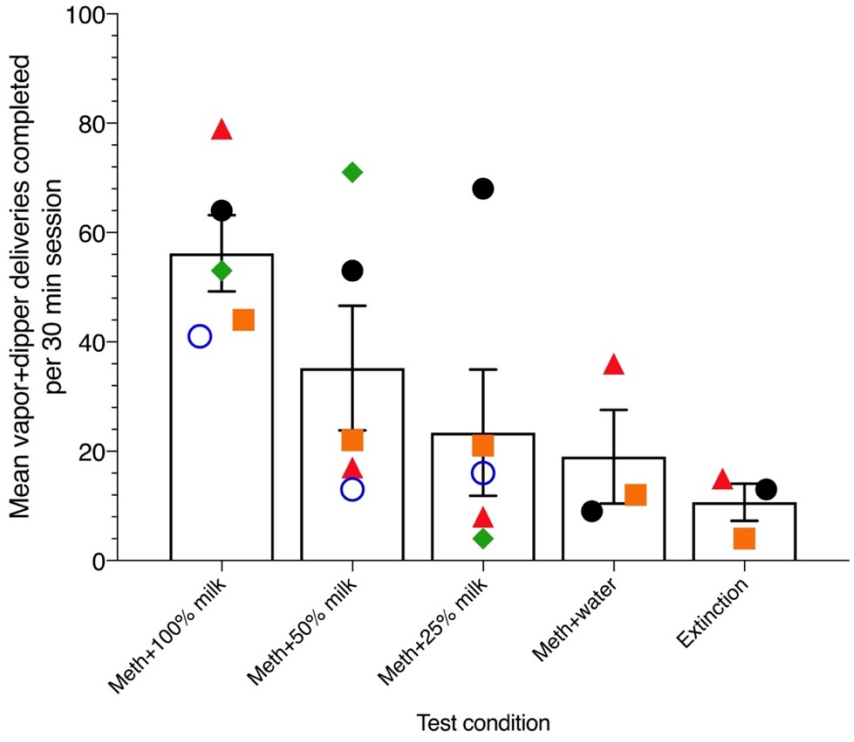

Figure 11. Mean Vapor and Dipper Deliveries Under Various Milk Conditions. The colored shapes are representative of individual rats. There are fewer rats included in the later test conditions (such as extinction) than in the earlier test conditions (such as Meth+100% milk) because some of the rats' response rates fell to zero and as such were not advanced to the subsequent test condition. Liquid reinforcement (milk and water) were altered while methamphetamine e-liquid concentration was kept constant to determine if methamphetamine vapor alone maintained rates of self-administration in the absence of the liquid reinforcer.

Locomotor Assay

Pretreatment with subcutaneous (SC) methamphetamine had a dose-dependent effect on distance traveled (Figure 12, upper panel). There was a significant main effect of dose [$F(3,27)= 15.94$, $p<0.0001$] but no significant main effect of time segment [$F(2,18)=2.949$, $p=0.0780$] nor a significant dose x time interaction [$F(6,54)=0.3602$, $p=0.9008$] Post hoc Fisher's tests revealed that doses of 1 and 1.7 mg/kg SC methamphetamine significantly increased distance traveled compared to the saline control condition. Methamphetamine administered via vaporizer also had a concentration-dependent effect on distance traveled (Figure 12, lower panel). There was a significant main effect of concentration [$F(4,36)= 7.776$, $p=0.0001$] but no significant main effect of time segment [$F(2,18)=1.805$, $p=0.1930$] nor a concentration x time interaction [$F(8,72)=0.98961$, $p=0.4541$]. Post hoc Fisher's tests revealed that methamphetamine e-liquid concentrations of 25, 50, and 100 mg/ml significantly increased distance traveled compared to the vehicle vapor control condition. There were no obvious non-significant trends in total distance traveled.

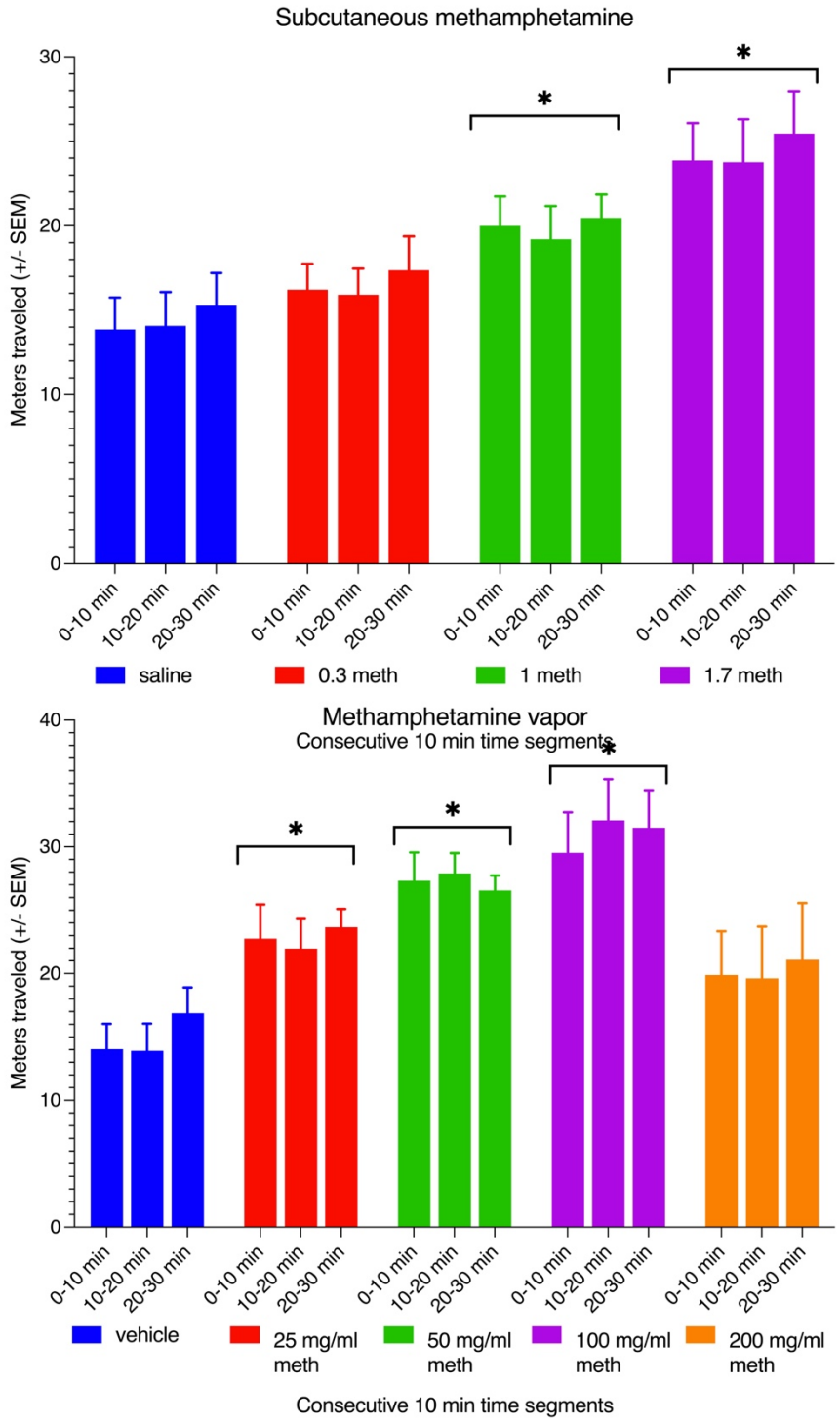

Figure 12. Time-Dependent Analysis of Locomotion: Total distance traveled. Total distance traveled during the 30 min locomotor session was assessed after administration of subcutaneous methamphetamine at doses of 0.3, 1, and 1.7 mg/kg (top). This was compared to SC saline.

Total distance traveled was also assessed following administration of methamphetamine vapor at e-liquid concentrations of 25, 50, 100, and 200 mg/ml. This was compared to vehicle vapor. Time-dependent changes in locomotion was also assessed by dividing the 30 min session into three 10 min sessions and assessing differences in locomotion across each of these 10 min intervals. * indicates statistical significance.

The number of freezing episodes was also assessed for drug and time-dependent changes in locomotion. Pretreatment with SC methamphetamine had a dose-dependent effects on the number of freezing episodes (Figure 13, upper panel). There was a significant main effect of dose [$F(3,27)= 4.392$, $p=0.0122$] and a significant effect of time segment [$F(2,18)=11.87$, $p=0.0005$] but no dose x time interaction [$F(6,54)=1.825$, $p=0.1115$]. Post hoc Fisher's tests revealed that none of the SC methamphetamine doses examined individually significantly affected the number of freezing episodes compared to the saline control condition. There was a general trend showing the greatest number of freezing episodes occurred in the second 10 min time interval at all SC methamphetamine doses but post-hoc analysis failed to demonstrate statistical significance. Pretreatment with methamphetamine vapor also had concentration-dependent effect on number of freezing episodes (Figure 13, lower panel). There was a significant main effect of concentration [$F(4,36)= 501$, $p=0.0047$] as well as a significant main effect of time segment [$F(2,18)=7.136$, $p=0.0052$] but no concentration x time interaction [$F(8,72)=0.5699$, $p=0.7992$]. Post hoc Fisher's tests revealed that all methamphetamine e-liquid concentrations tested, 25, 50, 100, and 200 mg/ml significantly reduced the number of freezing episodes compared to the vehicle vapor control. Furthermore, in every methamphetamine e-

liquid concentration tested, the first 10 minute time interval showed the fewest number of freezing episodes, although this trend was not significant.

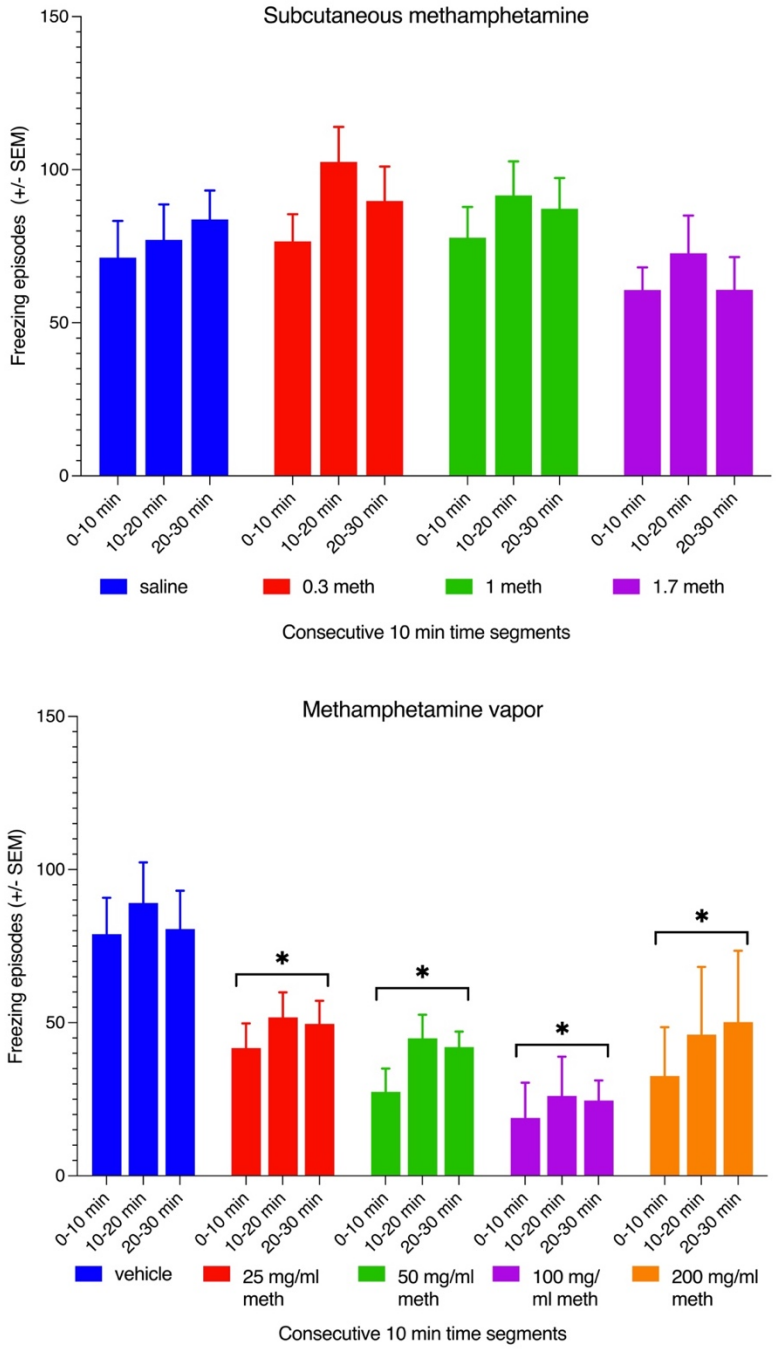

Figure 13. Time-Dependent Analysis of Locomotion: Number of Freezing Episodes. The number of freezing episodes during the 30 min locomotor session was assessed after

administration of subcutaneous methamphetamine at doses of 0.3, 1, and 1.7 mg/kg (top). This was compared to SC saline. The number of freezing episodes was also assessed following administration of methamphetamine vapor at e-liquid concentrations of 25, 50, 100, and 200 mg/ml. This was compared to vehicle vapor. Time-dependent changes in locomotion was also assessed by dividing the 30 min session into three 10 min sessions and assessing differences in locomotion across each of these 10 min intervals. * indicates statistical significance at the $p<0.05$ level.

The number of line crossings was also assessed for methamphetamine and time-dependent changes in locomotion. Pretreatment with SC methamphetamine had a dose-dependent effect on the number of line crossings (Figure 14, upper panel). There was a significant main effect of dose [$F(3,27)= 11.13$, $p<0.0001$] as well as a significant main effect of time segment [$F(2,18)=4.983$, $p=0.0190$] but no dose x time interaction [$F(6,54)=1.056$, $p=0.4001$]. Post hoc Fisher's tests revealed that doses of 1 and 1.7 mg/kg SC methamphetamine significantly affected the number of line crossings compared to the saline control condition. Pretreatment with methamphetamine vapor also had concentration-dependent effects on the number of line crossings (Figure 14, lower panel). There was a significant main effect of concentration [$F(4,36)= 8.261$, $p<0.0001$] but no significant main effect of time segment [$F(2,18)=0.2814$, $p=0.7580$] nor a concentration x time interaction [$F(8,72)=1.856$, $p=0.0805$]. Post hoc Fisher's tests revealed that all methamphetamine e-liquid concentrations tested, 25, 50, 100, and 200 mg/ml significantly increased the number of line crossings compared to the vehicle vapor control condition.

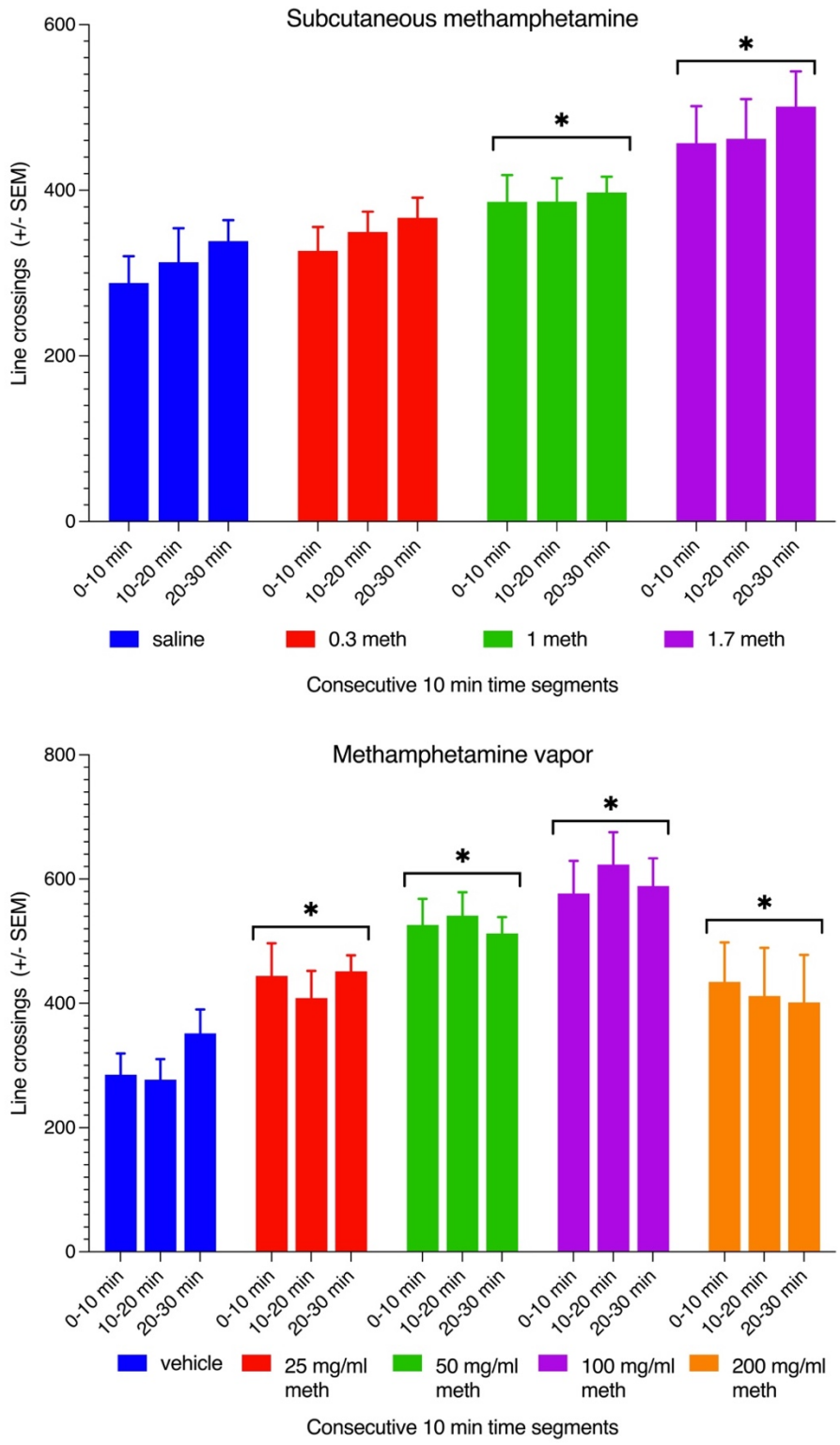

Figure 14. Time-Dependent Analysis of Locomotion: Number of Line Crossings. The number of line crossings during the 30 min locomotor session was assessed after administration of

subcutaneous methamphetamine at doses of 0.3, 1, and 1.7 mg/kg (top). This was compared to SC saline. The number of line crossings was also assessed following administration of methamphetamine vapor at e-liquid concentrations of 25, 50, 100, and 200 mg/ml. This was compared to vehicle vapor. Time-dependent changes in locomotion was also assessed by dividing the 30 min session into three 10 min sessions and assessing differences in locomotion across each of these 10 min intervals. * indicates statistical significance at the $p<0.05$ level.

Changes in total time mobile between each of the 10 minute intervals was also examined to assess methamphetamine and time-dependent changes in locomotion. Pretreatment with SC methamphetamine had a dose-dependent effect on time mobile (Figure 15, upper panel). There was a significant main effect of dose [$F(3,27)= 7.232$, $p=0.0010$] but no significant main effect of time segment [$F(2,18)=2.728$, $p=0.0923$] nor a dose x time interaction [$F(6,54)=0.8720$, $p=0.5215$]. Post hoc Fisher's tests revealed that doses of 1 and 1.7 mg/kg SC methamphetamine significantly increased time mobile compared to the saline control condition. Pretreatment with methamphetamine vapor also had a concentration-dependent effect on time mobile (Figure 15, lower panel). There was a significant main effect of concentration [$F(4,36)= 6.820$, $p=0.0003$] as well as a significant main effect of time segment [$F(2,18)=11.75$, $p=0.0005$] but no concentration x time interaction [$F(8,72)=0.7620$, $p=0.6371$]. Post hoc Fisher's tests revealed that methamphetamine e-liquid concentrations of 25, 50, and 100 mg/ml significantly increased time mobile compared to the vehicle vapor control condition. While there was a main effect of time segment, post-hoc Fisher's tests failed to show any differences in in time mobile within individual concentration conditions.

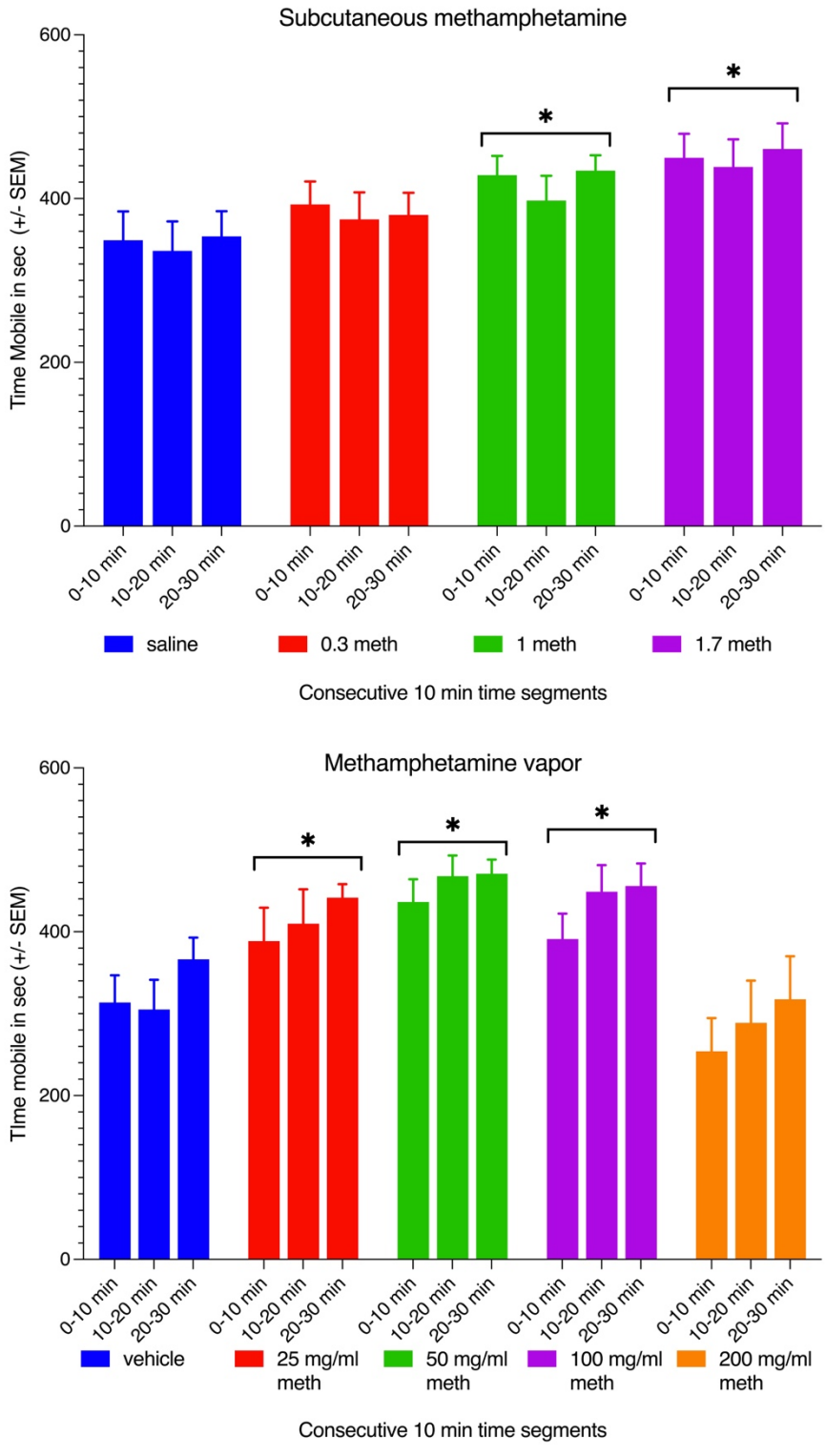

Figure 15. Time-Dependent Analysis of Locomotion: Total Time Mobile. Total time mobile during the 30 min locomotor session was assessed after administration of subcutaneous

methamphetamine at doses of 0.3, 1, and 1.7 mg/kg (top). This was compared to SC saline. Total time mobile was also assessed following administration of methamphetamine vapor at e-liquid concentrations of 25, 50, 100, and 200 mg/ml. This was compared to vehicle vapor. Time-dependent changes in locomotion was also assessed by dividing the 30 min session into three 10 min sessions and assessing differences in locomotion across each of these 10 min intervals. * indicates statistical significance at the $p<0.05$ level.

Lastly, the amount of time spent in zones along the wall was assessed for drug and time-dependent changes in locomotion. Pretreatment with SC methamphetamine had no dose-dependent effects on time spent in zones along the wall (Figure 16, upper panel). There was no significant main effect of dose [$F(3,27)= 2.373$, $p=0.0924$] but there was a significant main effect of time segment [$F(2,18)=6.863$, $p=0.0061$]. However, there was a significant dose x time interaction [$F(6,54)=1.465$, $p=0.2078$]. Post hoc Fisher's tests revealed that none of the SC methamphetamine doses tested significantly affected time spent along the peripheral zones compared to the saline control condition. Post-post hoc tests failed to reveal any significant change in time spent along the wall across successive time segments in individual treatment conditions. Pretreatment with methamphetamine vapor had a concentration-dependent effect on time spent along the periphery (Figure 16, lower panel). There was a significant main effect of concentration [$F(4,36)= 10.47$, $p<0.0001$] as well as a significant main effect of time segment [$F(2,18)=14.50$, $p=0.0002$] but no concentration x time interaction [$F(8,72)=1.603$, $p=0.1390$]. Post hoc Fisher's tests revealed that the two highest methamphetamine e-liquid concentrations tested, 100 and 200 mg/ml, significantly increased affected time spent in zones along the wall compared to the vehicle vapor control. Post-post hoc tests failed to reveal any

significant change in time spent along the wall across successive time segments in individual treatment conditions.

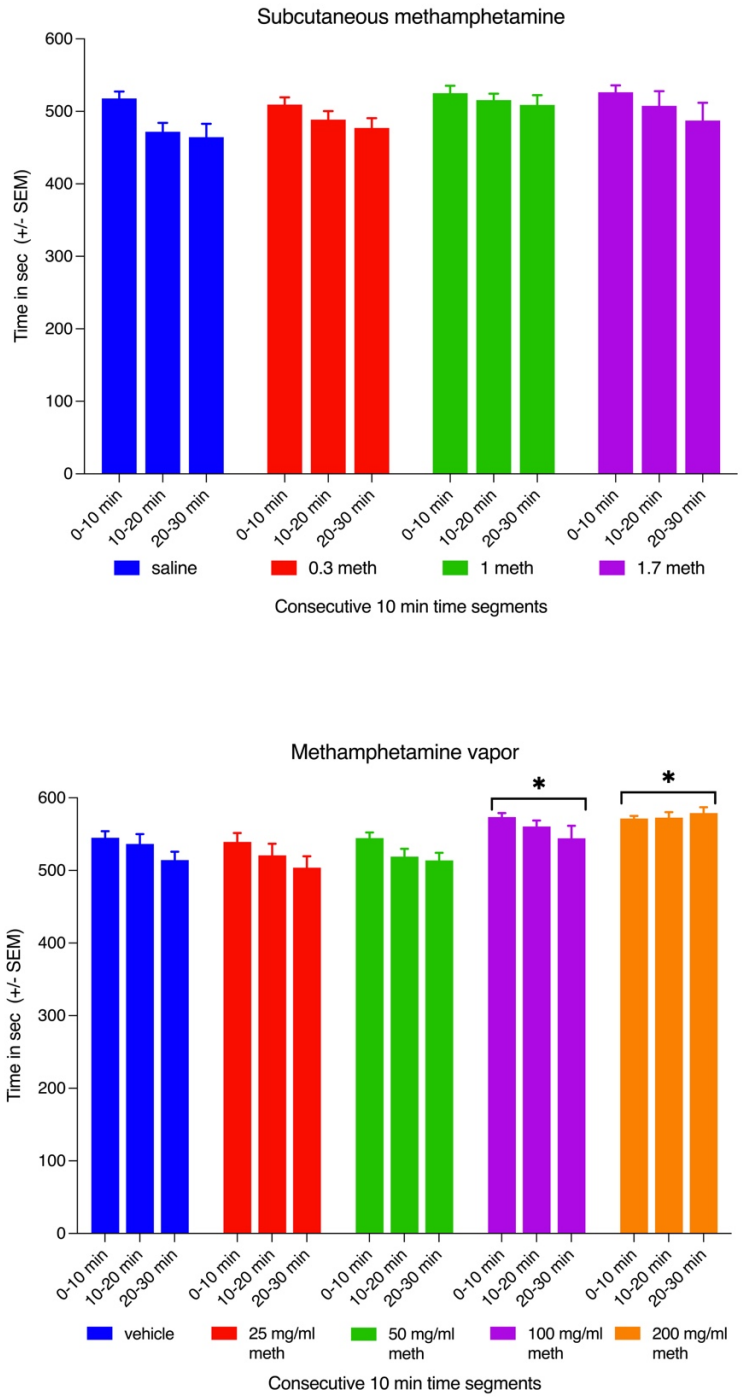

Figure 16. Time-Dependent Analysis of Locomotion: Total Time Spend in Zones Along the Wall. Time spent in zones along the wall during the 30 min locomotor session was assessed

after administration of subcutaneous methamphetamine at doses of 0.3, 1, and 1.7 mg/kg (top). This was compared to SC saline. Time spent in zones along the wall was also assessed following administration of methamphetamine vapor at e-liquid concentrations of 25, 50, 100, and 200 mg/ml. This was compared to vehicle vapor. Time-dependent changes in locomotion was also assessed by dividing the 30 min session into three 10 min sessions and assessing differences in locomotion across each of these 10 min intervals. * indicates statistical significance at the $p<0.05$ level.

After all experimentation was completed, sensitization was assessed to determine if repeated exposure to methamphetamine resulted in locomotor sensitization (Figure 17). Pretreatment with SC methamphetamine had a significant main effect on total distance travelled [$F(2,18)=6.612$, $p=0.007$]. Post hoc Fisher's tests revealed that SC methamphetamine tested at the beginning and end of the locomotor assay were significantly greater than saline ($p<0.05$) but that there was no significant difference between the first and second doses of 1 mg/kg methamphetamine indicating that sensitization did not occur during locomotor experimentation.

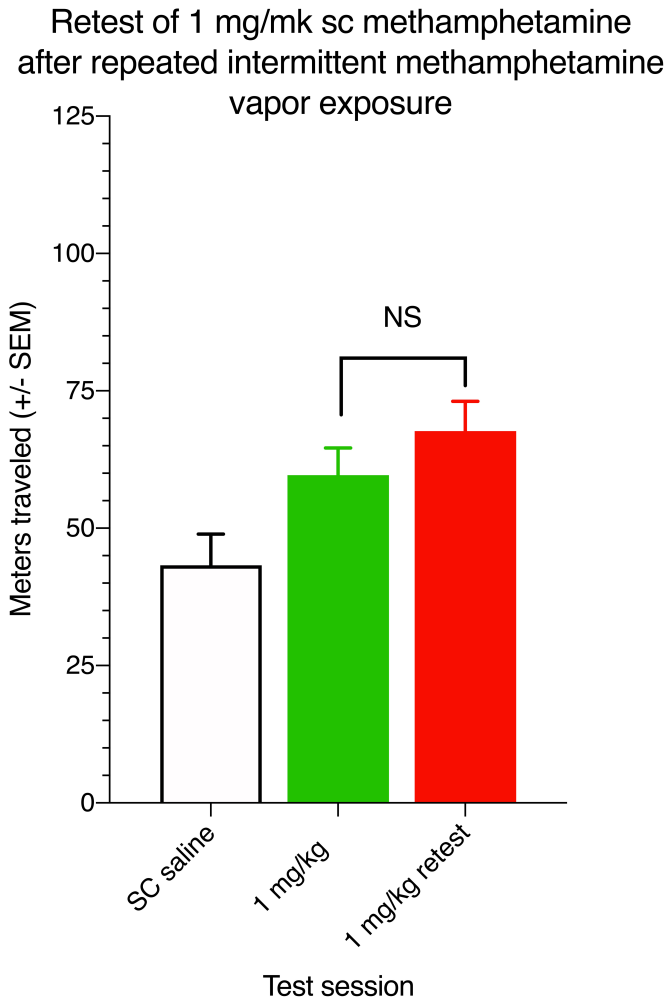

Figure 17. Testing for the Occurrence of Sensitization During Locomotor Assay. Locomotion after 1 mg/kg methamphetamine given subcutaneously at the beginning of the testing was compared to a repeated test of 1 mg/kg methamphetamine conducted after all other testing had been completed. *NS indicates non-significant ($P<0.05$) difference between the first and second test with 1 mg/kg s.c. methamphetamine.

Discussion

Vapor Analysis

The first experiment carried out to quantify and assess the amount of amphetamine being vaporized and delivered under our experimental conditions was with the wet vapor trap. This experiment indicated that increasing wattage from 18 W to 36 W while puff time and flow rate remained at standard conditions resulted in a dramatic increase in the amount of d-amphetamine captured from a mean of approximately 0.1 mg to 0.46 mg. Flow rate also increased the amount of d-amphetamine captured. Increasing flow rate from 1 L/min to 1.25 L/min while puff time and wattage remained at standard conditions resulted in a 249% increase in the amount of d-amphetamine captured. Decreasing puff time while wattage and flow rate were kept at standard conditions showed a linear decrease in the amount of d-amphetamine captured. A puff time of 3 s showed the lowest amount of d-amphetamine captured, a puff time of 5 s showed slightly higher amounts, and standard conditions with a puff time of 6 s showed the highest amount. These data are consistent with those findings from the literature with other drugs such as nicotine (Peace et al., 2018).

The results from testing 10 puffs of d-amphetamine e-liquid using the wet vapor trap were highly variable. Across the 3 replicates, a mean of 84 mg of d-amphetamine was captured during these 10 puff tests under standard conditions. However, the standard error for these three samples was ±83 mg. Specifically, one sample showed 250 mg of d-amphetamine captured while the second and third samples showed 1.192 and 0.897 mg of d-amphetamine captured, respectively. The data seem to indicate that the wet vapor trap experiments result in an

unacceptably high amount of variability between replicate samples so the data must be interpreted with a great deal of caution. As one 10 puff sample was almost 250x higher than the other two, we postulate that the outlier was somehow contaminated during the collection process. If this is true, increasing the number of puffs did not produce the expected increase in total d-amphetamine vaporized as the mean in the one puff condition was roughly comparable to the 10 puff condition with the outlier excluded.

The inconsistent results in the wet vapor trap experiment could partially be due to the hydrophobicity of the e-liquid components. As mentioned previously, the drug is suspended in a vehicle comprised of vegetable glycerol and propylene glycol, both of which are very hydrophobic substances that do not dissolve or disperse evenly in distilled water. As a result, if the drug is still suspended in microdroplets of glycerol or glycol after aerosolization, the drug may not be dispersed in the water well. In fact, it may not even be captured to any great extent. However, wet vapor trap experiments have been utilized previously to assess the amount of drug present in e-cigarette vapors composed of glycerol and propylene glycol (Peace et al., 2018; Krakowiak et al., 2019). In those studies, the results appear to be more consistent than those we show here. It was, however, conveyed to us in a personal communication from an author on one such paper that wet vapor traps have considerable variability, even in the hands of investigators with considerable expertise in their use. It could therefore be the case that some procedural factors such as flask size, water amounts in each flask, washing protocol or multiple combined factors played a role in the variability observed and more consistent results could be generated if the apparatus was better optimized.

There were several other inconsistencies in the data generated through the wet trap procedure. First, when vehicle vapor alone was tested under standard conditions, a small amount

of d-amphetamine was reported to be present in the sample. This suggests that there may be some residual d-amphetamine that was not washed out of the tubing or flasks completely from previous tests. However, the levels are so low that they are far from behavioral relevant doses. Based on these data from the wet vapor trap experiment, we can see that wattage, flow rate, and puff time all appear to influence drug delivery, all three of which appear to increase drug delivery as they increase under our conditions. However, given the great deal of variability and error in many of these samples, further testing in a more reliable manner should be carried out to verify these results.

Given the inconstant data with the wet vapor trap experiment, we altered our methods to employ a dry particulate filter trap modelled after a prototype nicotine e-cigarette automated vaping machine developed in the VCU School of Pharmacy (Personal communication, Dr. Matthew Halquist). We also chose to switch our test drug to methamphetamine because of its much greater solubility than d-amphetamine in the glycerol/propylene glycol vehicle. Briefly, we empirically determined that whereas the maximum solubility of d-amphetamine was limited to 50 mg/ml, methamphetamine could be solubilized to a concentration of at least 200 mg/ml and perhaps higher.

Under the dry filter condition, one 6s puff of 100 mg/ml methamphetamine at 18 W output resulted in a mean of 0.1829 mg of methamphetamine being captured by the dry filter. The 5 puff set of conditions resulted in 2.6402 mg of methamphetamine being captured on average. If the assumption is made that each puff yields an equal amount of capture methamphetamine, which is questionable based on the wet trap data examining 1 vs 10 puffs, this would suggest that each puff produces approximately 0.53 mg of methamphetamine. Based on the theoretical calculations, we estimated that about 1.826 mg of methamphetamine would be

vaporized in one puff. Therefore, the actual amount of methamphetamine captured under the one puff conditions is less than a third of our predictions based on the assumption that total amount of e-liquid vaporized would directly equate to amount of methamphetamine vaporized.

The inconsistencies between our theoretical calculations and the measured outcomes as well as the high degree of variability in measurements across replicates could be the result of many possible factors. One possibility is that the theoretical calculations are flawed. A number of assumptions were made in the equations used to generate the theoretical values and if any one of these was incorrect, the calculations would have been inaccurate. For instance, the assumption was made that methamphetamine would be vaporized in quantities proportional with the amount of e-liquid vaporized. There is little data addressing this of which we are aware. Another consideration is that the dry filter vapor capture system may not be effective at capturing all of the drug being vaporized. It was communicated to us by the developer of the dry trap system that, when examining nicotine, the efficiency of capture was in excess of 90%. It might well be that nicotine, which is present in e-juice at much lower concentrations relative to the concentrations of methamphetamine we used, has different aerosolization characteristics either due to the molecular characteristics of methamphetamine or the relative dissolved concentrations of drug. Additional detailed studies would be necessary to address these questions.

Lastly, the discrepant results may have been a function of the liquid chromatography-mass spectroscopy assay procedure. There is considerable supporting evidence for this hypothesis. First, blind quality control replicates of previously assayed samples were reanalyzed to confirm our findings. In the wet vapor trap experiments, these samples did not yield consisted replicable results. In the dry trap experiments, the result of reanalyzed samples was generally

better replicated but unexplained inconsistencies were still present. Second, in both the wet trap and dry filter studies, spiked quality control standards in which know quantities of amphetamine and methamphetamine were dissolved did not show the predicted concentrations of either drug. While the wet vapor trap results were substantially less consistent and less reliable compared to the dry filter results, data indicated that increasing voltage, puff time, and flow rate will increase exposure to vaporized drug. In order to better understand the extent which these factors play in drug delivery, these tests should be repeated with the dry filter system.

Ultimately, other methods of vapor analysis should also be considered given the inconsistent results obtained in our experiments. The dry filter experiment did not necessarily solve this problem of consistent drug dispersion within the solvent as the filters were placed in a solution of 80% methanol and 20% water after the vapor had been passed through it. Further studies of vapor analysis should focus on more reliable methods of vapor capture while LC-MS appears to be a reliable method of measuring amphetamine levels in vapor samples.

These results indicate that all three variables, puff time, wattage, and flow rate appear to contribute to and influence drug delivery and consequently the amount of amphetamine to which the animals will be exposed, presumably increasing drug delivery. This interpretation is consistent with published studies examining nicotine and other drugs (Peace et al., 2018; Krakowiak et al., 2019; Mulder et al., 2019). Therefore, obtaining behaviorally active vaporized doses of amphetamines in preclinical experiments should be more likely by appropriate alteration of these variables. Likewise, the reinforcing effects of methamphetamine vapor in humans would become more likely under conditions of high e-liquid drug concentrations and high wattage or voltage which can be more easily be generated using 3rd generation e-cigarettes. This supposition is consistent with the anecdotal data from drug users discussed in the introduction.

ICSS

Due to the Covid-19 pandemic, the research laboratory was closed and ICSS testing was halted for several months after the mice started training, resulting in numerous promising subjects, which had already undergone surgery and initial training, being rendered unusable. Consequently, a great deal of data was lost and the sample size of the study was far lower than expected. Briefly, only a fraction of the mice responded to ICSS at all after returning from the hiatus, roughly 1/3 of mice in our experiment. Of these mice that did respond adequately for ICSS, some failed to demonstrate reliable facilitation with cocaine. As a result, the number of subjects that were actually included in the experiment relative to the number of starting subjects is quite small. Although the reason underlying this phenomenon is unclear, our practical experience with mice as subjects in ICSS studies in the laboratory has shown that it is not uncommon for mice to have a limited experimentally-useful longevity. Furthermore, there was also a substantial difference in the amplitude of electrical stimulations that proved to be effective in each mouse and in the aging mice, the amplitude of electrical stimulations often times needed to be increased or decreased as a mouse would become more or less sensitive to stimulation. When a mouse needed to have its stimulation intensity changed, testing needed to be restarted with intraperitoneal cocaine injections again before testing intraperitoneal d-amphetamine injections and vaporized d-amphetamine to establish new baselines and control rates of responding.

Given a limited number of subjects were available, we chose to examine behavior of each subject individually in the tradition of classical experimental analysis of behavior. Furthermore, data was collapsed across the three components taking the average of the three components and

graphing them as a function of the change in stimulus frequency. This was done because there were no time-dependent changes in ICSS responding across the three components. As stated previously, cocaine was administered intraperitoneally at doses of 10 mg/kg and 17 mg/kg and showed an increase in ICSS responding in all four subjects at either one or both doses tested. Cocaine given at a dose of 17 mg/kg showed greater increases in ICSS responding in three of the four subjects compared to a dose of 10 mg/kg, which is to be expected based on previous studies done on ICSS rates of responding after cocaine administration (Gilliss et al., 2002; Kenny et al., 2003; Fish et al., 2010; Riday et al., 2012; Bauer et al., 2014; Tracy et al., 2014). These data demonstrate that the ICSS test procedure and electrode placements were adequate, at least when the mice were challenged with a highly efficacious facilitator of performance.

When the mice were given d-amphetamine intraperitoneally, at doses of 1.7 mg/kg and 3 mg/kg, two of the subjects showed an increase in ICSS responding relative to saline. One subject showed a frequency-dependent change in responding characterized by a general decrease in ICSS responding after being administered 3 mg/kg of d-amphetamine intraperitoneally at all but the highest frequency where there was an increase in ICSS responding. The fourth animal did not show any response at the 1.7 mg/kg dose and lost their electrode implant before they could be tested with 3 mg/kg. Overall, the degree of facilitation produced by d-amphetamine was lower we had anticipated based on the literature. We are not aware of any previous studies done on rates of ICSS responding in mice when administered d-amphetamine but there are several that have been done in rats (Borowski & Kokkinidis, 1992; Akhiary et al., 2018). These studies all showed a substantial increase in ICSS responding in rats following d-amphetamine administration, while our study in mice did not. There are a number of potential reasons why d-amphetamine did not produce a robust response. First, d-amphetamine was tested after cocaine

and it might be that the general degradation of performance exhibited by the subjects as a function of age had continued. However, this explanation seems unlikely since the baseline performance of the subjects on non-test days met the testing criteria. Second, the doses chosen, pretreatment time, or route of administration might have been less well optimized as compared to cocaine. The d-amphetamine doses chosen were based on the literature but the available studies in the literature were in rats and mice might require dosage adjustments (Dunnick & Elwell, 1989). Regardless, two subjects showed fairly robust facilitation of ICSS at the 3 mg/kg dose, with performance across all 10 frequencies being higher, in some cases more than double that of the saline control condition. In the one subject that showed a degradation of performance at the 3 mg/kg dose of d-amphetamine, it could be that subject could be more sensitive to d-amphetamine as the data from this subject are consistent with literature reports of high doses of d-amphetamine suppressing responding (Schaefer & Michael, 1988).

When the mice were administered vaporized d-amphetamine at an e-liquid concentration of 50 mg/ml, we saw a consistent increase in rates of ICSS responding across all frequencies in only one of the four subjects. Interestingly, this subject was also one of the two that demonstrated a consistent increase in ICSS performance following IP injected d-amphetamine. In the other subject that showed a robust faciliatory response following IP injected d-amphetamine, there was an increase in ICSS responding in the middle frequencies of 71-89 Hz with little change from vehicle conditions in the upper and lower ends of the frequency range. The remaining two subjects who did not show good facilitation following IP d-amphetamine also failed to show facilitation after administration of d-amphetamine vapor.

Given the small number of subjects and the single d-amphetamine vapor exposure conditions examine, the conclusions which can be drawn from this study are limited. Based on

the data from one subject that showed robust facilitation following both injected d-amphetamine and amphetamine vapor, there is at least some support for the hypothesis that d-amphetamine vapor had behavioral activity. This conclusion is, however, tempered by the data from the remaining subjects which showed less robust or indeed no response following exposure to 50 mg/ml d-amphetamine vapor. A prior study has demonstrated that exposure to 100 mg/ml methamphetamine vapor lowers ICSS thresholds (Nguyen et al., 2016). Methamphetamine and d-amphetamine have similar potencies (Hall et al., 2008). Therefore, it could be that increasing the total dose of d-amphetamine administered in our study might have produced more robust facilitation. However, the 50 mg/ml concentration of d-amphetamine utilized was the limit of d-amphetamine solubility in the VG/PG vehicle solution. As such, in order to produce an effectively higher dose it would have been necessary to increase either the exposure duration or the number of puffs administered to test this hypothesis. The limited solubility of d-amphetamine may suggest that it could have a lower relatively abuse liability by vapor administration than methamphetamine although this is somewhat speculative based on the limited amount of data available. In terms of other conclusions, the difficulties in this study suggest that future research on the impact of stimulant vapors on ICSS might be best done in in rats, which based on anecdotal evidence, tend to be more stable in the amplitude of the stimulation that is required to facilitate ICSS responding. Also, given their larger size, rats permit more accuracy and reliability that the electrode implantation is in the desired target region of the brain resulting in lower attrition of subjects and more efficient data collection.

Self-Administration

Rats could be readily trained to nose-poke for milk dipper reinforcers even when those reinforcers were paired with 4 second/18 W exposures to a low concentration methamphetamine vapor. Responding at the lowest 1 mg/ml methamphetamine condition resulted in approximately 375 nose-pokes and 87 dipper+vapor deliveries per 30 min session. The number of nose pokes and vapor deliveries during the session decreased dramatically in a linear fashion as the methamphetamine concentration of the e-liquid was increased. This could be due to a number of reasons. One possible reason is simply because methamphetamine vapor becomes increasingly aversive at higher concentrations, acting as a punisher for milk-reinforced operant responding. Punishers of all types decrease behavior and the present data are entirely consistent with that hypothesis (Poling et al., 2002). Methamphetamine has well-documented disruptive effects on operant responding so it is possible that the higher concentrations produced sufficient pharmacological effects to directly result in a decrease in responding for milk (Verhave, 1958; Borrelli et al., 2021). Another potential reason for the reductions in responding is that methamphetamine is causing an appetite suppressing effect (Evans, 1971). Given that the rats are responding for milk, any drug that acts as an appetite suppressant, such as methamphetamine and many other stimulants, may reduce responding for such a reward. Finally, it could be that the rats stop performing nose pokes in order to modulate their dose of methamphetamine and avoid becoming overstimulated.

In the paired-milk reinforcer fading portion of the self-administration experiment, there was a decrease in the number of vapor deliveries as the milk was diluted until being entirely replaced with water. The methamphetamine concentration during this portion of the experiment

was kept constant along with the conditions of the vaporizer so the only variable that is being changed is the liquid reinforcer. Because the vapor deliveries decreased each time the milk was further diluted with water, the milk appears to be the primary reinforcer with little evidence of transference to amphetamine vapor. This is more evident as response rates fall to almost zero when no liquid reinforcer is offered at all. Based on these data, methamphetamine vapor does not appear to have reinforcing effects under the conditions tested in this experiment. If methamphetamine vapor had reinforcing effects under these conditions, vapor deliveries and response rates would have remained elevated even in the absence of a paired milk reinforcer. The present data can only be directly compared to one other study of which we are aware (Nguyen et al., 2016). In that study, the authors concluded that full body exposure to methamphetamine was rewarding based a greater number of nose pokes for methamphetamine than when the animals were placed in extinction. However, the authors noted in supplementary data that the number of nose pokes for vehicle exceeded those for methamphetamine which would be evidence that either vehicle vapor puffs were more reinforcing than methamphetamine puffs but both were reinforcers, or that neither vehicle or methamphetamine were reinforcing. The most common definition of a reinforcer in a self-administration assay is that the number of drug deliveries exceed those for vehicle so the most consistent explanation for their data is that methamphetamine was not a reinforcer. In the present experiment, the mean number of vapor deliveries in the methamphetamine vapor+water dipper condition exceeded that for extinction but only for those subjects that had not already ceased responding before the milk solution had been entirely replaced with water. When combined, the results of both studies are not supportive of the hypothesis that methamphetamine vapor is reinforcing, at least under the test conditions which were explored.

It is certainly possible that other test conditions might have revealed that methamphetamine vapor has reinforcing effects. Based on the locomotor assay data, we found that methamphetamine vapor produced statistically significant increases in locomotion at e-liquid concentrations of greater than 25 mg/ml. Given that the highest concentration in the self-administration experiment was 25 mg/ml, it is possible that we were not yet achieving a cumulative dose that was high enough to be reinforcing in the self-administration experiments. We attempted to reach higher concentrations of methamphetamine in the vaping solution but the subjects would not tolerate exposing themselves to greater concentrations even when paired with milk reinforcers. This could be due to aversive taste, direct drug effects, or other factors but these factors are all unavoidable in rodent studies. While these variables are critical in rodent experiments, the aversive taste properties at least might be overlooked in humans so the present study does not rule out the possibility of human methamphetamine vapor abuse.

Locomotor Assay

The purpose of the locomotor assay was to determine if behaviorally relevant doses of methamphetamine were being achieved when being administered via vaporizer. The primary metric assessed was total distance traveled. The positive control of subcutaneously injected methamphetamine produced a dose-dependent statistically significant increase in total distance traveled at all three test doses, 0.3, 1, and 1.7 mg/kg, in a dose-dependent manner. This outcome and dose range is consistent with the effects of methamphetamine given by injection to mice in other published studies (Kelly et al., 2008; Good & Radcliffe, 2011). Methamphetamine vapor

also resulted in a concentration-dependent and significant increase in distance traveled. Negative control tests with vehicle vapor and air-only exposure confirmed that the results were not simply due to a locomotor agitation caused by the novel exposure chamber or stress-related response of being exposed to vapor itself and indicates that the vehicle vapor itself did not serve to alter locomotion or have any behaviorally relevant effects that influenced total distance traveled.

This data shows that, under appropriate exposure conditions, methamphetamine vapor produces locomotor activity increasing effects. This data is similar to that in a prior study in mice using a telemetry receiver locomotor assay and comparable methamphetamine vapor exposure concentrations (Nguyen et al., 2016). One interesting difference between the present study and that referenced is that the published study's locomotor assessment was conducted 20 min after the cessation of exposure and 40 min after initiation of exposure to methamphetamine vapor. It was our a priori hypothesis that methamphetamine vapor would have rapid onset of effects provided that the changes were due to uptake in the lungs as opposed to oral ingestion due to grooming vapor-contaminated fur. The present data confirm that the effects are indeed rapid in that the locomotor activating effects of methamphetamine vapor were at maximal levels within the first 10 min time block, which was collected beginning immediately after the cessation of exposure and did not diminish across the entire 30 min measurement period.

Unlike the prior study, in the present experiment we also assessed the positive control of injected methamphetamine in order to compare relative efficacies and potencies across routes of administration. Although the maximal locomotor stimulatory dose of injected methamphetamine may not have been achieved, total distance traveled at methamphetamine e-liquid concentrations of 50 and 100 mg/ml are both higher than the highest 1.7 mg/kg dose of methamphetamine given subcutaneously. This was unexpected given that the theoretical calculations predicted we would

be unlikely to reach a behaviorally-active total delivered dose of methamphetamine using our apparatus and test conditions. One possible explanation for this is that the theoretical vape dose calculations were inaccurate and the actual dose delivered is actually much greater. Another possible explanation is that methamphetamine vapor, when administered under our test conditions, is far more potent than we originally believed. Further studies that also examine blood plasma levels in the animals after exposure to methamphetamine can help determine the actual dose of methamphetamine they are receiving and give a better understanding of the pharmacokinetics of methamphetamine when administered with a vaporizer.

Methamphetamine vapor had an inverted U-shaped response across the tested concentration range. It appears that our vapor exposure test conditions captured the maximally stimulating inhaled dose of methamphetamine given that inhaled methamphetamine at an e-liquid concentration of 200 mg/ml did not produce a statistically significant increase in total locomotion compared to a lower concertation of 100 mg/ml. This reduction in total locomotion at the highest concentration is possibly due to stereotypy (Mueller et al., 1989; Milesi-Hallé et al., 2007). Stereotypical behavior, such as head twitching, behavior typically seen at high doses of amphetamines, can impede the mice's ability to walk and, in turn, reduce the total distance traveled (Sahakian et al., 1975). Another possibility is that the high dose of methamphetamine is producing anxiogenic-like effects, which also may reduce the total distance traveled as the mouse tends to remain stationary in the periphery of the locomotor chamber.

Although total distance traveled is the one of the most common measures in locomotor assays, we also assessed several other metrics that might be hypothesized to be impacted by methamphetamine. Measuring time spent in the zones along the wall can be a reflection of thigmotaxis, the tendency of an animal to remain close to the walls during a locomotor assay,

which has been hypothesized to be a rodent-specific index of anxiogenic-like effects (Simon et al., 1994). Anxiogenic drugs, such as methamphetamine, typically increase thigmotaxis while drugs that are anxiolytic, such as phenobarbital, will reduce thigmotaxis (Simon et al., 1994). Thigmotaxis increased (and time spent in the zones along the periphery increased) at a subcutaneous methamphetamine dose of 1 mg/kg. This was the only significant change in time in the peripheral zones with subcutaneous methamphetamine administration and may be indicative of some anxiogenic-like effects. The higher dose of 1.7 mg/kg may have also caused some mild anxiogenic-like effects but the stimulatory locomotive effects may have been large enough that they overpowered any anxiogenic-like thigmotaxis. Interestingly, while vehicle vapor alone did not alter total distance traveled, it did increase time spent in zones along the wall. This may indicate that the vehicle vapor itself is anxiogenic in nature. However, thigmotaxis is typically only a reliable indicator of anxiogenic-like effects when the environment is novel. Given that the mice were habituated to the locomotor chamber prior to drug testing, this may not be the strongest indicator of anxiogenic-like effects. Additionally, more than one experiment ot indicator of anxiety-like behavior may be necessary to more strongly support any conclusions. Furthermore, methamphetamine vapor also produced statistically significant increases in time spent along the wall. This could be for a few reasons. One possibility is that vaporized methamphetamine is causing anxiogenic-like effects. Thigmotaxis might increase time spent in the zones along the wall but not necessarily decrease total distance traveled as the mice may still be moving around the perimeter of the locomotor chamber at an increased rate. This may be the case at the methamphetamine e-liquid concentration of 100 mg/ml given that we see a significant increase in total distance traveled and a significant increase in the time spent along the wall. At the 200 mg/ml methamphetamine e-liquid concentration, we see a decrease in total distance

traveled and an increase in time spent in zones along the wall. The mice could be exhibiting stereotypical behavior at this dose which impedes gross locomotion in all zones. These results could certainly be a product of both the anxiogenic-like effects and stereotypical behavior in combination as well.

A final component of the locomotor assay was to ensure that sensitization to methamphetamine did not occur throughout the tests. Sensitization is a phenomenon that occurs as a product of frequent administration of a number of stimulants such as methamphetamine. These repeated administrations over time may result in a behavioral sensitization and increased locomotor response to methamphetamine (Wearne et al., 2015). As mentioned previously, doses of 1 mg/kg were given to the mice at the beginning of the locomotor assay testing and following the final vapor test session. There was no statistically significant change in locomotion between these doses indicating that sensitization was unlikely to have occurred.

As previously noted for total distance traveled, the time-dependent effects of methamphetamine on locomotion were examined across all metrics. There was little evidence for time-dependent effects across all metrics. There were some general trends, such as the observations that the highest number of freezing episodes during the second 10 minute time interval after administration of both vaporized methamphetamine and subcutaneous methamphetamine. However, none of the trends approached statistical significance.

In retrospect, the locomotor assay, which was conducted last in the series of studies, should have been one of the first in order to determine if we were in fact able to achieve behaviorally relevant doses of methamphetamine administered via a vaporizer. It was only conducted as a result of the generally negative data generated in the other assays. If the locomotor experiment was conducted first, it would have been informative to delineate those

exposure conditions which produced behaviorally-relevant effects. As a result, we carried out the ICSS and self-administration experiments without knowing what e-liquid concentration was required to achieve behaviorally relevant doses. Therefore, the conditions, specifically the methamphetamine or d-amphetamine e-liquid concentrations, were not consistent across the locomotor assay, ICSS, and self-administration. ICSS was carried out with a maximum d-amphetamine e-liquid concentration of 50mg/ml. While the locomotor assay showed increases in locomotion in a dose-dependent manner between e-liquid concentrations of 25 and 100 mg/ml, the maximum increase in locomotion was at an e-liquid concentration of 100 mg/ml. Additionally, one previous study that assessed vaporized methamphetamine's effects on ICSS and locomotion showed changes in ICSS threshold and locomotor activity at a methamphetamine e-liquid concentration of 100 mg/ml (Nguyen et al., 2016). Therefore, to ensure that the mice are receiving a dose of methamphetamine high enough to influence rates of ICSS responding, additional doses of methamphetamine should be administered at an e-liquid concentration of 100 mg/ml. Furthermore, the self-administration model only tested up to a maximum methamphetamine e-liquid concentration of 25mg/ml. A concentration of 25 mg/ml methamphetamine was the lowest that resulted in statistically significant changes in locomotion. As such, the locomotor data would suggest that further experimentation should potentially be carried out at the higher e-liquid concentrations in a self-administration model. However, this may prove to be problematic because it appears the rats may have found the methamphetamine vapor to be aversive, even at a concentration of 25 mg/ml. This raises the question as to whether the methamphetamine vapor has the potential of being reinforcing in rats at all if it is not engendering a set of conditions in which behaviorally-relevant doses can be achieved.

Additional studies using alternative methods of promoting tolerance to high concentration of methamphetamine vapor would be necessary to address this possibility.

Future Directions

Further studies in several directions might be carried out in order to better understand the abuse potential of vaping amphetamines. First, vapor analysis should be done with a more reliable method of vapor capture. The solubility of the drug and its vehicle should be taken into consideration when assessing alternate methods of vapor capture. Furthermore, the ICSS and self-administration experiments can be attempted with higher concentrations of methamphetamine e-liquid, up to 100 mg/ml, such that they can better predict the abuse potential given that a methamphetamine e-liquid concentration of 100 mg/ml was found to produce the greatest increase in locomotion based on several metrics including total distance traveled. In addition, ICSS should be carried out in rats rather than mice given the greater reliability of rats' ICSS performance compared to mice. Also, no lapses in experimentation should occur during these studies as it did with our experiments due to the Covid-19 pandemic. Additionally, with vaporized methamphetamine administration, it was impossible to determine whether the effects we were seeing were only from inhalation of the drug. Therefore, mucosal sampling of the nasal canal, to determine if nasal absorption is playing a role, as well as mucosal sampling of the esophagus, to determine if ingestion is playing a role, can be carried out. Also, an additional future direction that should be considered is other possible methods of inhaled methamphetamine. Another study assessing the abuse potential of inhaled methamphetamine

found that concentrations as low as 1 mg/ml, a fraction of the concentration we found to be effective in increasing locomotion, increased locomotion when the methamphetamine was administered via a nebulizer (Juarez-Portilla et al., 2017). Therefore, there are other systems that allow for administration of inhaled methamphetamine that may prove to be more potent and have greater abuse potential than our tested system. Finally, other experiments that better assess anxiogenic-like effects in rodents should be carried out to assess methamphetamine vapor's effects on mice. One possible experiment that could be carried out to assess anxiogenic effects is the elevated plus maze. The elevated plus maze has long been a reliable assessor of anxiety-like effects in rodents in general, after genetic alteration, and in pharmacological experiments (Komada et al., 2008; Kraeuter et al., 2019). This type of experiment has previously shown that methamphetamine has positive anxiogenic effects (Pometlová et al., 2012). Another possible experiment that could be carried out to assess the anxiogenic-like effects of methamphetamine vapor is the elevated T maze. The elevated T maze has also been used previously to study anxiety-like behavior and can prove beneficial over the elevated plus maze in also assessing panic (Jardim et al., 1999; Deacon & Rawlins, 2006). Both of these types of experiments could be used to better assess the anxiogenic-like effects produced by methamphetamine vapor.

Conclusion

Overall, the current data indicates limited support from our rodent studies that vaporized amphetamines are likely to have substantial abuse liability. What support is present appears to indicate that methamphetamine vapor is more likely to be abused than d-amphetamine vapor simply due to its greater solubility in e-liquid. Locomotor data indicated that behaviorally relevant doses of methamphetamine were being achieving under our test conditions and with our vapor exposure system. The experiments that were a stronger indicator of abuse potential, ICSS and self-administration, showed there is likely little abuse potential in vaping methamphetamine if the test conditions in our rodent models accurately reflect the usage characteristics of humans. However, it is possible that humans may ignore many of the aversive effects that could have impacted the data in rodents. Therefore, there is still a chance that inhaled methamphetamine has abuse potential when vaporized and administered with electronic cigarettes in humans. In conclusion, our data indicates that vaporized methamphetamine does not appear to have substantial abuse liability in rodent models of abuse but abuse potential in humans should still be considered a possibility and further research should be done to assess other conditions under which it may be administered.

APPENDIX

Theoretical Vape Dose Calculations:

d-Amph vape solution (mg/ml):	50
Vehicle weight lost (mg):	219.666667
Weight of 1ml Veh (mg):	1153.5
Weight of 1ml d-Amph sol (mg):	1203.5
Puff duration (s):	6
Puffs per session	10
Time before ventilation (s):	10
Time mouse exposed to single puff of vapor (s):	13
Seconds of puff per session	60
d-Amph vaporized per session (mg):	9.12615983
d-Amph vaporized per second (mg):	0.15210266
d-Amph per second x puff duration (mg):	0.91261598
d-Amph per second x puff duration (µmol):	5.31619928
Chamber Volume (cm^3):	12144.86
d-Amph distribution in one puff (µmol/cm^3):	0.00043773
Mouse Tidal Volume (cm^3):	0.15
Mouse Respiratory Rate (breath/min):	181
Volume inhaled per minute (cm^3/min):	27.15
d-Amph inhaled per puff (µmol):	0.00257496
d-Amph inhaled per session (µmol):	0.02574961

For reference, Methamphetamine ED50 via inhalation is 9.4 µmol/kg → 0.282 µmol for a 30g mouse

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Contributions

Data from all experiments was obtained from the laboratory of Dr. Keith Shelton at Virginia Commonwealth University. I thank Dr. Keith Shelton and Dr. Katherine Nicholson for their contributions to this project. I also thank Justin Poklis of the VCU pharmacology and toxicology labcore for analyzing the vapor samples with Liquid Chromatography-Mass Spectroscopy. Services in support of this research project were provided by VCU department of pharmacology and toxicology.

Vitae

Lucas Cameron Wohler grew up in Fredericksburg, Virginia. He attended the University of Virginia in Charlottesville, VA where he received a bachelor's degree in chemistry, graduating in 2018. He then attended the Premedical Graduate Sciences Certificate program at Virginia Commonwealth University, which he completed in 2019. Following this program, he pursued a master's degree in Pharmacology & Toxicology at Virginia Commonwealth University, studying under the supervision of Dr. Keith Shelton. Lucas plans to attend the West Virginia College of Osteopathic Medicine as part of the class of 2025.