Effects of Acute Ketamine on Operant Ethanol Self-Administration Over Time

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July 25, 2017

APPROVAL SHEET

Title of Dissertation:
"Effects of acute ketamine on operant ethanol self-administration over time"

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Date: 07/25/2017

THESIS AND ABSTRACT APPROVED:

DATE: 7/25/2017

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Abstract:

In this document I propose a set of experiments to determine if the long-term effects of ketamine exposure alter operant ethanol self-administration and ethanol consumption in ethanol dependent rats. Animals will be trained to self-administer ethanol, made dependent with ethanol vapor exposure, and treated with ketamine or related compounds. After drug treatment, measures of ethanol consumption will be examined for one week, in order to determine if the pre-established long-lasting effects of the drug ketamine will alter drinking behavior. In initial experiments, animals will be administered ketamine systemically, to determine it's long-term effects and viability as a treatment for alcohol use disorders. In a second experiment, animals will be treated with ketamine and compounds that antagonize some of its pharmacological actions, to uncover the potential mechanism of these effects. Additionally, ketamine will be microinjected into reward and motivated behavior associated regions of the brain, in order to determine which areas of the brain that ketamine may be acting on to alter drinking. It is my belief that these experiments could provide solid preclinical evidence for ketamine's potential as a treatment for alcoholism, as well as expanding our understanding of a commonly used drug's pharmacological effects on the brain.

Effects of Acute Ketamine on Operant Ethanol Self-Administration Over Time
A Research Proposal by Geoff Dilly

In the US, 15 million people suffer from alcohol use disorders (AUDs), leading to 88,000 deaths and 233 billion dollars in economic damage per year(Bouchery et al., 2011; Substance Abuse and Mental Health Services Administration. Center for Behavioral Health Statistics and Quality, 2016). Despite being some of the most damaging diseases, only three drugs have been approved as treatments for AUDs, and they all have significant limitations. The available pharmacological treatments tend to only be effective partially, or in specific genetic populations, and to have unpleasant side effects and low patient compliance, so the development of new treatments is of paramount importance. The NMDAR antagonist and anesthetic agent ketamine has been shown to rapidly induce long lasting changes in behavior, including having profound effects on depression(Garcia et al., 2008). Ketamine has been used in unpublished clinical trials as a treatment for depression and alcoholism in comorbid patients (Petrakis, 2015), and as an anesthetic during alcohol withdrawal. In animal studies ketamine treatment decreases ethanol self-administration in the short-term(Sabino et al., 2013).

Ketamine has been shown to induce lasting synaptic plasticity and long-term changes in behavior after a single low-dose exposure, likely through a homeostatic mechanism(Abdallah et al., 2016). These long-term effects have been shown to significantly decrease symptoms of depression, but their effects on other behavioral disorders are not well understood(Garcia et al., 2008). Evidence suggests that the long lasting effects of ketamine may reverse deficits that are present in alcohol consuming animals (Logrip et al., 2015; Stragier et al., 2015; Zhou et al., 2014), but only short-term changes in drinking behavior have been observed. For these reasons, we believe that the long-term effects of ketamine on ethanol consumption are worthy of further study.

It is not known how NMDAR receptor drugs with demonstrated long-term effects, such as ketamine, affect ethanol withdrawal and consumption. We propose a set of experiments to answer this question: How does ketamine exposure act on the motivational circuits of the brain, and how do these pharmacological manipulations affect ethanol consummatory behavior?

We propose the following aims to investigate this question:

Aim 1: To determine the long-term effect of a single administration of ketamine and pharmacologically related compounds on ethanol relapse.

A: Male Long-Evans rats will be trained to orally self-administer ethanol, exposed to ethanol vapor to promote dependence, and allowed to establish stable self-administration. Twelve hours before a self-administration session, animals will be injected with ketamine. Animals will be allowed to continue drinking each day for twelve hours, and measures of ethanol consumption will be compared between groups. These experiments will examine the potential of ketamine to act as rapid acting, extended-duration treatment to prevent alcohol relapse. We hypothesize that treatment with ketamine will induce a lasting neurological effect that will decrease ethanol consumption 12-24 hours, and up to one week after treatment.

B: We will perform experiments to uncover the potential mechanism of action of ketamine's effects on ethanol consumption. Drugs that are known to block particular pharmacological targets of ketamine will be injected simultaneously with ketamine, to determine which of these pathways is responsible for ketamine's effects. Ketamine, NMDAR competitive antagonist MK-801, and tropomyosin receptor kinase B (TrkB) antagonist ANA-12, and combinations of each will be administered with and without ketamine 12 hours before self-administration, and measures of ethanol consumption will be observed for seven days. We hypothesize that TrkB blockade will eliminate the long-term effects of ketamine on ethanol consumption.

Aim 2: To determine the long and short-term effects of ketamine in specific, reward-associated brain regions. Animals will be implanted with a microinjection cannula in selected brain regions of interest (e.g., the nucleus accumbens (NAcc), or medial prefrontal cortex (mPFC)), and undergo the self-administration and vapor exposure protocol described in Aim 1. During the test session the ROI will be infused with ketamine or saline. Measures of ethanol craving and consumption will be examined during the microinjection and on subsequent drinking days, and compared across groups. We hypothesize that acute ketamine in reward associated regions will have region dependent effects on ethanol consumption, during the initial session, and throughout the course of the following ethanol self-administration sessions.

These experiments would provide a solid rationale for the viability of ketamine and similar drugs as potential treatments for alcoholism, or lack thereof, and establish a framework that could be used to investigate the cellular and molecular effects of alcohol and ketamine further.

Significance:

Alcohol use disorder (AUD), or alcoholism, is a behavioral disorder that involves the continued consumption of alcohol, despite negative consequences on the affected individual. Criteria for the diagnosis of AUD are complex. The DSM-5 lists eleven criteria, at least two of which must be present for a positive diagnosis. Generally, AUD involves the development of cravings and withdrawal, excessive use, and an inability to quit drinking, despite the impact on the patient's life(American Psychiatric Association, 2013). AUD has a significant impact on the American populace, but current pharmacological treatments for alcoholism are generally not very effective.

The complexity of the disease suggests the possibility that a number of different therapies could be developed to treat the various aspects of AUD, but so far only three drugs have been approved for its treatment. Unfortunately, none of these drugs have been particularly effective. Considering prevalence and economic impact of alcoholism, additional treatments would be immensely beneficial.

The pharmacological effects of ethanol are complex. It has been shown to act on many types of molecular targets within the body, including ligand-gated ion channels, voltage-gated ion channels, metabotropic receptors, and G-protein coupled receptors (GPCRs) (Krystal et al., 2003; Trudell et al., 2014). Two of its primary targets, which are thought to be responsible for the majority of ethanol's intoxicating effects are the $GABA_A$ receptor ($GABA_AR$), and the NMDAR (Harris et al., 2008; Trudell et al., 2014). Ethanol acts on the $GABA_AR$ as a positive allosteric modulator, boosting chloride ion flow through the channel of the inhibitory receptor, and increasing inhibitory tone in the affected neuron (Harris et al., 2008; Kumar et al., 2009). Conversely, ethanol has opposing effects on NMDA receptors, acting as a negative allosteric modulator, and inhibiting the activity of the excitatory NMDAR (Harris et al., 2008). These mechanisms of action provide us with potential new avenues for the treatment of AUD. Interestingly, none of the currently approved treatments act on these targets directly, and instead affect receptors with poorly understood relationships to alcoholism, or have unknown mechanisms of action. Alcoholism itself is a disease that seems to arise from long term forms of aberrant synaptic plasticity that are induced by the pharmacological effects of ethanol (Trudell et al., 2014), so drugs that may be able to induce similar or opposing long-term plasticity may be of particular value.

One drug that may have use in the treatment of alcoholism is the anesthetic ketamine. Ketamine is a noncompetitive NMDAR antagonist, but like alcohol, it has many additional molecular targets, and has some effects that are unique among NMDAR antagonists. One of the most noteworthy unique effects of ketamine is its rapid-acting, long lasting antidepressant effect (Browne and Lucki, 2013; DiazGranados et al., 2010; Garcia et al., 2008; Murrough et al., 2013b; Serafini et al., 2014). A single, sub-anesthetic dose of ketamine has been shown to rapidly eliminate depressive symptoms in clinical trials (Berman et al., 2000; Murrough et al., 2013a; Serafini et al., 2014; Zarate et al., 2006), and in experiments with rodent models of stress and depression (Garcia et al., 2008; Holleran et al., 2016; Tizabi et al., 2012; Zhou et al., 2014). The mechanism of this effect is not well understood. Some experiments have demonstrated "rapid-acting" antidepressant effects from other NMDAR antagonists (Li et al., 2011; Maeng et al., 2008), albeit less robustly than ketamine, indicating that the effect may be mediated by NMDA antagonism, likely via the disinhibition of pyramidal neurons in the prefrontal

cortex (Browne and Lucki, 2013). However, evidence suggests that NMDAR inhibition is not the only possible driver of ketamine's antidepressant activity. Ketamine has been shown to induce the expression of BDNF and activation of mTOR dependent plasticity in brain regions associated with depression and other motivated behavior, particularly in the prefrontal cortex, hippocampus, and nucleus accumbens, through an AMPA receptor mediated mechanism (Zanos et al., 2016; Zhou et al., 2014). Interestingly, some of these changes that ketamine induces parallel and contrast with the long-term changes that are present in the alcoholic brain.

Chronic alcohol use has been shown to decrease BDNF levels in the prefrontal cortex (Logrip et al., 2015, 2009; Tapocik et al., 2014), paralleling the decrease in BDNF seen after stress and during the development of depression (Browne and Lucki, 2013; Shirayama et al., 2002), and decreased BDNF expression has been associated with increased drinking (Jeanblanc et al., 2014, 2009; Yan et al., 2005). Acute alcohol may cause an increase in BDNF release in some relevant regions, which is thought to have a protective effect against compulsive drinking(Logrip et al., 2015). Previously, the administration of BDNF to the prefrontal cortex has been shown to decrease drug-seeking behavior in cocaine dependent animals self-administering cocaine (Berglind et al., 2007), which may also apply to alcohol seeking and consuming. Interestingly, depression-like signs are also present during ethanol withdrawal in dependent animals, a symptom that can be reduced by ketamine administration, and which may be mediated by BDNF signaling (Holleran et al., 2016). These data seem to indicate that ketamine's effects on BDNF may have a significant, unique, and potentially long-lasting effect on drinking behavior, a hypothesis that is worth of further investigation. Experiments have demonstrated that ketamine decreases ethanol self administration in the short term via an mTOR dependent, and potentially BDNF associated mechanism (Sabino et al., 2013), but the longer term effects, and the direct BDNF mediated effects of ketamine on drinking are not well understood.

In addition to its effects on BDNF, ketamine's direct NMDAR antagonist effects may be able to effectively relieve ethanol withdrawal, an effect that would also be beneficial in an AUD treatment. Previous studies have demonstrated the ability of NMDAR antagonist drugs, such as memantine or ketamine, to acutely decrease self-administration of ethanol. In fact, ketamine has been investigated as a potential anesthetic for use during severe ethanol withdrawal, but its efficacy in human patients was unclear (Schmidt et al., 2016; Wong et al., 2015). A treatment that can decrease the effects of withdrawal and decrease drive to consume intoxicating substances is somewhat of a holy grail in the treatment of addiction. With actions on BDNF and NMDAR that may affect alcohol-dependent animals in precisely this manner, ketamine has high potential as a treatment for AUD. We seek to perform experiments that will determine the long-term (for the purpose of this proposal, long term refers to effects that last seven days or longer *after* drug exposure) effects of ketamine on alcohol dependent animals, will uncover the role of NMDAR antagonism and BDNF activation in those effects, and will examine brain regions that may mediate them.

Innovation:

Previous experiments have demonstrated that ketamine and other NMDAR antagonists can have acute effects on ethanol consumption (Alaux-Cantin et al., 2015; Malpass et al., 2010; Sabino et al., 2013), and preclinical studies have demonstrated the ability of ketamine to relieve the symptoms of alcohol withdrawal (Holleran et al., 2016), but none of these studies examined alterations in drinking behavior for longer than one day post-ketamine exposure, used NMDAR antagonists other than ketamine with different pharmacological profiles, and had other limitations. Some of these studies used

non-dependent, ethanol-preferring rat models (Malpass et al., 2010; Sabino et al., 2013) which may be less relevant to the human disease of alcoholism than our proposed vapor-exposure model. Other experiments demonstrated that the NMDAR antagonists memantine can alter drinking behavior for up to 30 hours after exposure (Alaux-Cantin et al., 2015), but memantine lacks the antidepressant-effects of ketamine (Möykkynen and Korpi, 2012) In these proposed experiments we will examine the ability of ketamine to influence ethanol drinking over the course of seven days, longer than any previous studies and roughly the length of ketamine's antidepressant effects, and attempt to dissect which of two possible mechanisms, activation of TrkB-B by BDNF and NMDAR antagonism, is responsible for effects on drinking. This will allow us to examine the withdrawal averting NMDAR antagonist effects of ketamine on drinking, along with the never before examined effects of ketamine's longer lasting antidepressant effects on ethanol consumption, on a time scale that has never before been attempted, in an animal model that is highly relevant to human alcoholism.

Approach:

Operant ethanol self-administration has been used in a number of rodent experiments, and is thought to serve as a model of the human disease of drinking (Alaux-Cantin et al., 2015; Doherty et al., 2016; Howard et al., 2009; Sabino et al., 2013; Vendruscolo and Roberts, 2014). In this study, we will use operant self-administration to examine the ability of ketamine to alter ethanol consumption in a dependent rat model. However, operant self-administration alone is not sufficient to produce the compulsive drinking and withdrawal that are associated with alcoholism (Vendruscolo and Roberts, 2014). To overcome this, we will make animals dependent using chronic intermittent ethanol vapor exposure. Ethanol vapor exposure enables animals to reach higher blood-alcohol concentrations over a long period of time, and is sufficient to induce compulsive operant self-administration. Previous examinations of the effect of ketamine on ethanol consumption have shown short and medium-term decreases in operant ethanol self-administration (Sabino et al., 2013), but longer-term observations in dependent animals have not been made. We will examine ethanol consumption daily for seven days after ketamine exposure, in order to examine the long-term and shorter-term effects of the drug in the same experiment. We will examine the effects of systemic ketamine alone, we will attempt to dissect the effects of ketamine by administering another NMDAR antagonist in its stead, and by administering both of these drugs in combination with a TrkB antagonist to uncover the potential mechanisms that ketamine may alter drinking through. Also, we will microinject ketamine into two regions of interest, the medial prefrontal cortex and the nucleus accumbens, to determine how the action of ketamine on a particular brain region will affect drinking. These experiments will examine the long-term effects of local ketamine injections, as well as the acute effects that ketamine has on drinking.

Aim1: To determine the long-term effect of a single administration of ketamine and pharmacologically related compounds on ethanol relapse.

In these experiments, we will examine the effect of systemic ketamine on drinking behavior, one to seven days after drinking. Initially, animals will be trained to self-administer ethanol by lever pressing for access to a sweet solution, and 10% ethanol will be added to the solution, then sweeteners as previously described(Funk et al., 2006). After self-administration of 10% ethanol with no sweeteners has been established, animals will be exposed to ethanol vapor for four weeks. Ethanol vapor exposure has been shown to induce dependence and withdrawal in animals that would not become dependent via self-administration alone (Vendruscolo and Roberts, 2014). Studies have demonstrated that vapor exposed animals are performing an operant task for ethanol must learn the task before vapor exposure, otherwise the association between the operant task and relief from withdrawal symptoms will be difficult to instill

(Hunter et al., 1974; Vendruscolo and Roberts, 2014). After vapor exposure, animals will be allowed to self-administer 10% ethanol to establish a baseline level of drinking. Animals will then be treated with ketamine or a related drug regimen, just after the final baseline drinking session. This ensures that none of the self-administration sessions are directly affected by the acute effects of the ketamine, and minimizes the risk that memory altering effects of NMDAR antagonism will affect the experiment. Drinking will then be observed over the course of seven days, and any changes in drinking behavior will be observed. In the second set of experiments, the role of TrkB in these effects will be examined. TrkB is the primary receptor of the neurotrophic factor BDNF, which is shown to be upregulated by ketamine exposure (Browne and Lucki, 2013; Zhou et al., 2014). We will administer ketamine and the NMDAR receptor antagonist MK-801, with and without the TrkB antagonist drug ANA-12, along with ANA-12 alone, and examine the effects of these drugs on ethanol consumption in ethanol dependent animals, again over the course of a week.

Methods:

Animals:

Male Long-Evans rats will be obtained from Charles River laboratories. Animals will be ordered at 8-weeks old, and allowed to habituate to their housing for one week before undergoing any experimental procedures. All animals will be housed on a 12-hour light/dark cycle. Lights in animals housing will be turned off and on at 7:00pm and 7:00am respectively. Animals will have ad libitum access to food and water in their home cages throughout the course of the experiment. Animals will be handled and lightly restrained throughout the course of the experiment, to acclimate them to handling and restraint that may be required during procedures.

Operant Chamber:

Self-administration will be performed in Med Associates operant chambers, equipped with two retractable levers, two retractable sipper tubes with 50ml bottles, a house light, fan, cue light, and lickometers. Med-PC software will be used to control the chamber, and to collect data from the lever and lickometers. The lickometer will count licks an animal takes for each bottle, counting up every time the circuit between the bottle and electrified cage floor is completed (when the tongue touches the bottle). The fan will be active throughout the session, in order to provide white noise and dampen external sounds. Successful lever presses will result in brief (0.5 second) illumination of the cue light, and once the response requirement is reached, the sipper tube will extend into the chamber, allowing the animal to consume the drinking solution. Food will be placed in the chamber in a small dish during the long operant session, so that animals can consume food ad-libitum.

Acquisition of Ethanol Self-Administration:

For all behavioral experiments, animals will be initially trained to self-administer ethanol using a modified sweetener fading protocol developed as a hybrid of previously used protocols (Howard et al., 2009; Walker and Koob, 2008). All drinking solutions will be held in 50ml cylindrical tubes, with sipper tubes. Prior to the session, tubes will be filled with 50ml of drinking solution or water. Animals will initially be trained to administer a highly palatable solution consisting of 3% glucose and .125% saccharin in solution (SuperSacc solution). SuperSacc will be administered in the operant chamber, with a fixed ratio response requirement of 1 lever press. Each successful response will extend the sipper tube into the chamber for 5, allowing the animal to drink, and will activate the cue light. If necessary, the experimenter will guide the animal toward the lever and bottle, using the back end of a cotton swab, to allow the animal to efficiently connect the reward with the lever. Training sessions will last for 60 minutes each day. On the fourth day of training, the animals will instead be allowed to self-administer SuperSacc solution with 10% ethanol, or water. Each sipper tube will be activated by one of the two levers in the operant chamber, and the position of the levers and bottles will alternate with every session.

After four days of SuperSacc + 10% ethanol, the glucose will be removed from the solution, and after four more sessions the saccharin will be removed from the drinking solution. From this point on, animals will consume a 10% ethanol solution (10E). After seven additional daily sessions of operant self-administration, the animals will be exposed to ethanol vapor. This procedure adapted from Funk et al. (2006)(Funk et al., 2006).

Ethanol Vapor Exposure:

To induce ethanol dependence, animals will be exposed to ethanol vapor in an ethanol vapor chamber. Animals will be exposed to intermittent ethanol vapor daily over the course of four weeks, fourteen hours each day, from 6:00pm to 8:00am, during the animal's dark cycle. Blood will be collected twice a week via tail vein puncture and blood alcohol concentrations will be analyzed, to determine if a consistent state of intoxication is being maintained. Vapor will be generated by warming 95% ethanol and distributed throughout the chamber through flowing air, at 11 liters per minute, as has previously performed for experiments in the Koob lab, in order to maintain blood alcohol concentration of 150-200mg% (Walker and Koob, 2008). Blood alcohol levels will be analyzed during a vapor session at the end of every other week, in order to assess the effectiveness of the vapor chambers.

Ethanol Self-Administration Testing:

After the conclusion of vapor exposure, animals will undergo a self-administration session once daily. Each session will proceed as follows: Initially, animals will be placed in the operant chamber, with fan on and levers retracted, for ten minutes in order for allow them to acclimate to the chamber. After a short exploration period, the levers will extend, allowing the animals to self-administer 10E or water. The bottles will be presented on an FR1 schedule, where each successful lever-press will extend the corresponding bottle into the chamber for 10 seconds, and briefly activate the cue light. This session will last for 12 hours, the entirety of the animal's dark cycle. After 12 hours, the cage fan will turn off, and the animals will be removed from the operant chamber and returned to their home cage. Animals will be allowed to self-administer as described above for as many days as are required until stable levels of drinking have been attained (± 20% across three sessions of drinking (Funk et al., 2007)). The final three days of prior to drug administration will be used to establish baseline drinking and lever pressing for each animal. Prior to drug administration, animals will be separated into one of the applicable experimental groups. After drug administration, animals will be allowed to self-administer 10% ethanol for seven days (the test portion of the experiment). The first day of the test portion, where the self-administration session takes place starting 12 hours after drug administration and ends 24 hours after drug administration will be regarded as the 1-day time point. Subsequent sessions will be numbered accordingly.

As an additional experiment in Aim1a, a group of animals will be exposed to a progressive ratio (PR) operant self-administration schedule. Animals will be trained on an FR1 schedule, as described above, but after vapor exposure, the self-administration protocol will switch to PR. Animals will be administered drug when they display a steady break point (± 20%) for three days. Animals will be allowed to lever press for access to 10% ethanol, but the number of lever presses for a successful response will increase by one for every two successful requirement (e.g.: 1,1,2,2,3,3,4,4,5,5…). Each successful response will extend the sipper tube for 10 seconds. The break point will be defined as the last successful response requirement 30 minutes after the last successful response. One hour after the animal has stopped responding, the sipper tube will extend back into the chamber for the remainder of the session, allowing the animals to consume ethanol ad-libitum, in order to prevent the animals from going into severe withdrawal. In both cases, videos will be recorded to observe behavior.

Drug Administration:

Aim 1a: After stable levels of drinking have been attained, ketamine or saline will be administered to animals immediately after a self-administration session. The animals in the FR1 group will receive 3, 10, or 25mg/kg body mass ketamine. Animals in the PR group will receive either saline, or a dose of ketamine that will be determined by FR1 experiments. Drugs will be administered intraperitoneally (i.p.) in 0.9% saline, in a volume equivalent to 7.5 milliliters per kilogram of body mass. Saline animals will receive 7.5 milliliters per kilogram body mass of 0.9% saline(Zanos et al., 2016).

Aim 1b: After stable levels of drinking have been attained, animals will be injected with saline, ketamine (the ketamine dose will be determined by the previous experiment, either 3, 10, or 25 mg/kg of body weight of ketamine), the non-competitive NMDAR antagonist MK-801 (0.1 mg/kg), the tropomyosin receptor kinase B (TrkB) antagonist ANA-12 (prepared in 1% DMSO, 0.5mg/kg), or a combination of ANA-12 and MK-801 and ketamine. Animals will be injected with drug intraperitoneally in 7.5ml/kg volume of vehicle immediately after the final baseline drinking session.

Data collection:

Data will be collected on each day of the training, baseline, and test sessions. For FR1 schedule animals, volume of solution consumed, number of lever presses, latency to lever press, number of licks, and latency to lick will all be measured during each self-administration session. For PR schedule animals, break point, latency to break point, number of licks, latency to lick, and volume of solution consumed will be measured during each self-administration session. Lick and lever press data will be acquired and recorded by Med Associates Med-PC software. Volume of solution consumed will be measured by hand with a motorized pipette filler and plastic pipette. Measures of drinking will be recorded during every drinking session, from the start of training through the conclusion of the experiment.

Data Analysis:

Volume solution consumed, break point (for PR), and number of lever presses (for FR1) will be analyzed using separate mixed model repeated measures of variance ANOVA, with drug condition as a between subjects factor (Saline, 3, 10, 25 mg/kg ketamine for aim 1a; saline, ketamine, MK-801, ketamine plus ANA-12, and MK-801 plus ANA-12) and day as a within subjects factor. Time points will separated into two phases, baseline and post-drug (ten days total, three days of baseline self-administration, seven days of post-drug self-administration). Bonferroni corrections will be applied if a significant effect across groups is observed, in order to identify the particular divergences. Data will be analyzed as absolute values and as percent of baseline. To examine the time course of drinking throughout a particular session, the latency to lick and latency to lever press may be examined, if necessary.

Results and Interpretation:

We hypothesize that measures of drinking, including lever pressing and total volume of ethanol consumed, will be decreased in ketamine treated animals throughout the course of the test phase of the experiment, via the induction of BDNF dependent plasticity by ketamine. This hypothesis would be supported by a ketamine-induced decrease in lever pressing and ethanol consumption after exposure to ketamine in the first experiment, and if the TrkB antagonism in the Aim 2 experiments blocks the effect of ketamine. Break point will determine the direction of this effect. If ketamine leads to an increased breakpoint, it is likely that ketamine is decreasing the reward that is provided by the pharmacological effects of the ethanol. If breakpoint is decreased, it would seem that ketamine is instead decreasing the

motivation to consume ethanol. In Aim 1b, if MK-801 is able to replicate these effects, it is likely that direct NMDA inhibition is sufficient to alter drinking behavior, otherwise another mechanism may need to be explored (such as the generation of AMPA agonist metabolites of ketamine, which may have a role in ketamine's antidepressant effects (Zanos et al., 2016)). Alternative results are possible. Because ketamine and alcohol share some pharmacological mechanisms, it is possible that acute ketamine would relieve the symptoms of alcohol withdrawal and decrease ethanol consumption acutely, which has been demonstrated (Sabino et al., 2013), but that any withdrawal or homeostatic effects may actually increase the symptoms of withdrawal, and increase ethanol consumption. This finding would not support the use of ketamine as a treatment for alcoholism, but would be interesting in its own right. It is also possible that ethanol will have no effect on ethanol consumption. Some changes that may be beneficial in depression, such as elevated motivational states, could increase the animal's drive to consume ethanol. This finding would add particular importance to the regional injection experiments in Aim 2, which may be able to parse out any differential effects of ketamine on different regions of the brain, even if systemic administration has no net effect.

Limitations:

The main limitation of this study is the time and difficulty of the dependence protocol, which limits the number of experiments that can reasonably performed. From start to finish, the animals will be undergoing procedures seven to eight weeks. However, we feel that this protocol maximizes relevance to the clinical treatment of alcoholism. Wild-type animals are made to drink heavily via vapor exposure, as opposed to using animals that are predisposed to drinking. This allows us have a more natural baseline level of drinking, which treatments could theoretically revert the animal to. Measuring ethanol consumption volumes may be difficult over the course of a twelve-hour session because of spillage, but this method will stress the animals significantly less than drawing blood to test blood alcohol levels, and will give us the opportunity to examine total drinking over the course of the session in a way that blood alcohol concentrations would not.

Aim 2: To determine the long and short-term effects of ketamine in specific, reward-associated brain regions.

In these experiments, we will examine effects of ketamine on two brain regions that are altered by exposure to ethanol (Barker et al., 2015) and the antidepressant effects of ketamine (Abdallah et al., 2016; Li et al., 2010), the nucleus accumbens and the medial prefrontal cortex. Ketamine will be microinjected into each of these regions just prior to a self-administration session. Unlike Aim1 experiments, the acute effects of microinjection of ketamine have not been investigated, so these acute effects will be examined. After the acute drug session, seven daily sessions of self-administration will be performed, and will be analyzed as in Aim 1.

Methods:

Acquisition of Self-Administration

Acquisition of self-administration and vapor exposure will be performed in cannulated animals, as described above in Aim 1. Training sessions will begin no earlier than one week after cannulation surgery.

Cannula Implantation:

Nine-week old animals will be implanted with bilateral 26 gauge stainless steel guide cannulas into one of two brain regions of interest (nucleus accumbens, and the medial prefrontal cortex). During surgery, anesthesia will be induced with 5% isoflurane, and maintained with 3% isoflurane throughout the procedure. Surgery will be performed on a Kopf rat stereotax. mPFC cannulas will be implanted so

that the tip is positioned just dorsal to the mPFC, +3.0mm A/P, ±0.6mm M/L, and -3.0mm D/V relative to bregma. Nucleus accumbens probes will be implanted in the vicinity of the nucleus accumbens shell, at +2.2mm A/P, ±0.9mm M/L, and -7.0mm D/V relative to bregma (Paxinos and Watson, 2006). Cannula will be held in place by a dental acrylic cap. Opturators will be kept in place throughout the course of the experiment in order to maintain the patency of the cannula.

Microinjection:

Microinjectors will be constructed as demonstrated in Poland et al. out of 33 gauge microinjection needle tubing (Poland et al., 2015). 75 nanograms of ketamine in 0.5ul saline will be bilaterally microinjected into the brain region of interest (medial prefrontal cortex (mPFC) or nucleus accumbens). Microinjections will be performed 1 hour before the first test self-administration session, after three days of stable drinking, and animals will be allowed to self-administer for seven additional days after treatment. The injection will be performed by a syringe pump that is connected to the microinjector via 26g plastic tubing.

Ethanol self-administration testing:

Testing will be performed as described in Aim 1, on an FR1 schedule. Unlike Aim 1 experiments, animals will be injected with drug one hour prior to the first self-administration session, in order to determine the acute effects of local ketamine administration. The self-administration session on this day will be categorized separately from the other, as the "acute drug-administration" session.

Cannula Placement Verification:

After the conclusion of the testing phase of self-administration, animals will be sacrificed and perfused with 4% paraformaldehyde solution. Brains will be extracted and sliced on a vibratome. Brains will be imaged and the position of the cannula in the brain will be verified.

Data Collection:

Data will be collected as previously described in Aim 1, using Med-PC. Volumes of ethanol consumed will be measured by hand with a motorized pipette filler and plastic pipette, accounting for spillage.

Analysis:

Volume of solution consumed, and lever presses will be compared across groups with separate mixed model repeated measures of variance ANOVA, with drug condition (mPFC saline or mPFC ketamine, and NAcc saline and NAcc ketamine) as a between subjects factor, and time as a within subjects factor. Eleven total time points will be broken up into 3 periods, (3 baseline-consumption days, 1 acute drug administration day, and 7 post-drug self-administration days. Bonferroni corrections will be applied if a significant effect across groups is observed, in order to identify the particular divergences. Data will be analyzed as absolute values and as percent of baseline. Animals with mPFC cannula will not be compared to animals with NAcc cannula; so two separate comparisons will be performed. In addition, the number of licks from the first three hours of each acute drug-administration session will be binned and analyzed by unpaired T-test.

Results and Interpretation:

We hypothesize that ketamine microinjected into the prefrontal cortex will decrease drinking acutely, via NMDAR inhibition by active ketamine, and long-term. Decreased BDNF levels in the prefrontal cortex have been shown to be associated with an increased preference for drinking (Logrip et al., 2015, 2009; Tapocik et al., 2014), and mPFC BDNF has been shown to decrease in response to ethanol (Logrip et al., 2015; Pickering et al., 2015). Meanwhile, we hypothesize that ketamine

microinjected into the NAcc will also acutely decrease ethanol consumption, but may increase or decrease drinking in the long term. Experiments have shown that ethanol increases glutamatergic activity in the NAcc (Barker et al., 2015), but the exact role of NAcc glutamatergic signaling in alcoholism is not yet understood. If one brain region replicates the longer-term effects of systemic ketamine, it is likely that homeostatic responses to ketamine are strengthening activity in that region, causing the majority of ketamine's effect. If microinjections of ketamine in the NAcc and mPFC have opposing effects, it is possible that plasticity induced in one region is strengthening components of a feedback loop between the mPFC and NAcc, or otherwise overpowering the region with effects that don't match with systemic ketamine.

Limitations:

Because numerous brain regions are involved in alcohol self-administration, motivated behavior, and the development of alcoholism, it is likely that any effect that ketamine has on drinking is going to be dependent on the action of more parts of the brain than we could feasibly examine in the course of this study. In addition, there are distinct regions of the mPFC and NAcc. The mPFC is composed of the infralimbic prefrontal cortex (IL) and the prelimbic prefrontal cortex (PL), and the NAcc is composed of the shell and core, all of which have distinct functions and connectivity (Barker et al., 2015). Because the effects of NAcc and mPFC microinjected ketamine on operant ethanol self-administration has never before been examined, we will target these regions generally, but future experiments targeting the individual subregions of mPFC and NAcc could prove useful.

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