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Opioid System's involvement in Ketamine's Antidepressant-like Effects

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Abstract

Depression is one of the most debilitating disorders in the world. The currently available medications typically have a 2-4 week delay in their therapeutic effects and are ineffective for about 40% of patients. In 2000, a subanesthetic dose (0.5 mg/kg i.e.) of the dissociative anesthetic ketamine was reported to have both rapid and robust antidepressant effects in in treatment-resistant depressed patients. However, the mechanisms responsible for ketamine's antidepressant effects remain unclear. In 2018, a clinical study reported that pretreatment with the nonselective opioid antagonist naltrexone attenuated the rapid antidepressant effect of ketamine in depressed patients. The current study investigated the potential role of the opioid system in ketamine's antidepressant-like effects in two different preclinical behavioral assays. Mice were tested in the differential-reinforcement-of-low-rate responding (DRL) 72 sec task and tail suspension test (TST). Locomotor activity also was measured to determine if ketamine and/or naltrexone produced any changes in locomotor behavior that could influence the results in the DRL and TST assays. Ketamine and the nonselective opioid antagonist naltrexone were tested alone and in combination in C57BL/6 mice. The current study found that ketamine alone produced an acute antidepressant-like effect in the DRL 72 sec task at a dose of 32 mg/kg and TST at a dose of 10 mg/kg, and that this antidepressant-like effect was blocked by pretreatment of 2 mg/kg naltrexone in both behavioral assays. These results suggest that ketamine's antidepressant effects may involve activation of the opioid system.

Key Words: Antidepressant, Ketamine, Naltrexone, Mice, Opioid, DRL, TST

Opioid System's involvement in Ketamine's Antidepressant-like Effects

Major Depressive Disorder (MDD) is a debilitating mental health disorder characterized by symptoms including depressed mood, fatigue, feelings of worthlessness, and suicidal ideation or attempt (Uher et al., 2014). It is currently the most prevalent mental illness worldwide, affecting 5.9% United States citizens in 2017 and affecting an estimated 9.4% of United States citizens during their lifetime (Kessler, 2012; World Health Organization [WHO], 2017). According to data collected in 2017 by the Substance Abuse and Mental Health Services Administration (SAMHSA), 64% of MDD patients suffer severe impairment due to the disorder, and among adolescent patients, this number is 71% (NIMH , 2019). In 2016, depressive disorders were responsible for about 13,043,000 emergency department visits, accounting for 9.4% of all chronic conditions at emergency department visits (NCHS, 2017). In 2017, 47,173 people died due to intentional self-harm or suicide in the United States, which accounted for 1.7% of total deaths, and it was the tenth leading cause of death in the country. The number of deaths increased by 3.7% from the previous year and counted for the fifth most substantial increase in deaths from the previous year (Kochanek et al., 2019). From 2011-2014, 12.7% of persons aged 12 and over from the United States filled an antidepressant prescription (Pratt et al., 2017). Among all adult patients who experienced a major depressive episode, 50% received some type of medication as treatment, and 6% of them received medications as the only type of treatment (NIMH , 2019). Currently available antidepressant drugs are undoubtedly a crucial component for the treatment of MDD; however, they are only effective in approximately 60% patients that are suffering from MDD, (Krystal et al., 2019). Thus, treatments that are more effective are needed for this growing epidemic, and pharmacological therapies are an essential component of the treatment of depressive symptoms. Ketamine is a novel antidepressant that has showed great

promise among treatment-resistant depression patients (DeWilde et al., 2015). The present study investigated the novel antidepressant ketamine and explored the role of opioid mechanisms in its antidepressant-like effects in the differential-reinforcement-of-low-rate responding (DRL) operant procedure and tail suspension test (TST) in C57BL/6 mice. Ketamine and the opioid antagonist naltrexone were tested alone and in combination in both tasks.

A brief history of the development of antidepressants.

Serendipity has played a major role in the development of drugs for the treatment of depression. Before the 1950s, the symptoms of depressive disorders were believed to be produced by internal personality conflicts, and there were very few attempts to investigate the biological etiology of depressive disorders (Lopez-Munoz & Alamo, 2009; Pereira & Hiroaki-Sato, 2018). The discovery of the monoamine oxidase inhibitor, iproniazid and the tricyclic, imipramine antidepressants in the 1950s marked the beginning of the pharmacological approach for the treatment of depression (Hillhouse & Porter, 2015; Lopez-Munoz & Alamo, 2009).

Iproniazid is a drug that was developed initially as an antituberculosis medication. During its initial clinical trials, elevated mood and hyperactivity was observed in the tuberculosis patients (Pleasure, 1954). In 1957, the first clinical trial with iproniazid in patients with psychotic and depressive disorders found that 70% of the participants exhibited an elevated mood and a more positive worldview. One year after this finding was reported, over 400,000 patients suffering from depression were treated with iproniazid; thus, iproniazid became the first official pharmacological antidepressant treatment. Thereafter, several medications based on the pharmacological properties of iproniazid were introduced to the market, such as tranylcypromine and phenelzine (Lopez-Munoz & Alamo, 2009). These drugs are classified as monoamine oxidase inhibitors (MAOIs) based on their inhibitory actions on monoamine oxidase enzymes.

This inhibition prevents the breakdown of monoamine neurotransmitters, including norepinephrine (NE), epinephrine (EPI), dopamine (DA), and serotonin (5-HT). This results in their increased availability in the nervous system (Quitkin, 1979).

Around the same time, also through fortuity, imipramine was found to have antidepressant properties after it failed to be introduced as an antipsychotic in 1958. Imipramine belongs to a class of drugs called tricyclics. This class of drugs is named after their unique chemical structure, which contains three rings of atoms. After discovering imipramine's antidepressant effect, most tricyclic medications were repurposed as antidepressants and classified together under the name, tricyclic antidepressants (TCA) (Lopez-Munoz & Alamo, 2009). The TCAs have a very complex pharmacological profile. They can inhibit the reuptake of serotonin and norepinephrine, as well as antagonizing histamine, adrenergic, muscarinic, and serotonin 5-HT_{2A} receptors post synaptically. While most TCAs have their highest binding affinity at histamine receptors, the inhibition of serotonin and norepinephrine reuptake is thought to be primarily responsible for their antidepressant effects (Gillman, 2007; Hillhouse & Porter, 2015).

The discovery of these two classes of drugs with antidepressant properties lead to the monoaminergic theory of depression, which postulates that a functional deficiency of noradrenergic and serotonergic neurotransmission in the central nervous system is the primary cause of the pathologies of depression. Within the umbrella of the monoamine theory, two theories were developed and competed at that time. One is the catecholamine theory, which proposed that norepinephrine, epinephrine, and dopamine systems are responsible for the antidepressant effects of the existing antidepressants; the serotonin theory, on the other hand,

attributed the antidepressant effect of those drugs to the serotonin system (Lopez-Munoz & Alamo, 2009).

The serotonergic hypothesis gained considerably more attention in the late 1960s and 1970s after findings indicated that MAOIs' and TCAs' antidepressant properties can be blocked by the inhibition of serotonin synthesis (Pereira & Hiroaki-Sato, 2018). A new class of drugs – selective serotonin reuptake inhibitors (SSRI) were developed, which led to the birth of fluoxetine--the first class of drugs that were created with the direct intention for treating depressive disorders (Lopez-Munoz & Alamo, 2009). The SSRIs, as their name suggests, were engineered to prolong serotonin's availability and to enhance neurotransmission by selectively blocking the active reuptake of serotonin in presynaptic nerve endings (Baghai et al., 2006). SSRIs have achieved immense success and remain one of the first-line treatments for depressive disorders today (Dale et al., 2015).

As successful as SSRIs are in the market today, they do not greatly improve the success rate for pharmacological treatment of depression as compared to MAOIs and TCAs. Based on meta-analyses conducted in the 1990s, the success rate for SSRI treatment was 59.6% for the inpatient group and 61.4% for the outpatient group, only 0.4% and 2.1% higher, respectively than for TCAs (Doyle et al., 2001). In addition, these medications have a two to eight weeks delay in the onset of their therapeutic effects, making them often unsuitable for patients with severe depressive symptoms and for patients with intense suicidal ideations (American Psychiatric Association, 2016; Machado-Vieira et al., 2010; Uher et al., 2011). Several new classes of antidepressants have been developed in the hope of resolving this problem. Examples include serotonin and norepinephrine reuptake inhibitors (SNRIs) that selectively block the reuptake of 5-HT and NE in the presynaptic neurons, and atypical antidepressants such as the

"multimodal" vortioxetine, which is a serotonin 5-HT_{1A} receptor agonist, 5-HT_{1B} receptor partial agonist, 5-HT_{3A} and 5-HT_7 receptor antagonist, and a potent serotonin reuptake inhibitor that can also block dopamine and norepinephrine transporters (Bang-Andersen et al., 2011; Dale et al., 2015; Hillhouse & Porter, 2015). Unfortunately, these new drugs offer no significant improvements in antidepressant efficacy. A major limitation of the monoamine theory is that the acute increase of monoamines in the synaptic cleft does not correspond to an immediate reduction of depressive symptoms in humans (Locher et al., 2017; Pereira & Hiroaki-Sato, 2018).

Finally, while many different types of antidepressant drugs are currently on the market, approximately 40% of patients remain treatment-resistant and do not respond to the currently available mediations (Hillhouse & Porter, 2015; Krystal et al., 2019). Treatment-resistance and the long delay in the onset of therapeutic effects is still an immense obstacle (American Psychiatric Association, 2016; Machado-Vieira et al., 2010; Uher et al., 2011).

Ketamine as an acute and lasting antidepressant

Berman et al.'s (2000) discovery that a single, low dose infusion of ketamine (0.5 mg/kg, i.v.) produced both *rapid* and *prolonged* antidepressant effects in depressed patients offered new hope for the treatment of depression and the first new molecular target since the discovery of monoaminergic drugs in the 1950s. (Hillhouse & Porter, 2015; Iadarola et al., 2015; Serafini et al., 2014). Since then, ketamine has been proven to be a reliable antidepressant in both preclinical and clinical studies of depression. In the majority of patients with treatment-resistant major depressive disorder, ketamine produces a rapid reduction in suicidal ideation (DiazGranados et al., 2010; Price et al., 2014; Zigman & Blier, 2013). Ketamine ((*RS*)-ketamine) is a racemic mixture of equal proportions of two isomers, (*S*)-ketamine (esketamine) and (*R*)-

ketamine (arketamine). Its *S* (+) enantiomer, esketamine, was approved by the U.S. Food and Drug Administration for use as an antidepressant for treatment-resistant depression in March 2019 as a nasal spray with the brand name SpravatoTM® (FDA, 2019; Salahudeen et al., 2020). In addition, the *R* (-) enantiomer, arketamine, also has been shown to have promising antidepressant-like properties in preclinical studies (Chang et al., 2019; Fukumoto et al., 2017) and in a recent open-label clinical study with treatment-resistant depressed patients (Leal et al., 2020).

Despite the excitement it has generated, ketamine's use as an antidepressant has been limited due to its abuse liability (Bokor et al., 2014; Dotson et al., 1995; Sassano-Higgins et al., 2016), and its potential to produce strong psychosomatic and dissociative effects (Curran & Morgan, 2000; Jansen, 1993). Ketamine's neuropharmacology is complex (Kohrs & Durieux, 1998; White & Ryan, 1996), and the underlying mechanisms mediating its antidepressant effects remain undetermined (Sanacora & Schatzberg, 2015; Williams & Schatzberg, 2016). To increase the safety of ketamine use or to develop better and safer antidepressants, it is crucial to understand the mechanism(s) of action that mediate the antidepressant effects of ketamine. Ketamine's antagonism of glutamatergic N-methyl-D-aspartate (NMDA) receptors has been the focus of most research, which is based on the fact that ketamine has its highest binding affinity to those receptors (Mion & Villevieille, 2013). However, studies have shown that not all NMDA antagonists share the same rapid and long-lasting antidepressant effects that ketamine has (Hillhouse & Porter, 2014; Zarate Jr et al., 2006). At the same time, while some NMDA antagonists have been reported to exhibit antidepressant-like effects in preclinical studies, they also produce hyperactivity. This can produce false-positive results in preclinical behavioral tests that use a measurement of an animal's immobility when placed in an inescapable, stressful

environment (e.g., forced swim test, TST) for antidepressant-like effects (Maj et al., 1992; Carlsson & Carlsson, 1989). These findings question NMDA receptor antagonism as the primary mechanism of ketamine's antidepressant effects and suggest that other mechanisms of action may be responsible (Hillhouse & Porter, 2014).

Notwithstanding the interactions with NMDA receptors, ketamine also interacts with many other receptors, including agonistic actions on opioid receptors. There is a copious amount of evidence showing the opioid system's involvement in emotional regulation, and several opioids are potential candidates for antidepressant treatment (Berrocoso et al., 2009; Lutz & Kieffer, 2013; Nummenmaa et al., 2020). Therefore, the opioid system's role in ketamine's antidepressant-like effect needs to be further investigated.

The opioid system as a target for antidepressant treatment and ketamine's interaction with it

The use of opiates as mood-altering agents can be traced back to the earliest recorded history of many cultures (Brownstein, 1993). Nevertheless, it was not until the concept of specific opioid receptors was introduced a half-century ago that researchers started to understand this intriguing and complex neurochemical system (Snyder & Pasternak, 2003). Among the opioid receptors that have been identified, the three most studied are the mu opioid receptor (MOR), the delta opioid receptor (DOR), and the kappa opioid receptor (KOR). Many opioid drugs have been shown to have antidepressant-like properties and mood-altering attributes through distinct actions at these three receptors (Berrocoso et al., 2009).

The MORs are primarily activated by the endogenous opioid peptides endomorphins and beta-endorphin (Janecka et al., 2004). These receptors are the most investigated opioid receptors among the three receptors discussed here. On the other hand, they are also the most mysterious

among all three receptors. MOR agonists are known for their superior antinociception properties, as well as their role in mood-altering effects and strong abusive features of opioids. The idea of MOR agonists as potential treatments for depressive disorders can be traced back to 1904 in Emil Kraepelin's *Introduction to the Psychiatric Clinic* (Berrocoso et al., 2009). However, research has been limited in this area due to concerns of abuse liability, which has led to an inadequate understanding of the potential antidepressant effects of MOR agonists. Clinically, low dose buprenorphine (mixed agonist–antagonist opioid receptor modulator) has demonstrated potential for treating severe suicidal ideations (Yovell et al., 2016). With repeated administration or when used in combination with the MOR antagonist samidorphan, buprenorphine can significantly reduce the depressive symptoms of patients that were unresponsive to traditional antidepressant medications and electroconvulsive therapy treatments (Fava et al., 2016; Nyhuis et al., 2008). Using different MOR agonists, Stoll (1999) reported that oxycodone and oxymorphone helped patients with severe and refractory major depression achieve long-term remission. Preclinically, buprenorphine also produced a significant antidepressant-like effect in the delayed novelty-feeding test in mice. Interestingly, the more potent MOR agonist—morphine, failed to produce similar effects (Robinson et al., 2017). These findings may be explained, at least in part, by a comparative study of opioids and SSRIs in the TST (Berrocoso et al., 2013). In this study, MOR agonists such as morphine, codeine, and methadone and five different SSRIs all significantly reduced immobility time of mice in the TST; however, the MOR agonists and SSRIs produced very different effects in the mice. While the SSRIs significantly increased the mice's swinging behavior, it had no effects on their curling behaviors; in contrast, the MOR agonists did not affect the mice's swinging behaviors, but significantly increased their curling behaviors. Tianeptine, a mixed opioid receptor agonist/antagonist (approved as an

antidepressant in most European and Asian nations, but not in the United States), produces antidepressant-like effects in wild-type mice, but not in MOR deficient mice (Samuels et al., 2017). These findings provide evidence for MOR agonism as a potential target for development of new antidepressants.

DOR agonists also have attracted attention for their potential antidepressant effects. Endogenously, the DORs are activated by the opioid peptides met-enkephalin and leu-enkephalin. Also, β-endorphin has similar affinities for DORs and MORs (Pradhan et al., 2011). The antinociceptive effect of DOR agonists is not as robust as MOR agonists, but they have shown promise for their potential anxiolytic-like and antidepressant-like effects (Jutkiewicz, 2006). Anxiety-like and depressive-like behaviors also have been observed in mice depleted of DORs. When compared with wild type mice, mice depleted of DORs exhibited longer immobility time in the forced swim test (indicating depressant-like behavior), and spend less time in the open arm in the elevated plus maze test and less time in the light box of the light-dark box test, which both reflect anxiety-like behaviors (Filliol et al., 2000). Age-related depressive-like behaviors and anxiety-like behaviors in mice also are linked to cortical DOR dysfunction (Narita et al., 2006). DOR agonists such as RM 101 and SNC80 attenuate the conditioned suppression of motility and decrease the duration of immobility in the forced swim test in mice; an effect that can be suppressed by DOR antagonists such as naltrindole (Baamonde et al., 1992; Saitoh et al., 2004). One limiting factor for the use of DOR agonist treatment is its proconvulsant effects (Jutkiewicz, 2006; Pradhan et al., 2011). However, the enkephalin inactive endopeptidase inhibitor, opiorphin, has been shown to produce antidepressant-like effects without any proconvulsant issues. While DOR's proconvulsive properties have limited the potential of DOR

agonists for treatment of depression, the enkephalin inactive endopeptidase inhibitor-opiorphin may be a safer alternative (Javelot et al., 2010; Popik et al., 2010).

The KOR appear to function in an opposite manner than the other two opioid receptors. The activation of KORs is associated with stress and can trigger glucocorticoid production (Lalanne et al., 2014). Studies have shown that dynorphin, an endogenous ligand for the KORs, can increase immunoreactivity in the hippocampus and nucleus accumbens after stress induced by prolonged immobilization and learned helplessness, which result in increased depressive-like behaviors such as increased immobility time in the forced swim test. Such depressive-like effects can be reversed by nor-BNI, which is an antagonist for dynorphin and KORs (Shirayama et al., 2004; Lv et al., 2012). Intracerebroventricular administration of apelin-13 dose-dependently increases immobility time in the forced swim test and the TST in mice. Such depressive-like effects were readily blocked by nor-BNI (Lv et al., 2012). Moreover, the KOR agonist salvinorin A can cause rapid and long-lasting reduction of dopamine concentrations in the nucleus accumbens, which in turn causes reduced motivation to work for sucrose reward in the progressive ratio test, and increased immobility time in the forced swim test (Carlezon et al., 2006; Ebner et al., 2010). On the other hand, acute administration of the KOR antagonists nor-BNI and DIPPA significantly reduces immobility time in forced swim tests in rats (Carr et al., 2010; Mague et al., 2003). Due to these differences between the opioid receptors and their possible role in depression, future studies need to better delineate the exact relationship of each receptor for development of therapeutic drugs.

Studies have shown that many of the traditional treatments for depression have achieved therapeutic effects, at least in part through the opioid system. Inturrisi et al. (1982) reported that electroconvulsive therapy treatment significantly increased beta-endorphin plasma values.

Furthermore, Dziedzicka-Wasylewska & Rogoz (1995) found that prolonged treatment with electroconvulsive therapy or imipramine significantly increased the nucleus accumbens' met-enkephalin concentration. Moreover, the enkephalin-degrading peptidases inhibitors bestatin and thiorphan, when paired with the TCAs imipramine or iprindole, significantly enhance their antidepressant-like effects in the FST. Furthermore, the pretreatment of the MOR antagonist naloxone completely reversed this effect (de Felipe et al., 1989). This finding was supported in a study by Ide et al. (2010), which found that venlafaxine produced an antidepressant-like effect in the FST with the wild-type mice, but not with MOR-KO mice. The SSRI fluvoxamine has been shown to reverse decreased beta-endorphin levels seen in depressed patients (Djurovic & Milic-As, 1999). Similar findings also have been reported with SNRIs. Berrocoso et al. (2004) found that the non-selective opioid receptor antagonist naloxone at 2 mg/kg blocked the SNRI venlafaxine's antidepressant-like effect in the FST. However, selective MOR, DOR, and KOR antagonists did not produce the same blocking effect.

Ketamine acts as an agonist at all three opioid receptors, with its highest binding affinity at MORs and KORs, with a more moderate binding affinity at DORs (see Table 1). In addition, studies have also suggested that the activation of NMDA receptors can attenuate MOR signaling (Chartoff & Connery, 2014). Recently, Williams et al. (2018) reported that the nonselective opioid receptor antagonist naltrexone (50 mg/kg, iv) attenuated ketamine's low dose (0.5 mg/kg, iv) antidepressant effect in depressed patients. Interestingly, the dissociative effects of ketamine were not blocked by naltrexone. In a subsequent clinical study, Williams et al. (2019) reported that opioid receptor antagonism also was able to attenuate the antisuicidal effects of ketamine. Based on these results, the authors suggested that opioid system activation is necessary for ketamine's acute antidepressant effects to be evident. However, discrepant results were reported

in a clinical study by Yoon et al. (2019). In this study patients with major depressive disorder comorbid with alcohol dependence were treated with 380 mg injectable naltrexone 2-6 days prior to the 4 weekly 0.5 mg/kg ketamine intravenous treatments. In this study, naltrexone did not attenuate the antidepressant effect of ketamine, but 80% of patients reported reduced alcohol craving throughout the period of the treatments. Mixed results also have been reported in preclinical studies. Zhang and Hashimoto (2019) reported that 10 mg/kg naltrexone failed to block the antidepressant-like effects of 10 mg/kg ketamine in mice in the forced swim test after exposure to the chronic social defeat depression paradigm. In contrast, Klein et al. (2020), reported that 1 mg/kg naltrexone blocked 15 mg/kg ketamine's antidepressant-like effects in the forced swim test and the progressive ratio test in congenitally learned helplessness Sprague-Dawley rats. These conflicting results leave uncertainties as to the role of the opioid system in ketamine's antidepressant-like effects. Clearly, further research is needed in order to determine if these different results are due to procedural differences or to the use of different preclinical assays. The present study used the DRL 72 sec test and TST to assess the antidepressant-like effects of ketamine and the ability of naltrexone to block it.

Receptor/Target	Action	Affinity/Potency (μM)	Species	Tissue
NMDA	Antagonist	Ki = 0.58 ± 0.07	Mouse	Brain
		Ki = 0.42 ± 0.03	Human	Cortex
MOR	Agonist	Ki = 42.1	Human	Brain
KOR	Agonist	Ki = 28.1	Human	Brain
		Ki = 28	Rat	Brain
DOR	Agonist	Ki = 272	Human	Brain
		Ki = 100	Rat	Brain

Table 1. Ketamine's inhibition constant (Ki) value at NMDA and opioid receptors. Low Ki values indicate high binding affinity. Information adapted from Zanos et al. (2018).

Behavior Assays

To test the opioid system's potential involvement in ketamine's antidepressant-like effects, the DRL 72 sec operant procedure and TST were used in the present study.

In the DRL assay, the animals are trained to respond on a lever or nose poke to obtain a reward, but they have to wait a specific interval of time between lever presses in order to receive a reward. The DRL test is considered to be one of the more reliable antidepressant screening methods, as it is less vulnerable to false positives produced by stimulant-like locomotor effects (O'Donnell & Seiden, 1983; Seiden et al., 1985).

The DRL test was originally designed to measure impulsivity, which is defined as a difficulty in delaying responding for an immediate reward, instead of obtaining greater rewards with a delay, or as the inability to inhibit delayed gratification (Gerhart et al., 2013; Szuhany et al., 2018). Delayed gratification is a fundamental skill for a mentally and physically healthy lifestyle, and the lack of such skills are highly correlated with depressive symptoms (Gerhart et al., 2013; Hoerger et al., 2011). This idea is also supported in a study by Pulcu et al. (2014) that found participants suffering from MDD have higher discounting of delayed rewards than do healthy participants or participants that achieved remission from MDD. This might be explained by depression's negative influence on working memory capacity, according to Szuhany et al. (2018). Moreover, impulsivity is also highly correlated with depressive symptoms, such as suicide attempts and suicidal ideation (Dombrovski et al., 2012), eating disorders (Privitera et al., 2015), and alcohol use disorder (Dennhardt & Murphy, 2011). External validity for the DRL procedure as a test for impulsivity has been supported by a series of studies conducted by van

den Broek et al. (1987). They used the DRL procedure in human subjects and found when impulsive and non-impulsive human participants were asked to delay responding using an inter-response time (IRT) of 10s to earn a reward, the impulsive participants earned far fewer reinforcers than the non-impulsive participants in the absence of cues.

The DRL test was first used to screen antidepressant-like effects of drugs in rats in the early 1980s (McGuire & Seiden, 1980a; McGuire & Seiden, 1980b). In the DRL test, psychostimulants such as amphetamine decrease reinforcement rate and increase response rate; anxiolytic drugs also decrease reinforcement rate and increase response rate; ethanol will decrease both reinforcement rate and response rate (O'Donnell, & Seiden, 1983). McGuire and Seiden (1980a) found that tricyclic antidepressants produced a pattern of behavioral responding that was unique from other psychoactive drug classes, i.e. increased reinforcement rate and decreased response rate. In the following years, many antidepressants including tricyclic antidepressants, monoamine oxidase inhibitors, selective serotonin reuptake inhibitors, norepinephrine reuptake inhibitors, serotonin and norepinephrine reuptake inhibitors, various atypical antidepressants, and also electroconvulsive therapy have been tested in DRL. All of these produced a consistent behavioral profile of increased number of reinforcers and decreased number of responses (Castagné et al., 2009; O'Donnell, J. M., & Seiden, L. S., 1983; O'Donnell et al., 2005; Seiden et al., 1985). Ketamine also has been tested in the DRL task and produced a similar antidepressant-like behavioral profile in rats (Hillhouse & Porter, 2014).

In addition to the DRL task, the TST also was used to examine the opioid system's potential involvement in ketamine's antidepressant-like effects. Unlike the DRL task, the TST measures an animal's innate behavior. This test was developed by Steru et al. (1985) based on the theoretical assumption of behavioral despair. According to this theory, under the stress

produced by an inescapable situation, an immobile posture will be developed by the testing animal, and treatment with an antidepressant will promote escape-oriented behaviors, and in turn, decrease the time the animal spend immobile (Porsolt et al., 1978; Castagné et al., 2011). In the TST test, the inescapable stressor is created by suspending the rodent in the air by the tail for several minutes (Steru et al., 1986).

The TST has been proven to be sensitive to the majority of the antidepressants from traditional antidepressants, such as TCAs, MAOIs, SSRIs, SNRIs, and atypical antidepressants (Cryan et al., 2005; Steru et al., 1985), as well as non-pharmacological treatment such as electroconvulsive shock (Teste et al., 1990). More important to the present study, ketamine (da Silva et al., 2010; Koike et al., 2011), MOR agonists, and mixed opioid agonists (Berrocoso et al., 2009) have been consistently reported to produce antidepressant-like effects in the TST assay.

However, all of the behavioral despair theory-based antidepressant screening tests, including TST, rely on changes in the motor behavior of the animals, which makes these tests vulnerable to false positive results caused by drugs that have psychostimulant effects. Previous studies have found that psychostimulants such as amphetamine (Teste et al., 1993), d-amphetamine (van der Heyden et al., 1987), and caffeine (Teste et al., 1987) can produce an anti-immobility effect in the TST, which results in false positive results. Hence, it is important to examine possible changes in locomotor activity of test drugs when assays like DRL and TST are used.

Nevertheless, the TST has good predictability for the majority of antidepressants, and it is one of the most used behavioral tests for screening antidepressant-like effects in the rodent model (Castagné et al., 2011; Cryan et al., 2002; Nestler et al., 2002). In addition, while

antidepressant-like effects in the TST are marked by the decreased immobility or increased activity (Steru et al., 1985), the antidepressant-like effect in the DRL task is manifested by an *increase* in the number of reinforcers and decreased responses, which requires the animal to self-regulate level press behavior (O'Donnell & Seiden, 1983). As a result, the behavioral pattern for the TST assay is the opposite from that of the DRL tasks. Since the same drug is unlikely to produce false positive results in both assays, these two assays represent an ideal combination for studying the antidepressant-like behavior of new compounds.

Aim and Hypothesis

Aim 1 (Experiment 1): Differential-reinforcement-of-low-rate (DRL)

To determine if ketamine produces an antidepressant-like profile in the DRL 72-second task (DRL 72-sec) in C57BL/6 mice and to determine if pretreatment with the nonselective opioid receptor antagonist naltrexone can block the antidepressant-like behavior produced by ketamine.

Hypothesis 1: Ketamine will produce a significant increase in the number of reinforcers and a significant decrease in the number of responses as compared to the vehicle. The current study tested doses of 5.0, 10.0, 17.8, and 32.0 mg/kg ketamine. This dose range was based on a previous study in rats by Hillhouse et al. (2014). In that study ketamine demonstrated an antidepressant-like profile at 10.0 mg/kg (*R*, *S*)-ketamine, but not at 1.0 and 3.2 mg/kg doses of ketamine.

Hypothesis 2: The pretreatment of ketamine with naltrexone will significantly reduce the number of earned reinforcers and increase the number of responses significantly as compared to ketamine alone. The current study tested doses of 1.0 and 2.0 mg/kg naltrexone. These doses

were based on a previous study with mice that demonstrated naltrexone blocked the behavioral effects of ethanol in C57BL/6 mice (Kiianmaa et al., 1983).

Aim 2 (Experiment 2): Tail Suspension Test

In addition to the DRL task, the TST also will be used to determine if ketamine produces an antidepressant-like profile and if pretreatment with the nonselective opioid receptor antagonist naltrexone can block the antidepressant-like behavior produced by ketamine.

Hypothesis 1: ketamine will produce a significant decrease in the duration of immobility time as compared to the vehicle. The current study will test a single dose of 10 mg/kg ketamine. This dose was chosen based on previous studies with mice that demonstrated 10 mg/kg ketamine produced significant antidepressant-like effects in the TST (da Silva et al., 2010; Koike et al., 2011; Zhang and Hashimoto, 2019).

Hypothesis 2: the pretreatment of ketamine with naltrexone will significantly increase the duration of immobility time as compared to ketamine alone. The test dose of naltrexone was determined by the results from the DRL results.

Aim 3 (Experiment 3): Locomotor Activity

Considering that a change in motor behavior by ketamine could produce a false positive result in the DRL task or the TST, it is important to assess the motor effects of ketamine. For example, if ketamine produced hypoactivity, this could lead to a false positive result in the DRL task; whereas, if ketamine produced hyperactivity, this could lead to a false positive result in the TST. Hence, locomotor activity was measured to determine if ketamine produces any changes in locomotor activity in C57BL/6 mice. Ketamine was tested alone and in combination with naltrexone.

Hypothesis 1: Ketamine will produce a significant increase in the total distance traveled compared to the vehicle. The test dose will be determined by the results from the DRL and the TST studies.

Hypothesis 2: The combination of ketamine and naltrexone will not produce any significant change in the total distance traveled when compared to ketamine alone, but the combination will produce a significant increase in the total distance traveled compared to the vehicle. The test doses were determined by the results from the DRL and the TST studies.

Method

Animals

DRL

Twelve wildtype male C57BL/6 inbred mice (Envigo, Indianapolis, IN) weighing 24.2-37.1g were used in the DRL study. The sample sizes were determined based on previously published studies (e.g. Scott-McKean et al., 2008). Mice were maintained on a 12/12-hour light-dark cycle (lights on 0600 h, lights off 1800h) and were allowed one week to adapt to their home cage environments before experimentation. The mice were housed individually in a temperature-controlled vivarium at 22-24° Celsius. Nesting material and ad libitum access to water were provided in their home cage. For each mouse 100% body weight was established using the highest free-feeding body weight before the withdrawal of free access to food, and their weight was maintained at 90% of their 100% body weight via food restriction. Because this study lasted over 12 months, every six months, the animals were placed on free access to food and no training/testing for a week. New 100% body weights were established at the end of each free-feed period. The DRL study was performed in agreement with the guide for the Care and Use of

Laboratory Animals (National Research Council, 2011), and all procedures were approved by *the Institutional Animal Care and Use Committee at Virginia Commonwealth University* (IACUC Protocol AM10284).

TST and Locomotor Activity

A total of 36 adult C57BL/6 mice (Male = 21; Female = 16) were used for the acute (30 mins) TST effects. A total of 24 adult ICR (CD-1) mice (Male = 12; Female = 12) weighing between 25.0 and 45.0 g at the beginning of the experiment were used to conduct the locomotor activity experiment. All mice were at least 8 weeks old at the start of the experiment. Mice were either purchased from Envigo or bred in-house at University of Wisconsin Green Bay. Breeders were purchased from Envigo to avoid any differences between purchased and bred mice. Mice were group housed in standard plastic cages in same-sex groups (n=3). The mice were given conditions of a 12-hour light/dark cycle (lights on 0600 h, lights off 1800h) in a temperature (20-22°C) controlled vivarium. The experiments were performed during the light portion of the cycle. The mice had free access to food and water at all times except during their experimental sessions. All experimental procedures were approved by *the Institutional Animal Care and Use Committee at University of Wisconsin Green Bay* (IACUC Protocol Fall-20-04) prior to the start of the study and were conducted in accordance with *National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 2011)*.

Drug treatment

(*R, S*) Ketamine HCL (Sigma-Aldrich, St Louis, Missouri, USA) and naltrexone (Sigma-Aldrich, St Louis, Missouri, USA) were dissolved in 0.9% physiological vehicle. All drugs were administered subcutaneously at a volume of 10 ml/kg for the DRL experiment and

intraperitoneally for the TST and for locomotor activity. Doses and pretreatment times were based on previous studies in the literature (Hillhouse & Porter, 2013; Kiianmaa et al., 1983). Drugs, doses, and pretreatment times were as follows: ketamine (5.0, 10.0, 17.8, and 32.0 mg/kg; 5 min), naltrexone (1.0 and 2.0 mg/kg; 30 min).

Apparatus

DRL training and testing were conducted in six standard computer-interfaced operant conditioning chambers (15 cm L X 11.5 cm D X 17.5 cm H; Model ENV-307A, Med Associates Inc., St. Albans, VT) each containing two retractable levers in the left and right position (8 cm apart) on the front panel of the operant chamber. The levers extended 0.8 cm into the chamber and were positioned 2.5 cm above a grid floor constructed with parallel, stainless-steel rods. Centered between them was a recessed food trough into which a liquid dipper delivered 0.02 mL of sweetened milk (by volume: 80 mL powdered milk, 80 mL sugar, and 200 mL tap water). The test chambers were housed in sound-attenuating cubicles equipped with ventilation fans. MED-PC software (Version 4.2, Med Associates Inc.) was used to control the operant sessions and record data.

TST were conducted from a metal bar attached to a ring stand approximately 35 cm from a tabletop using paper adhesive tape. The mice were suspended on a metal bar one at a time. Cardboard dividers were placed between each mouse to prevent the mice from observing and interacting with other mice. A video camera was used to record test sessions.

Locomotor activity sessions were conducted in three standard Plexiglas open field arenas (29cm X 29cm X 20 cm) enclosed within sound alleviating cubicles (Model ENV-510S; Med Associates Inc.). Each chamber was equipped with three 16-beam IR arrays, which tracked

distance traveled (cm), time spent in the center (central square 20 cm X 20 cm) of the open field, and rearing. Med-PC Activity Monitor software (version 7; Med-Associates) was used to collect the data.

Procedure

Differential-reinforcement-of-low-rate training

All mice first received two sessions of magazine training, during which the house light was on, but no lever was extended. One reinforcer (i.e., sweet milk) was delivered non-contingently according to a fixed-time 60 sec schedule for 30 min. On the third day, a 20 min single level training session was conducted, during which the mice were trained to press either the left or the right lever under a fixed ratio 1 (FR 1) food reinforcement schedule, in which each level press resulted in the delivery of a reinforcer. The position of the lever associated with the FR 1 schedule was counterbalanced, with half of the mice assigned to the right lever and half assigned to the left lever. The criterion for passing the FR 1 training was to obtain 30 reinforcers in 20 min. On day 7, after all the mice met the training criteria, DRL training began.

Under the DRL schedule, a response produced a reinforcer only after a specified inter-response time interval had elapsed. Responses emitted before the end of the inter-response interval reset the timer and did not result in delivery of a reinforcer. All DRL training and testing sessions were set to end at 60 min or when the animal received 50 reinforcers. The inter-response interval was gradually increased from an initial value of 4.5 sec to a terminal value of 72 sec over 14 sessions. Specifically, mice were initially trained on a DRL 4.5 sec schedule for the first session, and all mice reached the 50-reinforcer goal within the 60 min session time. On the next day, the inter-response time was increased to 9 sec. Once again, all mice reached the maximum

number of reinforcers before 60 min. The DRL schedule was then increased to 18 sec for 2 sessions and then to 36 sec for 5 sessions. Finally, the DRL schedule was increased to the terminal value of 72 sec until performance stabilized, meaning the number of responses for each mouse did not vary by more than 10% of the mean for five of six consecutive sessions. Test sessions occurred twice weekly (typically Tuesday and Friday) with a minimum of one training session between each test session. A vehicle baseline, which consisted of two vehicle-test days, was established before and after each drug test. Due to inadequate performance or health reasons, three of the mice were removed from the DRL study.

Tail Suspension Test

There were four groups of mice: (1) vehicle plus vehicle group (n = 9, 5 male and 4 female); (2) vehicle plus 10 mg/kg ketamine group (n = 9, 5 male and 4 female); (3) 2 mg/kg naltrexone plus vehicle group (n = 9, 5 male and 4 female); and (4) 2 mg/kg naltrexone and 10 mg/kg ketamine (n = 10, 6 male and 4 female). The TST was conducted consistent with methods previously described (Can et al., 2012; Dripps et al., 2017; Steru et al., 1985). Briefly, the mice were suspended by their tails from a metal bar attached to a ring stand approximately 35 cm from a tabletop using paper adhesive tape. Test sessions were 6 min in duration and videotaped.

Locomotor Activity

Two separate groups of mice (N = 12 each; 6 male and 6 female) were used for the 10.0 mg/kg and 32.0 mg /kg ketamine locomotor activity studies. Prior to the start of drug testing, mice were given a 60 min acclimation session in the locomotor activity chambers. On drug test days, mice received an injection of vehicle or 2.0 mg/kg naltrexone and then were placed in the open field arena for 30 minutes. After the 30 min baseline session, mice were removed from the

open field arena and placed back in their home cages while the experimenter loaded the activity monitor program for the next session. Mice received an injection of vehicle or ketamine (10 mg/kg or 32 mg/kg depending on the group) and were immediately placed in the activity chamber for 30 mins. The chambers were cleaned with 50% ethanol between each mouse to control for any movement motivated by scent from mice previously in the chamber.

The treatment conditions for the first group were as follows: vehicle + vehicle, vehicle + 10.0 mg/kg ketamine, 2.0 mg/kg naltrexone + vehicle, and 2.0 mg/kg naltrexone + 10.0 mg/kg ketamine. The treatment conditions for the second group were as follows: vehicle + vehicle, vehicle + 32.0 mg/kg ketamine, 2.0 mg/kg naltrexone + vehicle, and 2.0 mg/kg naltrexone + 32.0 mg/kg ketamine. The order of drug treatments was counterbalanced using the Latin square method.

Data Analysis

In the DRL study, the dependent variables included the total number of earned reinforcers and the total number of responses during each test session. All data were expressed as means ± SEM. Response and reinforcer data were analyzed using one-way repeated-measures analysis of variance (ANOVA) with drug dose as a factor. In addition, combination test data for ketamine and naltrexone were analyzed using one-way repeated-measures ANOVA with treatment combination as the within-subjects factor. Multiple comparisons using Tukey's post-hoc tests were conducted after all significant ANOVAs, as appropriate. The criterion for significance was a p value equal to or less than 0.05.

In the TST study, the dependent variable was the duration of immobility time. Two trained blinded observers scored the immobility time (sec) for each 6 min test session from the

video recordings and the average scores from these two observers were used. Immobility was defined as mice hanging motionless without any escape-related behaviors. Escape-related behaviors were defined as strong shakes of the body, running with hind- and forelimbs, and attempts to reach the suspension bar. Small subtle movements of forelimb(s) without the movement of hindlimbs were not counted as escape-related behaviors and thus were counted as immobility. All data were expressed as means ± SEM. Immobility times were analyzed using one-way independent-measures ANOVA for the different treatment combinations. Multiple comparisons using Tukey's post hoc test were conducted after all significant ANOVAs, as appropriate. The criterion for significance was a p value equal to or less than 0.05.

In the locomotor activity study, distance traveled (cm), vertical rearing (beam breaks), and time (min) spent in the center area of the locomotor chamber were used as dependent variables. A two-way ANOVA was used to test the effects of different combinations of drug treatments at different times. One-way independent measures ANOVAs were used to evaluate differences in total distance traveled, time in the center, and rearing behavior. Multiple comparisons using Tukey's post-hoc tests were conducted after all significant ANOVAs, as appropriate. The criterion for significance was a p value equal to or less than 0.05.

Data analysis for all three studies were conducted with Prism version 9.0 for MacOS, GraphPad Software; GraphPad Software Inc., La Jolla CA. All significant differences were at $p \leq .05$.

Results

DRL

Figure 1 shows results for the ketamine dose response in the DRL task. A one-way repeated measures ANOVA revealed that ketamine treatment produced a significant effect on the number of reinforcers, $F\ (4, 32) = 9.43, p < 0.001$ (Figure 1, top panel). A Tukey post hoc test indicated that the 32 mg/kg dose of ketamine ($M = 5.11, SD = 2.09$) produced a significant ($p = 0.004$) increase in the mean number of reinforcers as compared to the vehicle treatment ($M = 1.33, SD = 0.87$). There were no other significant differences for mean number of reinforcers between the ketamine doses ($p > 0.05$). Ketamine also produced a significant effect on the mean number of responses, $F\ (4, 32) = 4.27, p = .01$ (Figure 1, bottom panel). A Tukey post hoc test indicated that the mice responded significantly less when they received the 32 mg/kg dose of ketamine ($M = 172.3, SD = 49.08$) as compared to vehicle ($M = 253.7, SD = 49.08, p = 0.03$). There were no other significant differences in mean number of responses between the ketamine doses ($p > 0.05$).

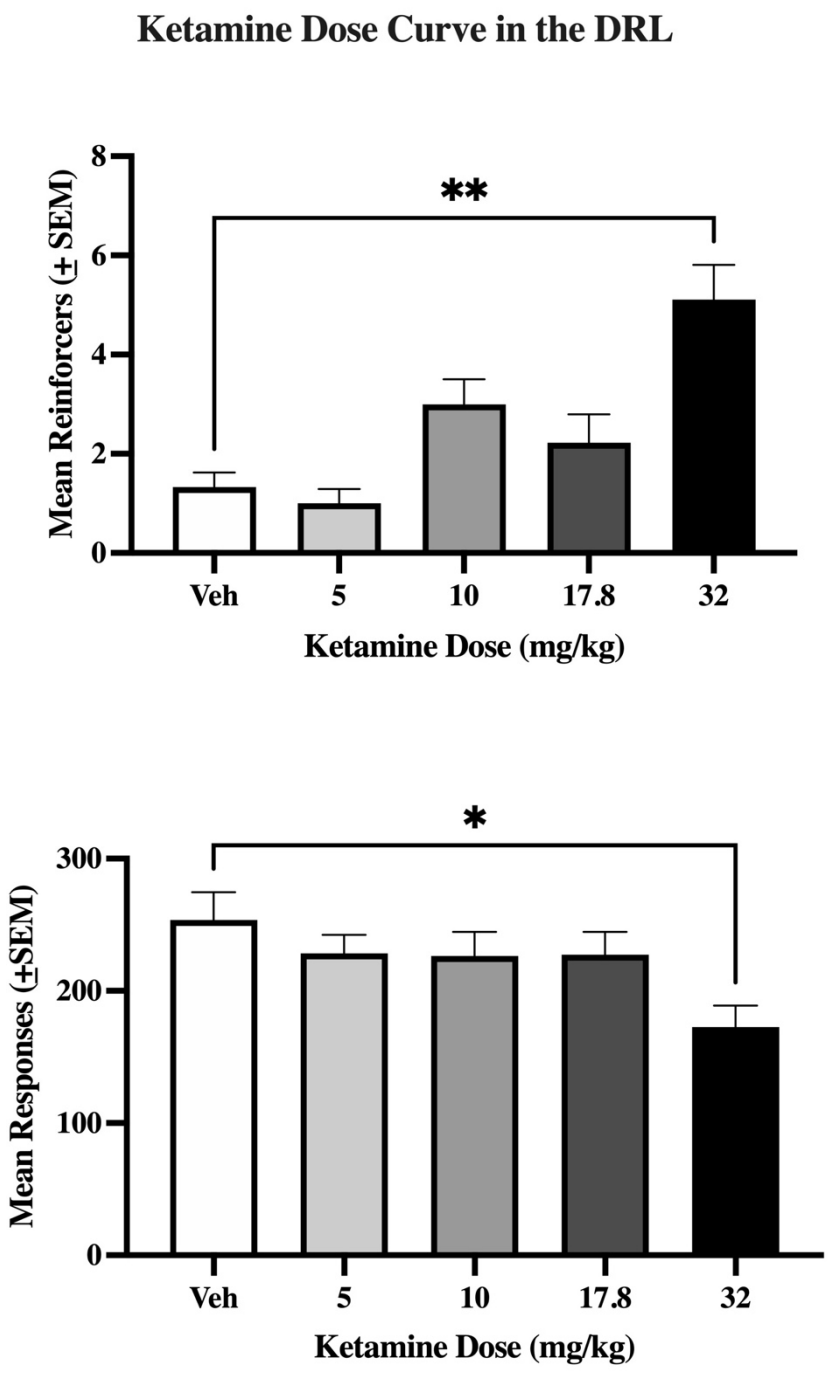

Figure 1. Effects of ketamine on mean number of reinforcers (top; n = 9) and number of responses (bottom; n = 9). Ket = ketamine, Veh = vehicle. Asterisks represent significant differences between the groups, and all data are expressed as means + standard error of the mean (+SEM). The alpha level was set to 0.05. For all graphs: * = $p < 0.05$, ** = $p < 0.01$.

Figure 2 shows the naltrexone control tests. For the mean number of reinforcers there was a small, but nonsignificant increase at the 2.0 mg/kg dose of naltrexone ($F(2, 16) = 3.02$, $p = 0.01$) (Figure 2, top panel). For responses, ANOVA revealed a significant effect ($F(2, 16) = 7.18$, $p = 0.007$) (Figure 2, bottom panel). A Tukey test showed that the 2.0 naltrexone dose ($M = 165$, $SD = 34.26$) produced small, but significant reductions in the mean number of responses as compared to both the vehicle plus vehicle ($M = 191$, $SD = 32.6$, $p = 0.025$) and 1.0 mg/kg naltrexone plus vehicle conditions ($M = 184.1$, $SD = 13.75$, $p = 0.036$).

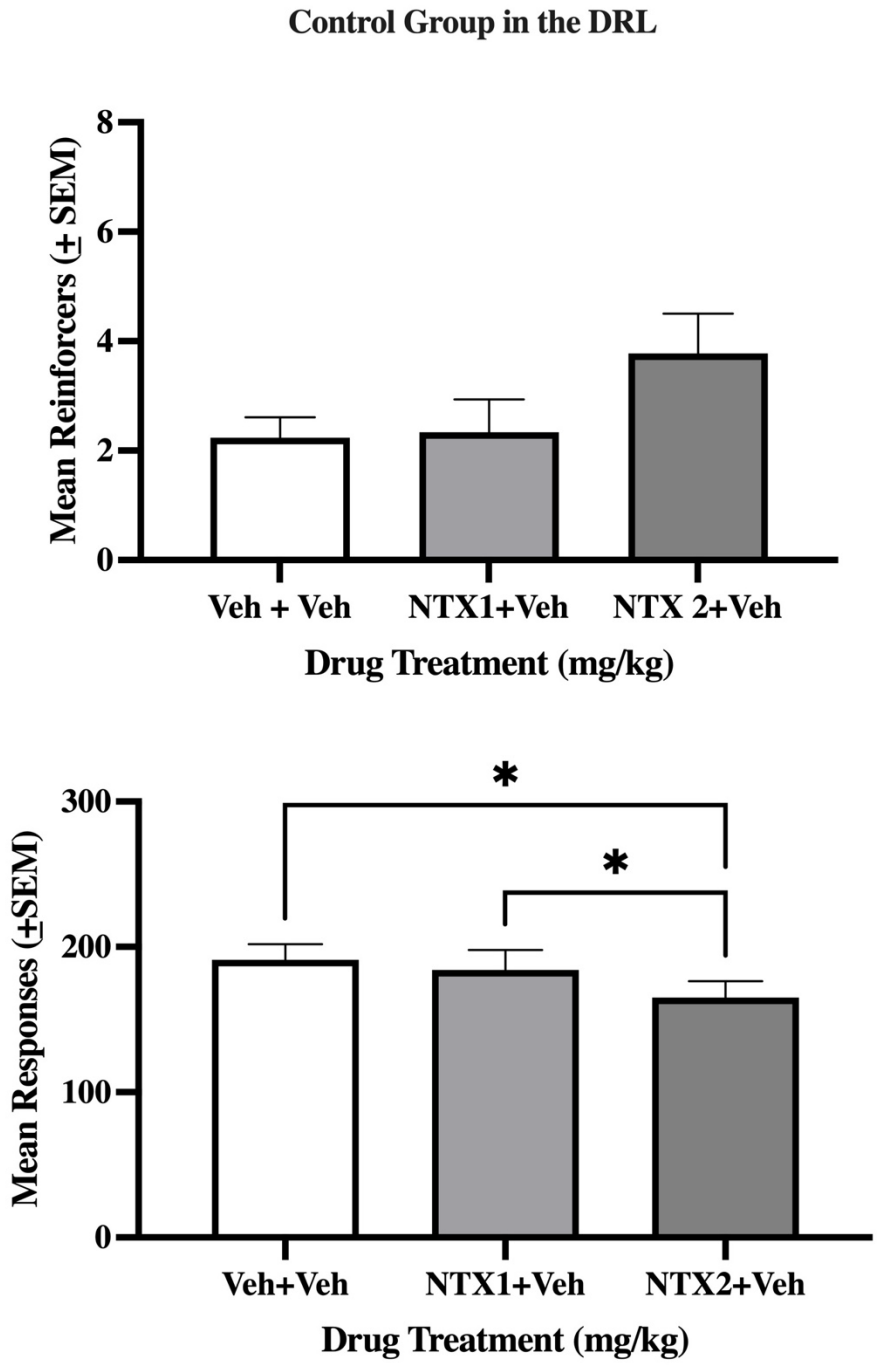

Figure 2. Effects of naltrexone on mean number of reinforcers (top; n = 9) and number of responses (bottom; n = 9). Veh = vehicle, NTX = naltrexone. Asterisks represent significant differences between the groups and all data are expressed as means + standard error of the mean (+SEM). The alpha level was set to 0.05. For all graphs: * = $p < 0.05$.

Figure 3 shows the results of combining the mixed opiate antagonist naltrexone with ketamine in the DRL task. A one-way repeated measures ANOVA on the mean number of reinforcements was significant, $F\ (3, 32) = 9.175, p = 0.001$ (Figure 3, top panel). A Tukey test indicated that the mice received significantly more reinforcers when they received the vehicle plus ketamine 32 mg/kg treatment ($M = 5.67, SD = 1.58$) compared to the vehicle plus vehicle treatment ($M = 2.24, SD = 1.11, p = 0.002$). When the 2 mg/kg dose of naltrexone was combined with ketamine, there was a significant reduction in the mean number of reinforcers ($M = 3.67$, $SD = 0.87$), as compared to the treatment combination of vehicle plus ketamine 32 mg/kg treatment ($M = 5.67, SD = 1.58, p = 0.0497$). There were no other significant differences between the treatment conditions ($p > 0.05$). ANOVA revealed that were no significant changes in the mean number of responses for any of the treatment combinations $F\ (3, 32) = 1.014, p =$ 0.37(Figure 3, bottom panel).

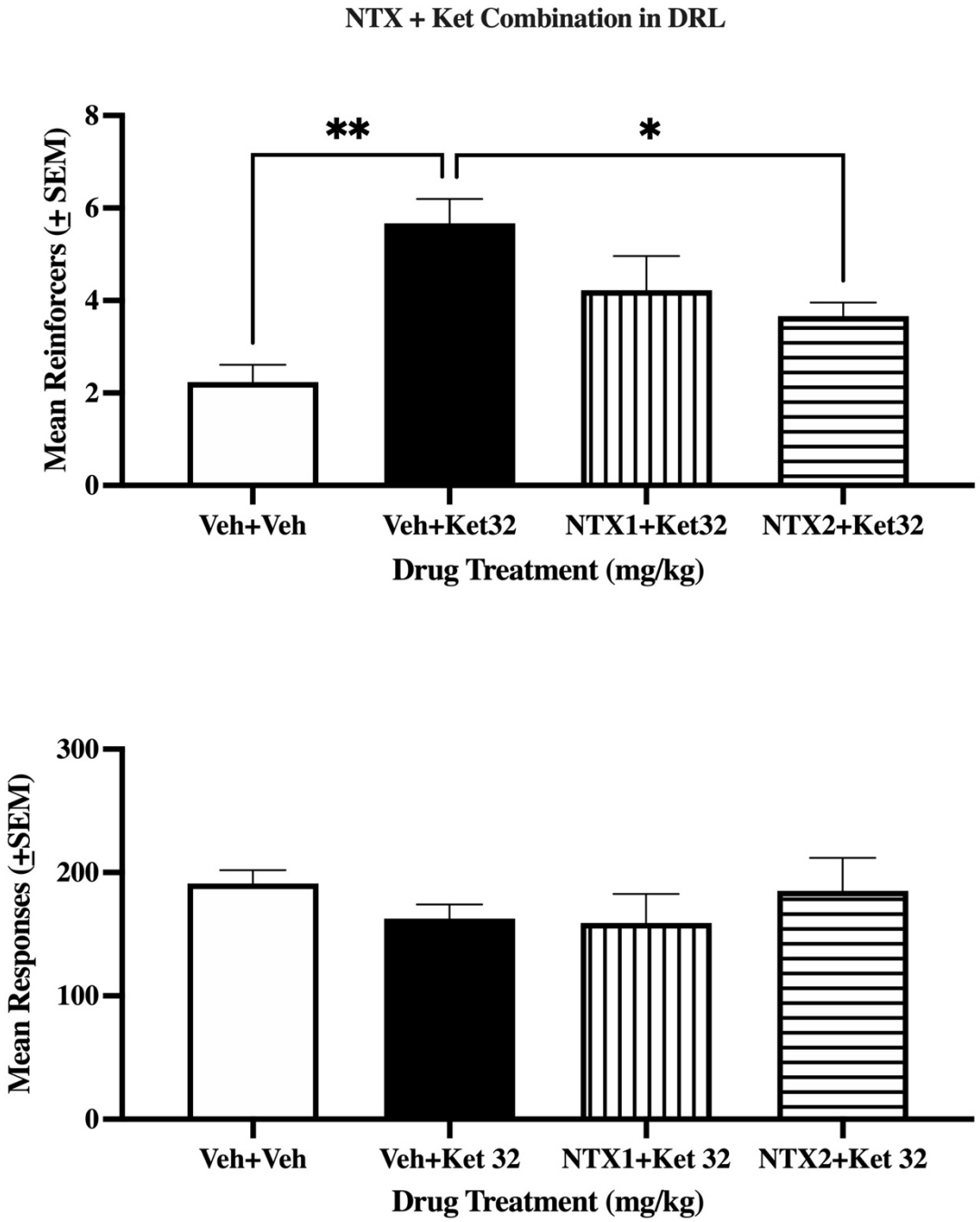

Figure 3. Effects of naltrexone and ketamine combination on mean number of reinforcers (top; n = 9) and number of responses (bottom; n = 9). Veh = vehicle, Ket = ketamine, NTX = naltrexone. Asterisks represent significant differences between the groups and all data are expressed as means + standard error of the mean (+SEM). The alpha level was set to 0.05. For all graphs: * = $p < 0.05$, ** = $p < 0.01$.

Tail Suspension Test

Figure 4 shows the results of combining the mixed opiate antagonist naltrexone with ketamine in the TST. A one-way ANOVA on immobility (sec) was significant, F (3, 33) = 6.212, p = 0.002. A Tukey post hoc test indicated that there was a significant decrease in immobility time after receiving the vehicle plus ketamine 10 mg/kg treatment (M = 141.7, SD = 44.31) compared to vehicle plus vehicle (M = 208, SD = 19.77, p = 0.002). When naltrexone 2 mg/kg was combined with ketamine 10 mg/kg (M = 196.8, SD = 38.05, p = 0.009, there was a significant increase in immobility time as compared 10 mg/kg ketamine alone (K10 + V), and it was not significantly different from vehicle plus vehicle.

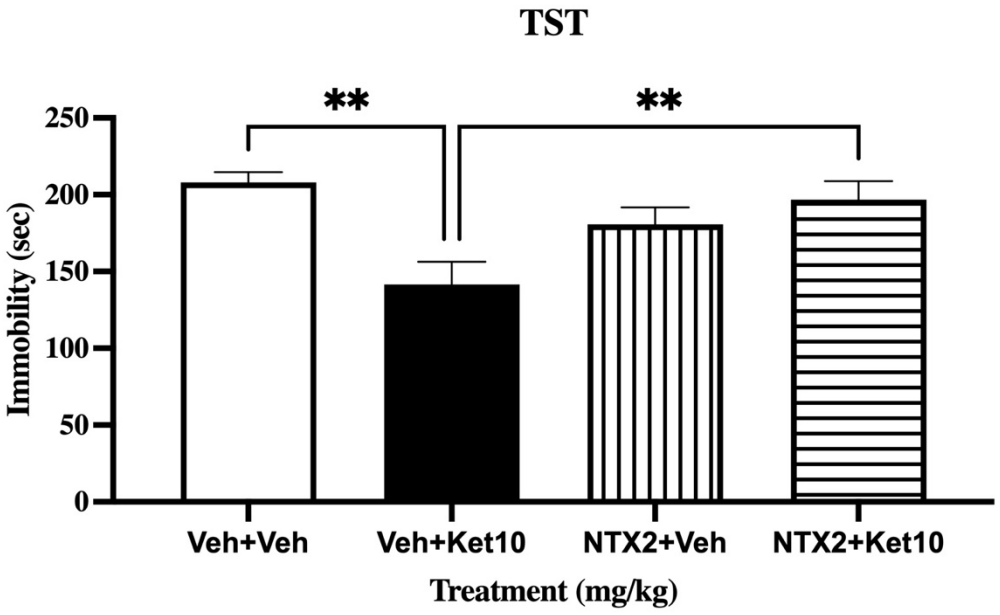

Figure 4. Effects of ketamine on mean time of immobility (n = 37). Veh = vehicle, Ket = ketamine, NTX = naltrexone. Asterisks represent significant differences between the groups, and all data are expressed as means + standard error of the mean (+SEM). The alpha level was set to 0.05. For all graphs: * = $p < 0.05$, ** = $p < 0.01$.

Locomotor Activity

Figures 5 and 6 show the results for combination testing with 2.0 mg/kg naltrexone plus 10 mg/kg ketamine and 2 mg/kg naltrexone plus 32 mg/kg ketamine in the locomotor test, respectively. The results for each are presented separately below.

10 mg/kg Ketamine

A one-way independent measures ANOVA was conducted to compare the mean of the total distance traveled between different treatment groups. ANOVA revealed that there was a significant difference in the mean of the total distance traveled between different treatment groups $F\ (3, 33) = 11.77, p < 0.001$(Figure 6, top left panel). A Tukey post hoc test indicated that the vehicle plus ketamine 10 mg/kg group ($M = 3803$, $SD = 1272$) produced a significant increase in distance traveled compared to the vehicles group ($M = 1635$, $SD = 882.6$, $p < 0.001$) and the naltrexone 2 mg/kg plus vehicle group ($M = 1419$, $SD = 822.1$, $p < 0.001$). The naltrexone 2 mg/kg plus ketamine 10 mg/kg group ($M = 2952$, $SD = 1571$) also produced a significant increase in distance traveled compared to the vehicles group ($M = 1635$, $SD = 882.6$, $p = 0.04$) and the naltrexone 2 mg/kg plus vehicle group ($M = 1419$, $SD = 822.1$, $p = 0.01$).

A two-way mixed ANOVA with treatment as a between-subjects factor and time as a within-subjects factor was conducted to compare the main effects of treatment and time as well as the interaction on the mean distance (cm) traveled in the locomotor test. A significant interaction effect was found between time and treatment ($F\ (33, 363) = 3.84, p < 0.001$) (Figure 6, top right panel). In addition, the main effect of time was significant ($F\ (11, 121) = 60.51, p < 0.001$, and the significant main effect of treatment was also found ($F\ (3, 33) = 9.76, p < 0.001$). Between-treatment group post hoc comparisons at each time bin revealed that during the first 5 minute bin after the habituation period, the vehicle plus ketamine 10 mg/kg group ($M = 1193$, SD

= 532.62) displayed a significant increase in distance traveled compared to the vehicle plus vehicle group (M = 535.5, SD = 259.24, p =0.007) and the naltrexone 2 mg/kg plus vehicle group (M = 481.9, SD = 227.49, p =0.004). The naltrexone 2 mg/kg group plus ketamine 10 mg/kg (M = 1033, SD = 441.12) also a significant increase in distance traveled compared to the vehicle plus vehicle group (M = 535.5, SD = 259.24, p =0.017) and the naltrexone 2 mg/kg plus vehicle group (M = 481.9, SD = 227.49, p =0.008). During the second 5 minute bin after the habituation period, the vehicle plus ketamine 10 mg/kg group (M = 802.3, SD = 595.74) displayed a significant increase in distance traveled compared to the vehicles group (M = 245.4, SD = 224.9, p =0.04) and the naltrexone 2 mg/kg plus vehicle group (M = 191, SD = 167.21, p =0.02). The naltrexone 2 mg/kg plus ketamine 10 mg/kg group (M = 685.7, SD = 419.8) also displayed a significant increase in distance traveled compared to the vehicles group (M = 245.4, SD = 224.9, p =0.02) and the naltrexone 2 mg/kg plus vehicle group (M = 191, SD = 167.21, p =0.009). During the third 5 minute bin after the habituation period, the vehicle plus ketamine 10mg/kg group (M = 445.1, SD = 222.71) displayed a significant increase in distance traveled compared to the naltrexone 2 mg/kg plus vehicle group (M = 180.1, SD = 146.63, p =0.013). During the fourth 5 minute bin after the habituation period, the vehicle plus ketamine 10mg/kg group (M = 537, SD = 445.23) displayed a significant increase in distance traveled compared to the vehicle mg/kg plus vehicle group (M = 205.5, SD = 166.80, $p < 0.001$) and the naltrexone 2 mg/kg plus ketamine 10 mg/kg group (M = 377.1, SD = 219.47) also displayed a significant increase in distance traveled compared to the vehicle plus vehicle group (M = 205.5, SD = 166.8, $p < 0.001$). During the fifth 5 minute bin after the habituation period, the vehicle plus ketamine 10mg/kg group (M = 493.9, SD = 340.86) displayed a significant increase in distance traveled compared to the vehicles group (M = 176.2, SD = 173.47, p =0.048).

A one-way independent measures ANOVA was conducted to compare the mean of the time spent (in minutes) in the center between different treatment groups. ANOVA revealed that there was a significant difference in the mean time (min) spent in center between different treatment groups $F\ (3, 33) = 4.24, p = 0.01$(Figure 6, bottom left panel). A Tukey post hoc test indicated that the vehicle plus ketamine 10 mg/kg group ($M = 631.1, SD = 322.1$) spent significantly more time in the center when compared to the naltrexone 2 mg/kg plus vehicle group ($M = 308.5, SD = 240.7, p = 0.03$), and the naltrexone 2 mg/kg plus ketamine 10 mg/kg group ($M = 617.9, SD = 372.1$) also spent significantly more time in the center when compared to the naltrexone 2 mg/kg plus vehicle group ($M = 308.5, SD = 240.7, p = 0.04$). A Tukey post hoc test indicated that there were no other significant differences in center time between any of the two groups ($p > 0.05$).

A one-way independent measures ANOVA was conducted to compare the mean of the number of rears between different treatment groups. ANOVA reveled that there was a significant difference in the mean of the number of rears between different treatment group $F\ (3, 33) = 1.6$, $p = 0.2$ (Figure 6, bottom right panel). A Tukey post hoc test indicated that there were no other significant differences rearing between any of the two groups ($p > 0.05$).

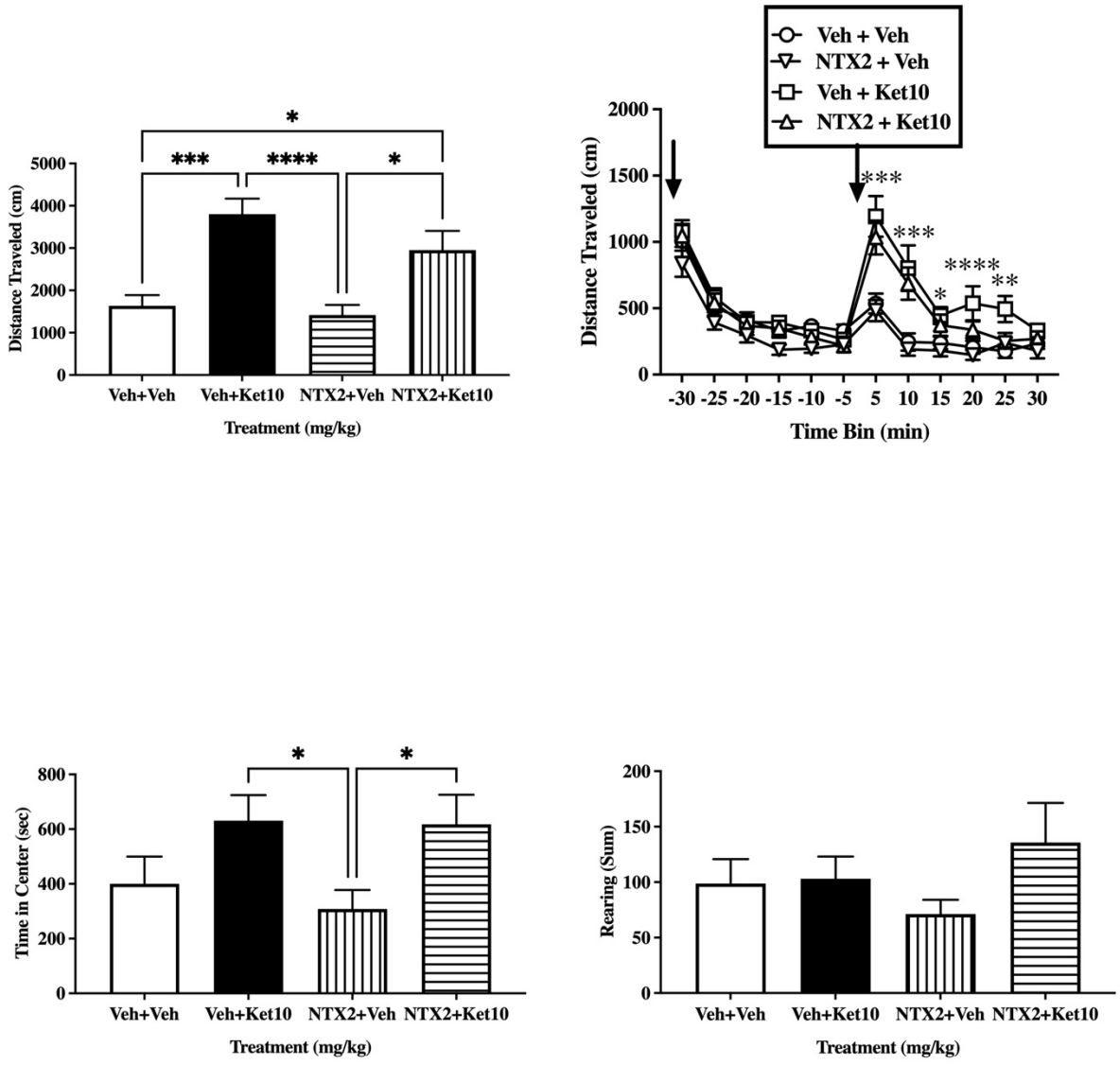

Figure 6. Effects of naltrexone 2mg/kg and ketamine 10 mg/kg on mean distance traveled (top left), distance traveled in 5 mins bins (top right), time in center (bottom left), and rearing (bottom right) (n = 12). Veh = vehicle, Ket = ketamine, NTX = naltrexone. Asterisks represent significant differences between the groups, and all data are expressed as means + standard error of the mean (+SEM). The alpha level was set to 0.05. For all graphs: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$.

32 mg/kg Ketamine

A one-way independent measures ANOVA was conducted to compare the mean of the total distance traveled (cm) between different treatment groups. ANOVA revealed that there was a significant difference in the mean of the total distance traveled between different treatment groups $F\ (3, 33) = 7.32, p < 0.001$(Figure 6, top left panel). A Tukey post hoc test indicated that 32 mg/kg ketamine plus vehicle group ($M = 3447$, $SD = 2653$) displayed a significant increase in distance traveled compared to the vehicles group ($M = 1080$, $SD = 933.8$, $p = 0.03$). This effect was not blocked when 2 mg/kg naltrexone was combined with 32 mg/kg ketamine ($M = 3299$, $SD = 2698$, $p = 0.04$). When 2 mg/kg naltrexone was combined with vehicle ($M = 481.7$, $SD = 471.2$), it was not significantly different from the vehicles group, but was significantly different from the ketamine 32 mg/kg plus vehicle group ($M = 3447$, $SD = 2653$, $p = 0.004$) and naltrexone 2 mg/kg plus ketamine 32 mg/kg group ($M = 3299$, $SD = 2689$, $p = 0.006$).

A two-way mixed ANOVA with treatment as a between-subjects factor and time as a within-subjects factor was conducted to compare the main effects of treatment and time as well as the interaction on the mean distance (cm) traveled in the locomotor test. A significant interaction effect was found between time and treatment ($F\ (33, 363) = 3.7, p < 0.001$) (Figure 6, top right panel). In addition, the main effect of time was significant ($F\ (11, 121) = 21.84, p < 0.001$, and the significant main effect of treatment was also found ($F\ (3, 33) = 9.12, p < 0.001$). Between-treatment group post hoc comparisons at each time bin revealed that during the first 5 minute bin after the habituation period, the vehicle plus ketamine 32 mg/kg group ($M = 1236$, $SD = 645.56$) displayed a significant increase in distance traveled as compared to the vehicles group ($M = 408.6$, $SD = 289.98$, $p < 0.001$) and the naltrexone 2 mg/kg plus vehicle group ($M = 310.5$, $SD = 188.04$, $p < 0.001$). The naltrexone 2 mg/kg plus ketamine 32 mg/kg group ($M = 1090$, SD

= 708.12) also displayed a significant increase in distance traveled as compared to the vehicles group ($M = 408.6$, $SD = 289.98$, $p < 0.001$) and the naltrexone 2 mg/kg plus vehicle group ($M = 310.5$, $SD = 188.04$, $p < 0.001$). During the second 5 minute bin after the habituation period, the vehicle plus ketamine 32 mg/kg group ($M = 815.2$, $SD = 613.38$) displayed a significant increase in the distance traveled compared to the vehicles group ($M = 140.4$, $SD = 145.09$, $p < 0.001$) and the naltrexone 2 mg/kg plus vehicle group ($M = 48.8$, $SD = 91.78$, $p < 0.001$). The naltrexone 2 mg/kg plus ketamine 32 mg/kg group ($M = 715.1$, $SD = 703.16$) also displayed a significant increase in the distance traveled compared to the vehicles group ($M = 140.4$, $SD = 145.09$, $p < 0.001$) and the naltrexone 2 mg/kg plus vehicle group ($M = 48.8$, $SD = 91.78$, $p < 0.001$). During the third 5 minute bin after the habituation period, the vehicle plus ketamine 32 mg/kg group ($M = 591.3$, $SD = 591.67$) displayed a significant increase in distance traveled compared to the vehicles group ($M = 119.3$, $SD = 166.87$, $p < 0.001$) and the naltrexone 2 mg/kg plus vehicle group ($M = 45.72$, $SD = 109.29$, $p < 0.001$). The naltrexone 2 mg/kg plus ketamine 32 mg/kg group ($M = 589.3$, $SD = 792.49$, $p < 0.001$) also displayed a significant increase in distance traveled compared to the vehicles group ($M = 119.3$, $SD = 166.87$, $p < 0.001$) and the naltrexone 2 mg/kg plus vehicle group ($M = 45.72$, $SD = 109.29$, $p < 0.001$). During the fourth 5 minute bin after the habituation period, the naltrexone 2 mg/kg plus ketamine 32 mg/kg group ($M = 463.5$, $SD = 699.45$) displayed a significant increase in distance traveled compared to the vehicles group ($M = 124.8$, $SD = 194.52$, $p = 0.02$), and the naltrexone 2 mg/kg plus ketamine 32 mg/kg group ($M = 463.5$, $SD = 699.45$) displayed a significant increase in distance traveled compared to the naltrexone 2 mg/kg plus vehicle group ($M = 51.65$, $SD = 95.2$, $p = 0.002$).

A one-way independent measures ANOVA was conducted to compare the mean of the time spent (in minutes) in center between different treatment group. ANOVA revealed that there

was a significant difference in the mean of the time spent in center between different treatment groups $F\ (3, 33) = 8.91, p < 0.001$(Figure 6, bottom left panel). A Tukey post hoc test indicated that the naltrexone 2 mg/kg plus ketamine 32 mg/kg group ($M = 583.6$, $SD = 406.7$) spent significantly more time in the center compared to the vehicles group ($M = 217$, $SD = 251.8$, $p = 0.006$). The vehicle plus ketamine 32 mg/kg group ($M = 387$, $SD = 216.6$) spent significantly more time in the center compared to the naltrexone 2 mg/kg plus vehicle group ($M = 74.19$, $SD = 129.1$, $p = 0.02$). The naltrexone 2 mg/kg plus ketamine 32 mg/kg group ($M = 583.6$, $SD = 406.7$) also spent significantly more time in the center compared to the naltrexone 2 mg/kg plus vehicle group ($M = 74.19$, $SD = 129.1$, $p < 0.001$).

A one-way independent measures ANOVA was conducted to compare the mean of the number of rears between different treatment groups. ANOVA reveled that there was a significant difference in the mean of the number of rears between different treatment groups $F\ (3, 33) = 1.47$, $p = 0.24$ (Figure 6, bottom right panel). However, a Tukey post hoc test indicated that there were no significant differences in rearing revealed for pair-wise comparisons between groups ($p > 0.05$).

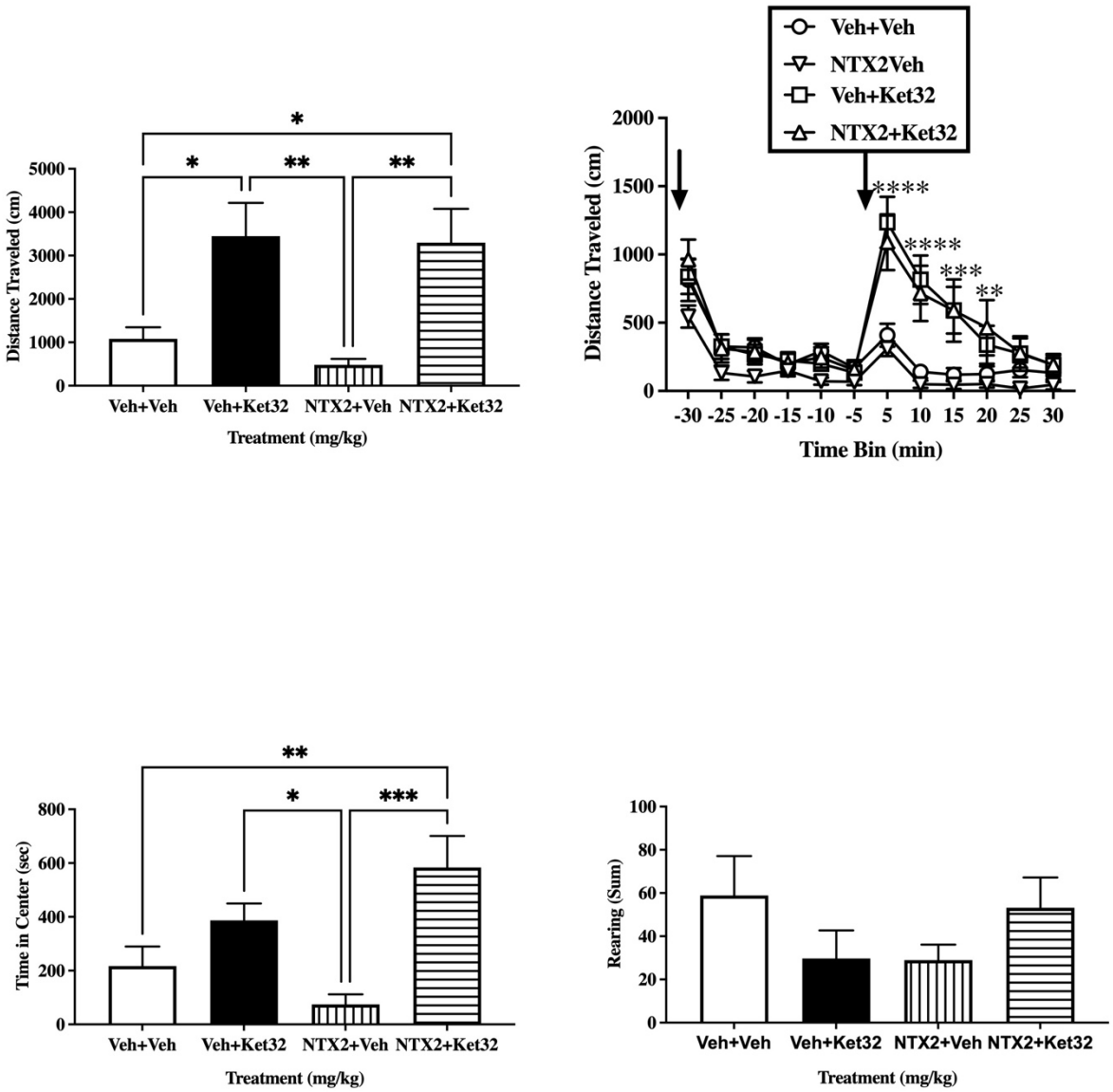

Figure 6. Effects of naltrexone 2mg/kg and ketamine 10 mg/kg on mean distance traveled (top left), distance traveled in 5 mins bins (top right), time in center (bottom left), and rearing (bottom right) (n = 12). Veh = vehicle, Ket = ketamine, NTX = naltrexone. Asterisks represent significant differences between the groups, and all data are expressed as means + standard error of the mean (+SEM). The alpha level was set to 0.05. For all graphs: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$.

Discussion

The present study is the first to demonstrate that the μ opioid antagonist naltrexone can block the antidepressant-like effects of ketamine in two preclinical animal models. Specifically, the present study tested ketamine in three preclinical behavior assays. DRL task, TST, and locomotor activity in adult, male and female C57Bl/6 mice. The DRL task and the TST measured the antidepressant-like effects of ketamine, and the locomotor activity test determined if the test drugs produced any changes in motor behavior that could have influenced results in the other two behavior assays. In the DRL task and the TST, it was hypothesized that treatment of ketamine alone would produce antidepressant-like effects, and that pretreatment with the μ opioid antagonist naltrexone would attenuate or block the antidepressant-like effects produced by ketamine. In the locomotor test, it was hypothesized that the ketamine alone would produce an acute hyperactivity effect, and that pretreatment with naltrexone would not affect ketamine's hyperactivity effect. As reported, the findings of this study supported all three hypotheses. First, ketamine produced an antidepressant-like effect at 32 mg/kg in the DRL task, significantly increasing the number of reinforcers and decreasing the number of responses. Naltrexone at 2 mg/kg attenuated ketamine's antidepressant-like effect. Second, ketamine produced an antidepressant-like effect at 10 mg/kg in the TST significantly increasing immobility. Additionally, 2 mg/kg naltrexone successfully blocked ketamine's antidepressant-like effect in the TST. Third, ketamine alone at 10 mg/kg and 32 mg/kg produced hyperactivity in the locomotor test, and 2 mg/kg naltrexone did not produce any significant changes in ketamine's locomotor effects.

The antidepressant-like effects found in the present study with C57BL/6 mice are consistent with previous studies demonstrating that ketamine produces an antidepressant-like

behavior profile - increased reinforcement and decreased response—in the DRL task with Sprague-Dawley rats (Hillhouse & Porter, 2014; Hillhouse et al., 2014). The results of these studies using the DRL task also are consistent with ketamine's antidepressive-like effects in other preclinical animal models like FST, TST, and novelty suppression of feeding (see Browne & Lucki, 2013). However, the present study did not find the antidepressant-like effects of ketamine in the DRL task with the mice in the most reported dose 10mg/kg and 15 mg/kg (Burgdorf., 2013; Garcia et al., 2008; Müller, 2013; Yang et al., 2012; Yang et al., 2013). Instead, a higher dose of 32 mg/kg ketamine was needed to produce an antidepressant-like profile in the DRL task. None the less, the fact that ketamine can still produce an antidepressant-like effect at this higher dose is consistent with previous studies that found ketamine produced acute antidepressant-like effect at 25 mg/kg and 50 mg/kg in the forced swim test (Cruz et al., 2009; Lindholm, 2012). For the present study, the lack of significant antidepressant-like effects of ketamine at lower doses probably reflects differences between the DRL task (which measures changes in a motivated operant response) and the FST and TST tasks (which measure behavioral immobility) (Castagné et al., 2009; Malkesman et al., 2009; O'Donnell et al., 2005). There is also a possibility that this phenomenon can be attributed to a potential floor effect. Note that in all 12 test sessions, the highest average number of earned reinforcers was 7 and the mean vehicle baseline reinforcers was 2.4. The range of this difference and the vehicle baseline are smaller as compared to the previous DRL studies that used rats (Hillhouse & Porter, 2014; Hillhouse et al. 2014). Also, with the relatively small sample size used in the DRL study, this increased the difficulty finding significant differences between groups with a smaller treatment effect size. The floor effect of the DRL task may be because the inter-response interval of 72 seconds is more difficult for mice (in contrast to rat DRL studies in which 72 sec is optimal for screening

antidepressant-like effects of drugs). There is some literature that suggests using a 36 second interval is more appropriate for mice in the DRL task (see Lueptow & O'Donnell, 2011). In future studies, we plan to use a 36 second interval with mice to determine if ketamine can produce an antidepressant-like behavioral profile at a lower dose.

The present study found that naltrexone at 1 mg/kg and 2 mg/kg did not significantly change the mean number or reinforcers in the DRL task. However, 2 mg/kg naltrexone produced a small, but significant decrease in the mean number of responses in the DRL task. Also, the current results are consistent with findings published by Berrocoso et al. (2013), who found that naltrexone alone at 0.5 mg/kg and 2.0 mg/kg, did not produce any antidepressant-like effects in the TST. In contrast, Almatroudi et al. (2015) reported that naltrexone at 1 mg/kg was able to significantly reduce mice's immobility time in the FST, and Robinson et al. (2017) reported that naltrexone at 1.0 mg/kg reduced latency in the Novelty-induced hypophagia test, but failed to increase food consumption in the same test. Thus, our understanding of the opioid system's role in depressive symptoms and antidepressant effects may be influenced by which preclinical assay is studied. While MOR and DOR antagonism is traditionally associated with depressive-like behaviors in humans (Light et al., 2017; Lutz & Kieffer, 2013; Nummenmaa et al., 2020), KOR antagonism has been demonstrated to have an antidepressant-like effect in preclinical studies. Considering that naltrexone can produce antagonism at all three opioid receptors, it is possible that at different dosages, naltrexone alone can produce very different effects on the behavior of animals (which obviously is also task dependent). Further exploration of naltrexone dose ranges needs to be tested in the different preclinical animal assays to provide more definitive information.

Most importantly in the present study, the DRL task demonstrated that at 2 mg/kg, naltrexone could significantly attenuate ketamine's antidepressant-like effects. This result is in agreement with clinical findings from Williams et al. (2018; 2019) that naltrexone attenuates ketamine's antidepressant effect and antisuicidal effects in patients with major depressive disorder. In contrast, the clinical study by Yoon et al. (2019), which found that injectable naltrexone (380 mg) 2-6 days prior to ketamine treatment did not block the therapeutic effects of four weekly 0.5 mg/kg ketamine intravenous treatments in depressed patients who also had alcohol use disorder. The discrepant results between these clinical studies may reflect the large number of methodological differences between them. In the two Williams et al. studies (2018; 2019), a double-blind cross-over design was used with sample sizes of 12 and 16, respectively; whereas, the Yoon et al. (2019) study used an open-label design with only 5 subjects. The Williams et al. studies (2018; 2019) used a single oral administration of 50 mg naltrexone (half-life of 4-13 hours) 45 minutes prior to a single 0.5 mg (i.v.) dose of ketamine. The Yoon et al. (2019) study used an injectable dose of 380 mg of naltrexone (half-life of 5-10 days, slow release into blood for about 30 days) 2-6 days prior to four 0.5 mg (i.v.) doses of ketamine given once weekly. As Heifets et al. (2019) argue, the open-label design in the Yoon et al. (2019) study may have allowed expectancy bias to play a major role in the results of that study, and additionally, more rigorous double-blind studies are needed to resolve these issues. There also are some discrepant results in preclinical animal studies. While Klein et al. (2020) have reported that naltrexone at 1 mg/kg blocked 15 mg/kg ketamine's antidepressant-like effects in the forced swim test, and the progressive ratio test in congenitally learned helplessness Sprague-Dawley rats; in contrast, Zhang and Hashimoto (2019), reported that 10 mg/kg naltrexone failed to block the antidepressant-like effects of 10 mg/kg ketamine in C57BL/6 mice in the forced swim test

after exposed to the chronic social defeat depression paradigm. The reason for the varied results in these preclinical studies is likely once again the result of the disparate methodologies. In the Klein et al. (2020) study with the rats in forced swim test and the current study with mice in DRL and TST, the dosage of naltrexone used was 1 mg/kg and 2 mg/kg. Both studies demonstrated that naltrexone was able to block or attenuate the acute antidepressant-like effects of ketamine. On the other hand, in the preclinical study that demonstrated opposite results, Zhang and Hashimoto (2019) used a much higher dose of naltrexone, 10 mg/kg. It appears, in the clinical studies and the pre-clinical study, naltrexone was only able to block ketamine's antidepressant-like effects at lower doses. Future studies should explore the role of naltrexone dose to gain a better understanding of these discrepancies.

The results of the TST are consistent with previous studies, which demonstrated ketamine alone can produce an acute antidepressant-like effect in the TST assay (da Silva et al., 2010; Pazini et al., 2016; Koike et al., 2011). The results of the TST are also consistent with the present DRL study that found ketamine's antidepressant-like effect in the TST was attenuated by naltrexone at 2 mg/kg and a previous study that reported that naltrexone at 1 mg/kg blocked 15 mg/kg ketamine's antidepressant-like effects in the forced swim test, and the progressive ratio test in congenitally learned helplessness Sprague-Dawley rats (Klein et al., 2020). In addition, as in the DRL study, naltrexone alone at 2 mg/kg did not produce any behavioral effects that were statistically different from the vehicle condition.

In the locomotor activity tests, it was found that both 10 mg/kg and 32 mg/kg ketamine elicited an immediate, but short-lasting hyperactivity effect. The increased locomotor activity that causes by 10 mg/kg and 32 mg/kg ketamine were both reduced to the vehicle groups' locomotor activity before the 30 minute test sessions were over. These results are consistent with

previous findings that demonstrated similar results (Imre et al., 2006; Hetzler & Swain Wautlet, 1985; Wilson et al., 2007). These results also confirmed that the antidepressant-like effects of ketamine in DRL was not a result of any reduction in activity. At the same time, it does raise the possibility that the increase in mobility produced by ketamine in the TST may be partially due to this hyperactivity effect. However, given that the DRL task and the TST have an opposite behavioral profile for antidepressant-like effects (O'Donnell & Seiden, 1983) and that ketamine was able to produce antidepressant-like effects in both assays, the increase in mobility observed in the TST is probably not a false positive result. Moreover, the locomotor activity tests also found that 2 mg/kg naltrexone did not produce any significant locomotor effects when given alone or when combined with the ketamine. These results indicate that naltrexone's attenuation of ketamine's antidepressant-like effects in the DRL task and the TST is not due to any effects of naltrexone on locomotor activity.

In addition to the hyperactivity effect measured by the distance traveled, the locomotor activity study also found that ketamine at both doses increased time spent in the center of the testing chamber by the mice (and thus a decrease in thigmotaxic behavior). A reduction in thigmotactic behavior and corresponding increase in time spent in the center of the test chamber by a drug is typically interpreted as an antianxiety-like effect (see Simon et al., 1994). Anxiolytic symptoms are highly correlated with depressive symptoms (Miller & Massie, 2006; Tiller, 2012) and the comorbidity between depressive disorders and anxiety disorders is as high as 90% (Gorman, 1996). While it is possible that ketamine at 10 mg/kg and 32 mg/kg produced an anti-anxiolytic effect, previous studies only found ketamine's anti-anxiolytic effects at lower doses from 0.625 mg/kg to 2.5 mg/kg in male ICR mice (Zhang et al., 2015) and at 1 mg/kg in female Swiss mice (Fraga et al., 2018), but not higher doses such as 8 mg/kg and 10 mg/kg (Fraga et al.,

2018). At the same time, it is well known that ketamine can produce a strong dissociative or psychotomimetic effect (Domino & Warner, 2010; Green & Krauss, 2000; Wolff & Winstock, 2006). Thus, it could also be argued that the increase in center time (reduction of the thigmotactic behavior) may be because the dissociative effects of ketamine made the mice less aware of their physical environment with regard to the sides and center of the testing area. Future studies should apply the same dosages of ketamine used in the present study to other anxiolytic behavioral assays to exam the nature of this phenomenon.

It has been 20 years since ketamine was found to have rapid and sustained antidepressant effects in patients with major depression disorder and recently, ketamine's S (+) enantiomer, esketamine (Spravato®) was approved as a treatment for treatment-resistant depression in a nasal spray formulation. Yet, the underlying mechanism for ketamine's antidepressant-like effect is still not fully understood (Sanacora & Schatzberg., 2015; Williams & Schatzberg., 2016). Based on the fact that ketamine has its highest binding affinity at NMDA receptors (Mion & Villevieille, 2013), it was first hypothesized that the source of ketamine's antidepressant-like effects are through its noncompetitive NMDA antagonism. However, other NMDA antagonists have failed to produce an antidepressant-like effect (Hillhouse & Porter, 2014; Machado-Vieira et al., 2017; Zarate Jr et al., 2006), which suggests that other mechanisms also may play a role in this effect. The findings by Williams et al. (2018) that naltrexone can attenuate ketamine's antidepressant effects have suggested a possible role for the activation of opioid systems in ketamine's antidepressant effects. Since then, one more clinical study (Williams et al., 2019) and one more preclinical study (Klein et al., 2020) have demonstrated the same effect. In addition, an earlier study demonstrated that the mixed opioid receptor agonist tramadol could amplify ketamine's acute antidepressant-like effects and increase levels of a brain-derived neurotrophic

factor in the hippocampus (Yang et al., 2012) - also providing evidence for the possible involvement of the opioid system in ketamine's antidepressant-like effects. The present study provided additional evidence for the opioid system's role in ketamine's antidepressant-like effect, finding that naltrexone attenuated the antidepressant-like effects of ketamine in two different assays (DRL and TST) in C57BL/6 mice.

Neither the present study nor previous studies, explain exactly how the opioid system plays a mediating role in ketamine's antidepressant-like effects. While based on the face value of these results, it is intuitive to think that these findings indicated that ketamine's antidepressant-like effect is produced at least partially through activation of the opioid system. However, the binding profiles of ketamine and naltrexone suggest otherwise. Naltrexone produces antagonistic actions on all three opioid receptors and has its highest binding affinity at MORs (Raynor et al., 1994). Ketamine, on the other hand, has agonistic actions on the same three opioid receptors, but its highest binding affinity is at KORs, for which has been demonstrated many times that its activation is associated with depressive-like behaviors (e.g., Berrocoso et al., 2009; Lalanne et al., 2014). Since ketamine's agonistic action on KOR is about 1.5-fold more potent than its agonistic action on the MOR (Chartoff & Connery., 2014; Zanos et al., 2018), it is illogical to think ketamine's antidepressant-like effect is derived directly through its MOR agonism. However, ketamine can affect MOR activation through its NMDA antagonistic actions. It is well established that NMDA antagonism has an inhibitory effect on MOR tolerance (Elliott & Inturrisi., 1995; Herman, 1995). A previous study also has found that ketamine can increase the affinity of MOR agonists morphine and fentanyl by blocking the MOR desensitization process, which in turn, increases the duration of MOR agonists' analgesic effect (Gupta et al., 2011). While previous studies have demonstrated that NMDA antagonism alone may not be sufficient

to produce similar antidepressant-like effect (see Hillhouse & Porter, 2014), it is possible that ketamine's antidepressant-like effects are in part the result of a combination of MOR agonism and the MOR sensitization mediated by its NMDA antagonism.

In addition to the possible involvement of the opioid and NMDA systems, ketamine's antidepressant-like effect also has been shown to be possibly associated with the AMPA system (Koike et al., 2011) and alpha 7 nicotinic acetylcholine receptors (Moaddel et al., 2013). Ketamine's pharmacology is very complex, so the etiology of its antidepressive effects also is likely to be very complex. It is possible that all the mechanisms mentioned here either directly or indirectly play a role in ketamine's antidepressant-like effects. Additional studies are needed to further delineate the role of these various molecular targets to determine their involvement in ketamine's antidepressive effects.

Limitations

For the DRL study, the present study did not find a significant antidepressant-like effect of ketamine at lower, more frequently reported doses of 10 mg/kg and 15 mg/kg (Burgdorf, 2013; Garcia et al., 2008; Müller, 2013; Yang et al., 2012; Yang et al., 2013). A shorter inter-response interval (e.g., 32 sec) should be tested in the DRL task to determine if robust antidepressant-like effects of ketamine could be produced with lower doses of ketamine using mice as the subjects. In addition, a previous study has reported that female mice are more sensitive to ketamine's acute and sustained antidepressant-like effects in the forced swim test (Franceschelli et al., 2015). Future studies should determine if the sex differences in the antidepressant-like effects of ketamine can be replicated in the DRL task and if naltrexone's attenuation effect of ketamine's antidepressant-like effects can be replicated in both sexes in the DRL task.

Another limitation in the present study is that 32 mg/kg ketamine was not tested in the TST; hence the TST didn't fully replicate the results of the DRL task. Future studies should examine if ketamine at 32 mg/kg can also produce an increase in mobility in the TST and if the pretreatment of naltrexone can still attenuate such an effect.

Finally, for both DRL and TST, higher doses of naltrexone should be tested. A previous study has reported opposite results using a much higher, 10 mg/kg dose of naltrexone (Zhang and Hashimoto, 2019). In addition, more selective MOR antagonists or rodents with MOR knock-out backgrounds should be used in the future studies to more fully explore the role of MORs in ketamine's antidepressant-like effects.

Conclusion

In conclusion, the present study demonstrated that ketamine's antidepressant-like effects in the DRL task and TST are attenuated by naltrexone at 2 mg/kg. Furthermore, the locomotor test confirmed that ketamine's antidepressant-like effects displayed in DRL and TST were not confounded by changes in locomotor activity. Finally, the present results, along with recent clinical findings, suggest that ketamine's antidepressant effects may involve activation of the opioid system.

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