ORIGINAL ARTICLE

Prolonged blockade of NMDA receptors and positive modulation of α5 $GABA_A$ receptors: no changes in depressive-like behavior, while the former slightly increased emotional reactivity in unstressed rats

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Submitted: 01 April 2025
Revised: 21 July 2025
Accepted: 05 August 2025
Online First: 22 August 2025
Published: 24 September 2025

Check for updates

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Summary

Introduction: Depression is a multifaceted disorder with a limited therapeutic repertoire. A significant breakthrough in depression research has been the discovery of fast-acting antidepressants that target the glutamate/GABA system, namely ketamine and neurosteroids. Positive modulation of $GABA_A$ receptors containing the α5 subunit (α5GABAARs) represents a promising new approach for targeted therapy of depression. The aim of this study was to investigate the effects of repeated administration of ketamine, an NMDA antagonist, and GL-II-73, a positive allosteric modulator at α5GABAARs, on behavioral despair, anxiety, and locomotor activity.

Material and Methods: The experiments were performed on adult male Sprague-Dawley rats. Animals were treated for 7 days with either 6 mg/kg or 10 mg/kg ketamine in an intermittent dosing regimen, 10 mg/kg GL-II-73, or solvent. Following treatment, we performed a battery of behavioral tests consisting of forced swim test (FST), spontaneous locomotor activity (SLA), and novelty suppressed feeding test (NSFT).

Results: No change was detected in any of the treatment groups regarding performance in the FST and NSFT. In SLA, all forms of treatment caused a decrease in the percentage of central time at both 10 and 30 minutes. Central distance was reduced at 10 and 30 minutes only after the 10 mg/kg dose of ketamine.

Conclusion: Ketamine at the higher dose (10 mg/kg) elicited behavioral changes consistent with a slight increase in emotional reactivity, while minor changes of the same quality in the lower-dose ketamine and GL-II-73 groups hardly reflect any anxiety-inducing influence.

Keywords: depression, anxiety, ketamine, α5 $GABA_A$ receptor modulation.

Cite this article as: Djorovic Dj, Ivanovic J, Jezdic K, Sharmin D, Arandjelovic J, M Cook J, M Savic M. Prolonged blockade of NMDA receptors and positive modulation of α5 $GABA_A$ receptors: no changes in depressive-like behavior, while the former slightly increased emotional reactivity in unstressed rats; Medicinska istraživanja 2025; 58(3):163-171; DOI: 10.5937/medi0-57913

INTRODUCTION

Depression is a multifaceted and widespread mental disorder that has a high prevalence worldwide and causes significant personal, social, and economic distress (1). Conventional treatments for depression, such as selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs), are slow-acting and take several weeks to achieve their full therapeutic effect (2). This delayed response, combined with the fact that a significant proportion of patients with major depressive disorder (MDD) experience inadequate symptom relief, highlights the urgent need for alternative therapies, particularly for treatment-resistant depression (TRD) (3). In this context, ketamine, a dissociative anaesthetic traditionally used in surgery, has emerged as a breakthrough intervention (4). Ketamine was initially studied for its anaesthetic properties. It was later found to have a rapid antidepressant effect when administered in subanesthetic doses (5). The mechanism of action is thought to be primarily related to N-methyl-D-aspartate receptor (NMDAR) antagonism, although alternative mechanisms have also been described (6). The beneficial effects are believed to be mediated by the ability to induce neuroplastic changes and improve neuronal connectivity, particularly in brain regions associated with mood regulation, including the prefrontal cortex and hippocampus (7).

Notably, the effects of ketamine on depression are not only rapid but also long-lasting, with some patients experiencing relief from depressive symptoms for up to a week or longer after a single infusion (8). This contrasts sharply with traditional antidepressants, which require prolonged use to elicit therapeutic effects. The potential for ketamine to offer rapid and sustained relief for individuals suffering from TRD has sparked significant interest and research in the field of psychiatric medicine (9). Studies have demonstrated that even in patients with severe, chronic depression who have not benefited from multiple rounds of other treatments, ketamine can produce significant improvements in mood, cognition, and overall functioning (9). In addition, there is evidence that ketamine may be effective in treating a range of depression subtypes, including patients with co-occurring anxiety and suicidality for whom other treatments have failed (10).

Despite its promising effect, the clinical use of ketamine in the treatment of depression is still fraught with problems. The optimal dosage, frequency of administration, and duration of effect are still under investigation (11). In addition, the safety profile of ketamine remains a concern, particularly with regard to its abuse potential, dissociative side effects, and long-term cognitive effects (12,13). The fact that ketamine produces psychedelic-like experiences in some individuals also raises questions about the psychological consequences of its use (14).

On the wave of the discovery of fast-acting antidepressants, innovative $GABA_A$ receptor (GABAAR) modulators have emerged as promising treatments for depression, particularly for patients who do not respond to traditional monoaminergic antidepressants (15). These modulators, such as brexanolone and zuranolone, target neurosteroid-sensitive GABAARs to enhance inhibitory neurotransmission and restore disrupted neural circuits implicated in depression (15). In contrast to conventional benzodiazepines, which predominantly potentiate synaptic GABAARs and thus enhance phasic inhibition, these novel modulators enhance both synaptic and extrasynaptic GABAAR function and thus potentiate phasic and tonic inhibition in parallel (16). Brexanolone, the first FDA-approved drug of this class, has demonstrated rapid antidepressant effects in postpartum depression, while zuranolone, an orally active analog, offers potential advantages in terms of accessibility and chronic use (17). By leveraging the role of GABAAR plasticity in stress-related disorders, these modulators represent a paradigm shift in antidepressant development, providing a mechanistically distinct and faster-acting alternative to traditional treatments (18).

GABAARs containing the α5 subunit (α5GABAARs) have garnered increasing attention in depression research due to their unique anatomical distribution and physiological role (19). Due to the ability of the α5 subunit to bind to key anchoring proteins located both synaptically and extrasynaptically, these receptors may mediate tonic inhibition in a regulated manner, which is critical for modulating network excitability and synaptic plasticity (19). Evidence suggests that augmenting α5GABAAR activity reverses age related neuronal atrophy and cognitive impairment (20). Preclinical studies indicate that selective positive allosteric modulators (PAMs) of α5GABAAR can exert antidepressant and procognitive effects and can reverse stress related neuronal pathologies (21-24). While there is open debate in the field about the apparent contradiction that ketamine and α5GABAAR PAMs induce antidepressant effects through differentially opposing mechanisms (15,25,26), a recent report indicating that time-dependent modulation of GABAergic activity is required for the sustained antidepressant-like responses induced by ketamine has shed new light on this topic (27).

Repeated administration of ketamine has been suggested to be more efficacious in inducing lasting antidepressant effects (28,29). However, ketamine use has been documented to cause structural brain changes (cortical atrophy) after chronic administration in drug abusers (30). Also, NMDAR antagonists such as phencyclidine and ketamine transiently induce symptoms of acute schizophrenia, and they have been reliably used to model schizophrenia features in rodents (31). Preclinical studies have drawn attention to the negative effects of chronic ketamine administration in rats, highlighting complex neurophysiological changes arising from the treatment (32). Having these facts in mind, it has been suggested that an intermittent dosing regimen of ketamine might

be a preferred treatment option in a protocol aimed to assess its antidepressant-like effects (32).

In this paper, we aimed to compare the behavioral effects of intermittent dosing of ketamine and repeated administration of GL-II-73, a widely used α5GABAAR PAM (21,23,24) in the domains of behavioral despair, anxiety, and locomotor activity in unstressed male rats.

MATERIAL AND METHODS

Animals

Male Sprague-Dawley rats (n=40), descended from a colony purchased from Charles River, Italy, were born and reared in the vivarium of the Faculty of Pharmacy, University of Belgrade, Serbia. The vivarium environment was maintained at a temperature of 22 ± 2°C and a relative humidity of 40-70%. The animals received food and water ad libitum and were exposed to a 12/12-hour day/night light cycle, with the light being switched on at 06:00. The research was conducted in accordance with European Union guidelines (Directive 2010/63/EU). It was approved by the Ethics Committee for Animal Experiments of the University of Belgrade – Faculty of Pharmacy, Serbia and the Ministry of Agriculture, Forestry and Water Management – Veterinary Directorate (323-07-10046/2020-05, 23/09/2020).

Drug treatment

In this study, four groups of 10-12-week-old animals weighing approximately 300-350 g were subjected to different treatments administered by intraperitoneal injection. The first group received the solvent (SOL) consisting of 14% (w/v) propylene glycol and 1% (w/v) polysorbate 80 dissolved in ultrapure water, which served as a control. The second and third groups were administered ketamine hydrochloride (Ketamidor, Richter Pharma AG, Vienna, Austria) at a dosage of 6 mg/kg and 10 mg/kg, respectively, with the ketamine diluted in physiological saline to achieve the desired dosage. The fourth group received GL-II-73, the imidazobenzodiazepine derivative synthesized by the research group of Dr. James M. Cook (Department of Chemistry and Biochemistry, University of Wisconsin-Milwaukee, Milwaukee, WI, USA). The GL-II-73 solution was prepared in SOL at a concentration of 2 mg/ml, which corresponds to a final dose of 10 mg/kg. GL-II-73 is a PAM at the benzodiazepine binding site that is selective for α5GABAARs (24).

Experimental design

Before starting the protocol (day -1), the animals were placed individually in a cage and a 15-minute training session for the forced swim test was performed on this day. The treatment was applied for 7 days (0 - 6 days of the protocol). The control group received SOL i.p. daily, the Ket 6 and Ket 10 groups received ketamine i.p. on 0, 2, 4 and 6 days while receiving SOL i.p. on the other days, and the GL-II-73 group received GL-II-73 i.p. on all 7 days. This was followed by a series of behavioral tests: forced swim test (day 7), spontaneous locomotor activity (day 8), novelty suppressed feeding test and home-cage feeding (day 9). The experimental design is shown in **Figure 1**.

Behavioral testing

During the spontaneous locomotor activity (SLA) test, each experimental animal was allowed a 30-minute exploratory period in an open arena of specific dimensions (60 cm × 50 cm × 35 cm) illuminated with indirect bright light (60 lx). The animals were gently placed in the arena and their behavior was monitored using AnyMaze software version 6.35 (www.any-maze.com). The following parameters were tracked: the percentage of time the animals spent in the central zone during the 30-minute duration of the SLA test, as well as during the 10-minute exploration period; the total distance travelled and the percentage of active time during both the 30-minute and 10-minute durations of the SLA test (33).

The forced swim test (FST) was performed as previously described for rats. Each rat was pre-swum for 15 minutes in a Plexiglas cylinder (30 cm diameter, 65 cm height) filled with water (25 ± 1°C, 45 cm depth). After 8 days, each rat was again placed in the swimming cylinder for 10 minutes and videotaped. The data were analyzed in a blinded manner, and the total duration of immobility during the entire 10-minute swimming period was assessed. The duration of immobility was assessed between

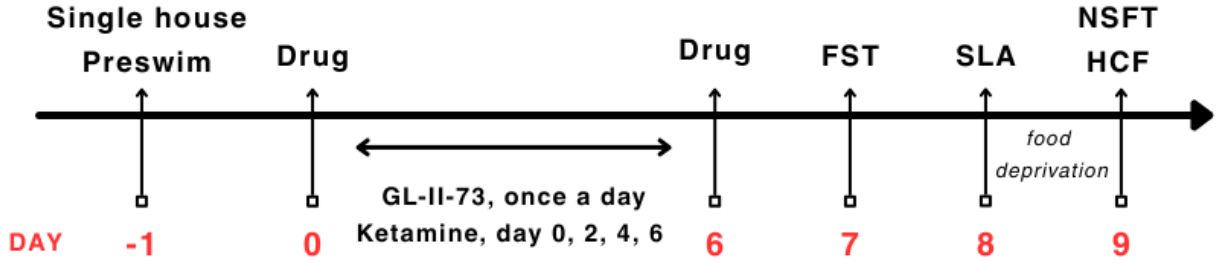

Figure 1. The schedule for conducting the experiment and the accompanying behavioral tests

2 and 6 minutes by an experimenter who was blind to the treatment groups. The parameters recorded included the time the animals spent in various activities during the 2-6 min period of the test: swimming, immobility, and struggling (34).

In the novelty suppressed feeding test (NSFT), the animals were deprived of food overnight and then placed in an arena with an open field (100 cm × 100 cm × 35 cm) and a small amount of food in the center. The latency time to the onset of food intake was recorded, with a maximum cut-off time of 15 minutes (34).

Following the NSFT, home-cage feeding (HCF) was assessed during a 10-minute period to determine the animals' motivation to eat (34).

Animal behavior was recorded with a camera and analyzed with AnyMaze software (Stoelting Co., Wood Dale, IL, USA).

Statistical analysis

Data were analyzed by Kruskal–Wallis test and Mann–Whitney test (GraphPad Prism software version 10.1.0; GraphPad Software, Inc., La Jolla, CA) with the Bonferroni-Holm correction for post hoc comparison (MultipleTesting.com) (35). A value of $p < 0.05$ was considered statistically significant. A technical outlier was observed in the SLA data due to a mistracking error in the AniMaze system.

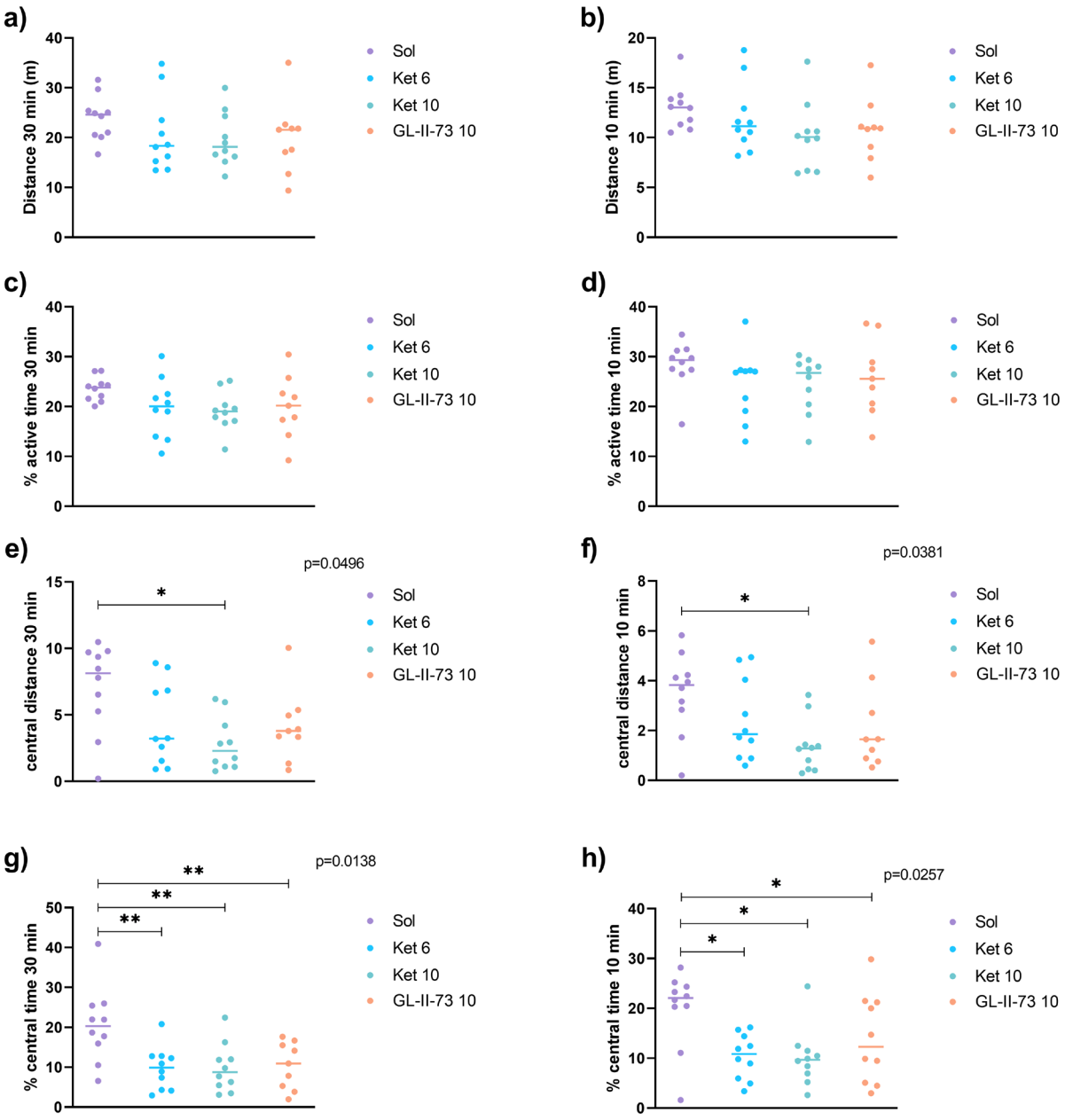

Figure 2. Assessment of locomotor activity and anxiety in male Sprague-Dawley rats in the spontaneous locomotor activity test. The total distance travelled in 30 minutes (a) and in 10 minutes (b), the percentage of active time in 30 minutes (c) and in 10 minutes (d), the central distance travelled in 30 minutes (e) and in 10 minutes (f), the percentage of time spent in the central zone in 30 minutes (g) and in 10 minutes (h) are shown 24 hours after the last application. Statistical significance is indicated in the graphs with * for $0.01 < p < 0.05$, ** for $0.001 < p < 0.01$.

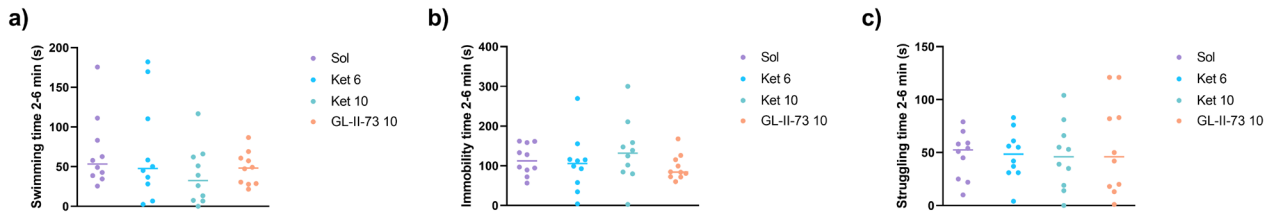

Figure 3. Results of the forced swim test. The data presented show the time the animals spent on different activities during the 2 and 6 minutes of the test: swimming (a), immobility (b) and struggling (c).

RESULTS

Spontaneous locomotor activity (SLA) test

The total distance travelled and the percentage of active time in 30 minutes **(Figure 2a, 2c)** as well as the total distance travelled and the percentage of active time in 10 minutes in the SLA test **(Figure 2b, 2d)** did not differ significantly in any of the groups.

A significant decrease in the central distance parameter was observed during both the 30 minutes **(Figure 2e)** and the 10 minutes **(Figure 2f)** of the test in the Ket 10 group compared to the control group of animals (p=0.007, respectively).

A significant reduction in the percentage of time the animals spent in the central zone during the 30-minute duration of the SLA test **(Figure 2g)** was observed in all three treatment groups, Ket 6, Ket 10, and GL-II-73 (p=0.007; p=0.009; p=0.008, respectively) compared to the control group. In addition, a significant decrease in the percentage of time the animals spent in the central zone during the 10-minute duration of the SLA test was observed in the three treatment groups (p=0.007; p=0.018; p=0.022, respectively) compared to the control group **(Figure 2h)**.

Forced swim test (FST)

There is no significant difference in any of the groups in swimming **(Figure 3a)**, immobility **(Figure 3b)** and struggling **(Figure 3c)** in FST.

Novelty suppressed feeding test (NSFT) and home-cage feeding (HCF)

There is no significant difference in any of the groups in the latency time to food intake in NSFT **(Figure 4a)** and food intake in HCF **(Figure 4b)**.

DISCUSSION

Behavioral studies that ensued 24 hours after the last administration of ketamine at a dose of 6 mg/kg or 10 mg/kg or GL-II-73 at a dose of 10 mg/kg showed that none of the treatments significantly decreased immobility time in the FST or latency to food intake in the NSFT, which were the previously demonstrated effects of 10 mg/kg ketamine (34). Analysis of SLA behavior showed that activity in the central parts of the chamber seemed to be differentially reduced in animals previously subjected to the treatments studied. Ketamine at doses of 6 and 10 mg/kg, as well as 10 mg/kg GL-II-7 decreased the percentage of central time at both 10 and 30 minutes. The central distance was reduced at 10 and 30 minutes only after the 10 mg/kg dose of ketamine.

When manifested in the central parts of an activity chamber, the decrease in locomotor activity may reflect an increase in emotional reactivity, whereas when manifested in the peripheral parts of a cage, such locomotor changes may rather reflect a vague "lack of well-being" (36) or a decrease in exploratory drive in general (cf. 37). On the other hand, the emotional reactivity (related to neophobia) disappears once the animal has become ha-

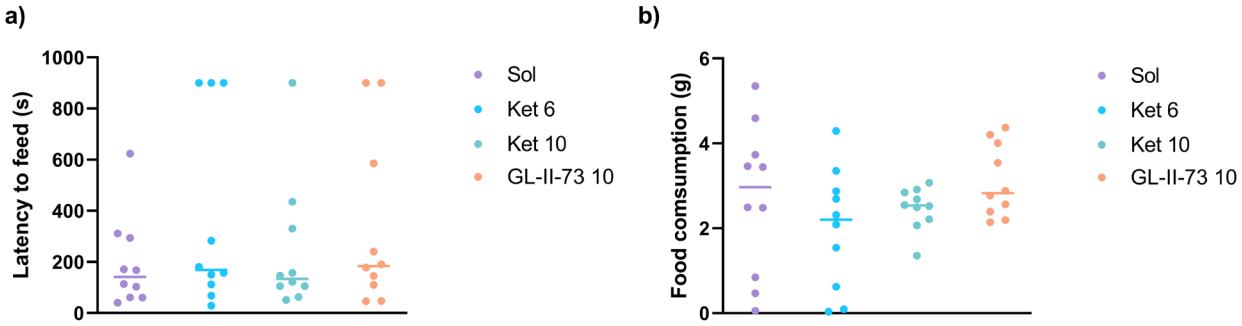

Figure 4. Results of a novelty suppressed feeding test and home-cage feeding. The data represented the time it took an animal to start eating (a) and the amount of food it consumed within 10 minutes in its home cage (b).

bituated to the enclosure (for about 10 minutes), and the behavior presented afterwards may rather reflect a general, spontaneous locomotor activity (38). With this in mind, there is a need to differently interpret increasing changes in SLA behavior, starting at 6 mg/kg ketamine and 10 mg/kg GL-II-73 with minor changes that hardly reflect any anxiety-inducing influence, up to ketamine at a dose of 10 mg/kg, at which a slight but consistent increase in emotional reactivity was revealed.

Our study was conducted in unstressed male rats with an intermittent administration regimen of ketamine. Wide variations in the effects of ketamine have been reported, related to dosage, treatment regimen, stress status, species, strain and sex of the animals, and time interval from the last dose (see 39,40). The stress-related dichotomy in the effects of ketamine appears to be particularly striking. It has been suggested that ketamine more reliably produces unwanted effects in non-stressed animals, whereas it has a beneficial effect in stressed animals (41). Fitzgerald *et al*. have shown that ketamine administered i.p. to C57BL/6J mice at two different doses (10 mg/kg and 30 mg/kg) produced depression-like behavior (decreased swimming and increased immobility) in the forced swim test in non-stressed rats 24 hours after injection, while the higher dose had the opposite effect in stressed rats, correcting the behavioral impairment caused by chronic, unpredictable stress (42). In another experiment, the same group showed no effect of 30 mg/kg i.p. ketamine in the forced swim test 72 hours after injection (43).

Similar phenomenon has been observed in humans. Namely, ketamine tends to exert prodepressive action in healthy volunteers, and the opposite in patients with major depressive disorder. This dichotomous effect has been documented in a series of randomized double-blind placebo-controlled studies led by the group of Carlos A. Zarate Jr. (44-47). They have shown that ketamine in healthy control subjects induced symptoms of anxiety, emotional blunting and anhedonia. This effect was related to increased gamma oscillations in multiple nodes related to the depression network, where presumably ketamine affected homeostatic plasticity in these regions, which resulted in altered homeostatic balance, and the effects were conditioned on the baseline functional state in the network (44). The authors also concluded that it differentially affected connectivity within the fronto-striatal network, which includes regions of the prefrontal cortex and striatum and is involved in cognition and affective processing (45). This contrasts with a previous report that postulated a global shift in connectivity (48). They also determined that the same effect of ketamine applies to functional connectivity during emotional processing tasks in healthy controls vs depressed patients (46,47).

It is important to note that there are studies on rats and mice that show behavioral changes in both directions and no change after ketamine injection in unstressed animals of the same strain (39,40). This variability is a recognized issue in the literature, and there are attempts to reach an explanation which connect it to different vendors for animal procurement, handling of experimental animals or even the sex of the experimenter (49).

Ketamine has been shown to induce anxiety in rats (50,51). Certain behaviours are sensitive to cumulative ketamine dose, suggesting a possible inverted U-shaped relationship (52). This aspect has not yet been adequately explored in ketamine research. Interestingly, there is an inverted U-shaped relationship between glutamate concentration and genetic excitation/inhibition (E/I) levels on the one hand and the temporal binding window (determinant of schizotypy) on the other, where both low and high E/I levels were associated with schizotypy and only optimal E/I levels contributed to favourable functioning (53). Intriguingly, this variability was shown in a cohort of healthy individuals, highlighting the importance of within-group variation that is overlooked in classical approaches.

Salome *et al*. compared the differences in cFOS expression after open field (OF) and open arm (OA) exposure in two lines of Wistar rats that were selectively bred for either high (HAB) or low (LAB) anxiety related behavior (54). They concluded that HAB rats had significantly higher cFOS expression in the medial and lateral hypothalamus and lower expression in the medial prefrontal cortex (mPFC) in response to both OF and OA exposure. These results indicate that the anxious behavior after ketamine injection in our experiment might be related to an mPFC mechanism.

Compelling line of evidence suggests that the influence of ketamine on interneurons (predominantly somatostatin (SST), or parvalbumin (PV) positive cells) is key for its function (55). α5GABAAR potentiation in our behavioral experiments mirrors, to a small extent, the proanxiety effects of ketamine. α5GABAARs are expressed in the prefrontal cortex on dendrites of pyramidal neurons innervated by SST+ interneurons and are key effectors of dendritic inhibition (56), and modulation of dendritic inhibition is an important target of ketamine (57). Very recent evidence has emerged that although ketamine initially decreases GABAergic function, contrary to traditional views it later increases GABAergic function, which is key for its sustained behavioral effects (27). It has also been shown that ketamine potentiates the function of extrasynaptic GABAARs (58,59). A single 10 mg/kg GL-II-73 dose can induce anxiolytic response in the elevated plus maze in unstressed mice (21). Also, a previous report from our group demonstrated a dose dependent bimodal influence of a different α5GABAAR PAM on various forms of memory, suggesting that α5GABAAR potentiation, similar to ketamine displays a very sensitive range for producing optimal effects (60). Additional studies are necessary to parse out the precise influence of α5GABAAR potentiation on anxiety. Opto-

genetic activation of excitatory neurons in the orbitofrontal cortex (OFC) induced increased anxiety, while activation of inhibitory neurons had an opposite effect (61). Also, fiber photometry of OFC neurons demonstrated lower activity of excitatory neurons during exposure to various anxiogenic stimuli including the center of the OF arena (61).

CONCLUSION

It is assumed that the mechanisms of ketamine and GABAAR modulators overlap to a certain extent. Intermittent administration of ketamine and prolonged α5GABAAR potentiation produced similar behavioral effects in rats. These results add to the growing literature suggesting that agents targeting the glutamate/GABA system may not appear to have beneficial effects in non-stressed rats. Finally, these results support the postulate that emotional reactivity has a sensitive relationship with the E/I state.

Acknowledgment: N. A.

Funding information: This research was funded by the Science Fund of the Republic of Serbia, Grant No. 7749108, Neuroimmune aspects of mood, anxiety and cognitive effects of leads/drug candidates acting at GABAA and/or sigma-2 receptors: In vitro/in vivo delineation by nano- and hiPSCbased platform – NanoCellEmoCog. We would also like to acknowledge the National Institutes of Health, USA (2R01 DA043204-06A1 (NIDA), R01 AA029023 (NIAAA) and R01 DA054177 (NIDA) subaward to JMC).

Conflict of interest: J.M.C. and M.M.S. are listed inventors on patents covering the synthesis and use of the compound.

Author contributions: The conception or design of the work, M.M.S.; The acquisition, analysis, or interpretation of data: all authors; Preparing the draft of the manuscript or interpretation of revised version of manuscript, Dj.Dj., J.I., K.J., J.A., M.M.S.; All authors have read and agreed to the submitted version of the manuscript.

Ethical approval: The research was conducted in accordance with European Union guidelines (Directive 2010/63/EU). It was approved by the Ethics Committee for Animal Experiments of the University of Belgrade – Faculty of Pharmacy, Serbia and the Ministry of Agriculture, Forestry and Water Management – Veterinary Directorate (323-07-10046/2020-05, 23/09/2020).

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DUGOTRAJNA BLOKADA NMDA RECEPTORA I POZITIVNA MODULACIJA α5 $GABA_A$ RECEPTORA KOD NESTRESIRANIH PACOVA: ODSUSTVO UTICAJA NA DEPRESIVNO PONAŠANJE UZ BLAGO POVEĆANJE EMOCIONALNE REAKTIVNOSTI POD UTICAJEM PRETHODNE

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Sažetak

Uvod: Depresija predstavlja tešku psihijatrijsku bolest sa ograničenim terapijskim repertoarom. Značajan prodor u istraživanju depresije predstavlja otkriće brzodelujućih antidepresiva, poput ketamina i neurosteroida. Pozitivna modulacija $GABA_A$ receptora koji sadrže α5 subjedinicu (α5GABAAR) predstavlja obećavajući novi pristup u ciljanoj terapiji depresije. Cilj ove studije bio je da se ispita dejstvo ponavljanog davanja ketamina, antagoniste NMDA receptora i GL-II-73, pozitivnog alosternog modulatora α5GABAAR na depresivno i anksiozno ponašanje, kao i lokomotornu aktivnost.

Materijal i metode: U eksperimentima su korišćeni adultni mužjaci *Sprague-Dawley* pacova. Životinje su izlagane sedmodnevnom tretmanu ketaminom svakog drugog dana u dozi od 6 mg/kg ili 10 mg/kg, dnevnoj primeni 10 mg/kg GL-II-73 ili rastvarača. Nakon tretmana sprovedena je baterija bihejvioralnih testova koja se sastojala od testa forsiranog plivanja (engl. forced swimm test-FST), spontane lokomotorne aktivnosti (engl. spontaneous locomotor activity - SLA) i testa supresije hranjenja (engl. novelty suppressed feeding test - NSFT).

Rezutati: Nijedan vid tretmana nije ostvario uticaj na ponašanje u FST i NSFT. U SLA, svi vidovi tretmana uzrokovali su smanjenje procenta vremena u centralnom delu prostora tokom 10 i 30 min praćenja.. Pređena distanca u centralnom delu prostora bila je smanjena samo kod 10 mg/kg ketamina tokom 10 i 30 min.

Zaključak: Ketamin u većoj dozi (10 mg/kg) prouzrokovao je promene ponašanja koje odgovaraju blago povećanoj emocionalnoj reaktivnosti, dok promene istog kvaliteta uočene nakon primene manje doze ketamina i u GL-II-73 grupi ne oličavaju jasan proanksiozni uticaj.

Ključne reči: depresija, anksioznost, ketamin, α5 $GABA_A$ receptorska modulacija

Primljen: 01.04.2025. | **Revidiran:** 21.07.2025. | **Prihvaćen:** 05.08.2025. | **Online First:** 22.08.2025. | **Objavljen:** 24.09.2025.

Medicinska istraživanja 2025; 58(3):163-171