

A rodent model of schizophrenia reveals increase in creatine kinase activity with associated behavior changes

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Schizophrenia is a debilitating mental disorder characterized by positive (delusions, hallucinations, disorganized speech) and negative (affective flattening, avolition and social withdrawal) symptoms as well as cognitive deficits. The frequency, severity and topography characterize the disorder as heterogeneous, the pathophysiology of schizophrenia is poorly understood. Sub-anesthetic doses of ketamine produce hyperactivity, stereotypy and abnormal social interaction and it is used as a model of schizophrenia. In this study, we induced an animal model by acute sub-anesthetic doses of ketamine and tested different behavioral parameters. We also evaluated the activity of creatine kinase (CK) in brain of rats treated with ketamine. Our results demonstrated that administration of 10, 25 and 50 mg/kg of ketamine induced an increase of covered distance in habituated and non-habituated rats to the behavioral apparatus. Ketamine administration induced significant social deficits and stereotypic behavioral in all doses tested. Finally we evaluated the effect of different doses of ketamine on creatinine kinase (CK) activity and we observed that CK activity is increased in specific regions of the brain. Our study suggests that our animal model may be used as a model of schizophrenia and that cerebral energy metabolism might be altered in the brain of schizophrenic patients, probably leading to alterations that might be involved in the pathogenesis of schizophrenia.

Introduction

Schizophrenia is a severe psychiatric disorder, it carries a lifetime risk of around 0.5–1% and its early onset and tendency to chronicity mean that its prevalence is relatively high. The social and economic impact of this illness is enormous and its impact on sufferers and their families can be devastating.¹ It is a complex disorder with a poorly defined cause. Pathophysiological findings in schizophrenia include an enlarged ventricular system accompanied by an overall reduction in brain volume, with regional decreases in the hippocampus, thalamus and frontal lobes. Neuroimaging and *post mortem* analyses have demonstrated subtle, but distinct, abnormalities in the schizophrenic brain particularly in the hippocampus and neocortex.² The classical symptoms of schizophrenia include positive symptoms, such as delusions and hallucinations and negative symptoms, such as blunted affect and social withdrawals, but aspects as awareness, orientation, perception, cognitive impairments, affect, thought and language and neurological signs are also important findings in this disease.³

In order to understand the pathologic mechanisms found in this disease, animal models have been used. These models are animals prepared that attempt to mimic a human condition of the psychopathology associated with the group of schizophrenia disorders.⁴ Many models have already been described including behavioral models, psychotropic drug-induced locomotor hyperactivity and prepulse inhibition.^{5–8} An animal model using ketamine is widely accepted; it is a dissociative anesthetic, affecting glutamatergic activity via blockade of the N-methyl-D-aspartate (NMDA) receptor.⁹ It is already known that ketamine causes individual psychoses in remitted schizophrenic patients and the use of this substance is also used to assess positive and negative symptoms of schizophrenia in healthy volunteers.⁸ These authors also reported that sub-chronically ketamine-treated animals give evidence that ketamine is a useful animal model of schizophrenia. Moreover, Geyer et al.⁴ described in their review that in rats and monkeys, noncompetitive NMDA antagonists, including PCP and ketamine, produce a range of behavioral abnormalities that have important relationships to schizophrenic symptomatology.

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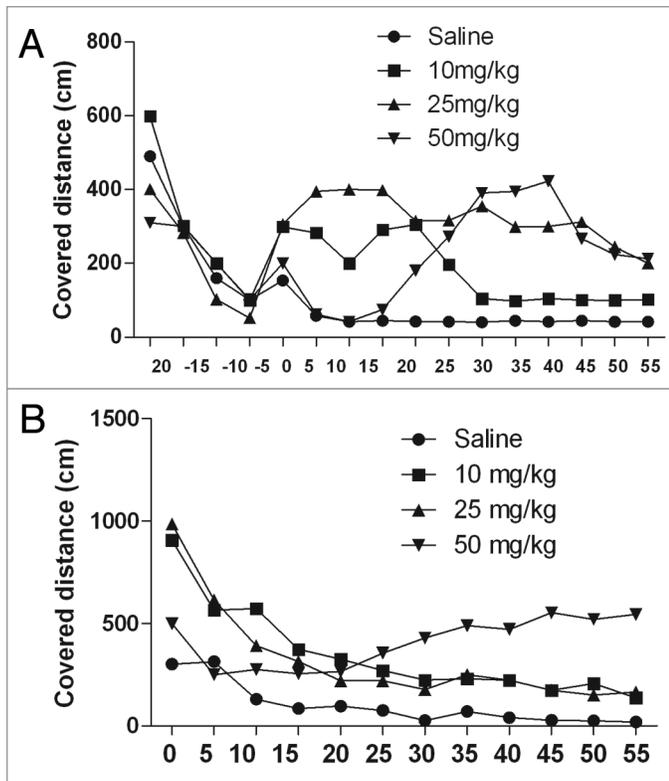


Figure 1. The open-field apparatus was used to assess locomotor activity. Covered distance was measured in open-field test during 60 minutes after acute administration of ketamine (control, 10, 25 and 50 mg/kg). We observed animals which had 20 minutes habituated in the apparatus (A) and animals that had no this habituated time (B). The distance was analyzed in 5 minute-blocks for 60 minutes. Control group is treated with saline 0.9%. Data were analyzed by ANOVA followed by Tuckey test. *A value of $p < 0.05$ was considered significant and different from control. Results were expressed as centimeters (cm).

Disturbs found in brain energy metabolism are important factors that contribute to neuronal damage in patients with schizophrenia.¹⁰⁻¹⁵ Creatine kinase (CK) is an important enzyme that participates of an important system that maintains the energy homeostasis, this regulation of cell homeostasis is done by isoforms mitochondrial (mtCK) and cytosolic (ctCK) and any alteration in the circuit of creatine-phosphocreatine can be an important step for the neurodegenerative process that leads to the loss neuronal in the brain.^{16,17}

Mitochondrial dysfunction with subsequent alteration in respiratory chain complexes is implied in the pathophysiology of schizophrenia.^{18,19} Once the CKmit enzyme acts as ATP energetic reserve, it can acquire a modulatory role to suppress cerebral energy demand.²⁰ This cerebral demand is altered in schizophrenia, as well as in other psychiatric disorders.^{21,22}

Considering that schizophrenia is a complex psychiatric disorder, we induced an animal model by chronic sub-anesthetic doses of ketamine and test different parameters of behavior rat tasks. It is important to provide new information on the mechanisms involved in the symptoms and development of the disease. Therefore, we also evaluated the effects of sub-anesthetic doses of

ketamine on creatine kinase (CK) activity, an enzyme responsible for energy buffering in tissues that use highly rates of energy such as the brain, in prefrontal cortex, hippocampus, cerebellum, striatum and cerebral cortex of animals.

Results

In this study, we verify the effect of different doses of ketamine in 60 days-old rats in order to evaluate behavioral parameters in an animal model of schizophrenia. **Figure 1** shows that habituated rats (A) have a different behavior when compared to non-habituated groups (B). We observed that the administration of 25 mg/kg ketamine induced an increase of covered distance in non-habituated group. We also observed an enhanced covered distance in habituated group in all doses tested [$F_{(7,91)} = 6.63$; $p < 0.01$]. An increase of rearings at 10 mg/kg ketamine was observed, either in the habituated or non-habituated groups, when compared to control [$F_{(7,92)} = 4.63$; $p < 0.01$] (data not shown). In **Figure 2** we compared these data to the accumulated covered distance of both groups in all doses tested.

Ketamine administration induced significant social deficits. There was no significant difference in the latency to start the interaction [$F_{(3,34)} = 0.203$; $p = 0.87$] (**Fig. 3A**). However, comparisons revealed significant differences among groups in number of contacts in 50 mg/kg [$F_{(3,34)} = 10.14$; $p < 0.01$] (**Fig. 3B**) and the amount of time spent engaged in social interaction in all doses tested [$F_{(3,34)} = 25.81$; $p < 0.01$] (**Fig. 3C**).

Next, we examined the stereotypy induced by the administration of ketamine. We showed that all the doses induced stereotypic movements in habituated and non-habituated rats (**Fig. 4**) [$F_{(7,89)} = 12.630$; $p < 0.01$].

Finally, it was evaluated the effect of different doses of ketamine on CK activity (**Fig. 5**). We observed an increase of CK activity caused by the administration of 25 and 50 mg/kg of ketamine all structures analyzed but hippocampus. In this structure, we observed an increase only when 50 mg/Kg of ketamine was administrated.

Discussion

Schizophrenia is a polygenic disorder and the involvement of factors, such as environment and development, complicates the study of the underlying mechanisms. However, there are some characteristic manifestations that mark this disease. The positive symptoms (delusions, hallucinations and disorganization) as well as the negative symptoms (loss of motivation and emotional vibrancy) and also disturbance in cognitive functions and memory are observed in schizophrenic patients.²³

Ketamine, amphetamine or dopaminergic psycho stimulants have faced validity for the stereotyped behavior induced in humans.⁴ In contrast to amphetamine, which mimics only the positive symptoms of the disease, subanesthetic doses of the NMDA antagonist ketamine have been reported to produce positive and negative symptoms and cognitive impairments consistent with those seen in schizophrenia. These models measure locomotor hyperactivity and have been used extensively to

characterize the effects of both amphetamine and NMDA in the animals.²⁴

It is already known that ketamine is an appropriate drug to induce psychotic behavior; however, there is not a consensus in literature that defines an adequate dose. There are evidences showing that ketamine in very low doses, such as 5, 10 and 15 mg/kg, could be an anti-depressive agent.^{25,26} To induce locomotor activity and cellular dysfunction it ranges from 10 to 50 mg/Kg^{7,27,28} and, in higher doses, ketamine is a potent anesthetic drug.²⁹

Previous studies from our group have also showed that acute administration of ketamine (15 mg/kg) reversed the inhibition of mitochondrial respiratory chain in cerebral cortex and cerebellum, in animals submitted to mild chronic stress.²⁶ This study is in agreement with other lines of evidence that affirmed that ketamine promotes a protective effect on cellular energy, maintained glucose metabolism and ATP production.³⁰ On the other hand, Rudin et al.³¹ showed that some sub-anesthetic doses of ketamine, ranging from 10 to 40 mg/kg, caused apoptosis, increasing the number of damaged neuron in neonatal mice, confirmed by DNA fragmentation, in cortex and cerebellum.

In the present study, our results showed that ketamine induces hyper-locomotor activity in rats subjected to sub-anesthetic doses. We tested the animals in a 20 minutes habituation period or without habituation. Our results showed differences between doses and habituation and they also demonstrated a hyperlocomotion of these animals when subjected to ketamine. We observed a hypolocomotion in 50 mg/kg in the first minutes of the analyses and we attributed this to a pre-anesthetic stage of ketamine.

We attributed the effect of habituation an important fact in the hyperlocomotion since we observed that the anxiety may be involved and interfere in the behavioral of the animals. A recent study showed that repeated use of ketamine in weekly intervals may result in neural sensibilization, especially when associated to diverse environmental conditions.⁶ Irufune et al.³² demonstrated that ketamine-induced hyperlocomotion is inhibited by the administration benzodiazepinic drugs, once it suppresses dopaminergic neuronal activation in *nucleum accubens* and striatum.

We also analyzed the social interaction (evident parameter observed in schizophrenic patients) between animals treated and non-treated with ketamine. The study showed that the time spent in social interaction was altered when compared to control groups in all doses tested. In this context, our rats presented an anti-social behavioral, corroborating with the symptoms found in schizophrenia.

Stereotyped behaviors are features of psychiatric disorders such as schizophrenia and obsessive-compulsive disorder,³³ and it is broadly defined, including a number of types of abnormal movements. We demonstrated that the habituated and non-habituated animals in all ketamine doses tested showed stereotypic movements when compared to controls. Together, our chronic model of administration of ketamine showed an alteration in the behavioral of rats that are in agreement with schizophrenic symptoms.

Although behavioral alterations are important parameters to understand the symptoms found in schizophrenic patients, there

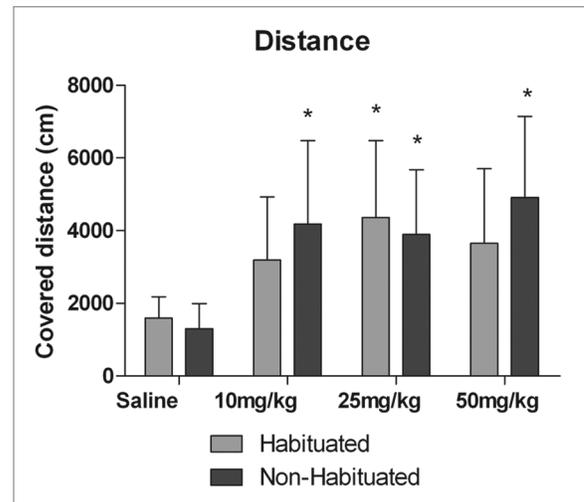


Figure 2. The open-field apparatus was used to assess locomotor activity and accumulated covered distance was measured in habituated and non-habituated rats to the open-field apparatus. The distance was analyzed by 60 minutes. Data were analyzed by ANOVA followed by Tuckey test. *A value of $p < 0.05$ was considered significant and different from control. Results were expressed as centimeters (cm).

are some evidence showing relevant biochemical and physiological abnormalities, including neuronal disorganization of the prefrontal cortex³⁴ and a reduction in cortical volume.³⁵ Alterations in γ -aminobutyric acid (GABA) neurons as well as dopaminergic³⁶ and glutamatergic alterations are also fundamental in the disease.³⁷

Habituation is one of the simplest ways to implicit learning and it is altered in schizophrenic patients, whom are capable to execute the routine tasks in a family place, but are not able to perform the same tasks in a different place.³⁸ A recent research showed that the subchronic treatment with sub-anesthetic doses of ketamine induces schizophrenia-related alterations.⁷ It has been also described cognitive and functional deficit such as alteration of declarative memory and surveillance.³⁹

CK activity is an important energy reserve in tissues that need a high demand of energy. Some studies related a hypometabolic activity in prefrontal cortex⁴⁰ and alterations in dopaminergic, cholinergic and serotonergic neurotransmission.⁴¹ We can observe in this study that the CK activity is increased in all brain structures tested, suggesting that 25 and 50 mg/kg may cause the increase of the energy demand to supply the necessity of ATP. Our study are in agreement with Littlewood et al.²⁷ which demonstrated evidences that determine the temporal and spatial neuronal activation profile of ketamine in the rat brain by indentifying changes in blood-oxygenation-level-dependent (BOLD) in different cerebral structures, using sub-anesthetic doses of 10 and 25 mg/kg. Both doses revealed specific activations in prefrontal cortex, hippocampus and limbic areas, after the injection of sub-anesthetic dose of 25 mg/kg of ketamine. In this context, Oliveira et al.⁴² observed that sub-anesthetic doses of ketamine at 4, 10 and 30 mg/kg, indicating an increase of lipid peroxidation, oxidative protein damage and decrease of enzymatic antioxidant defense. In this context,

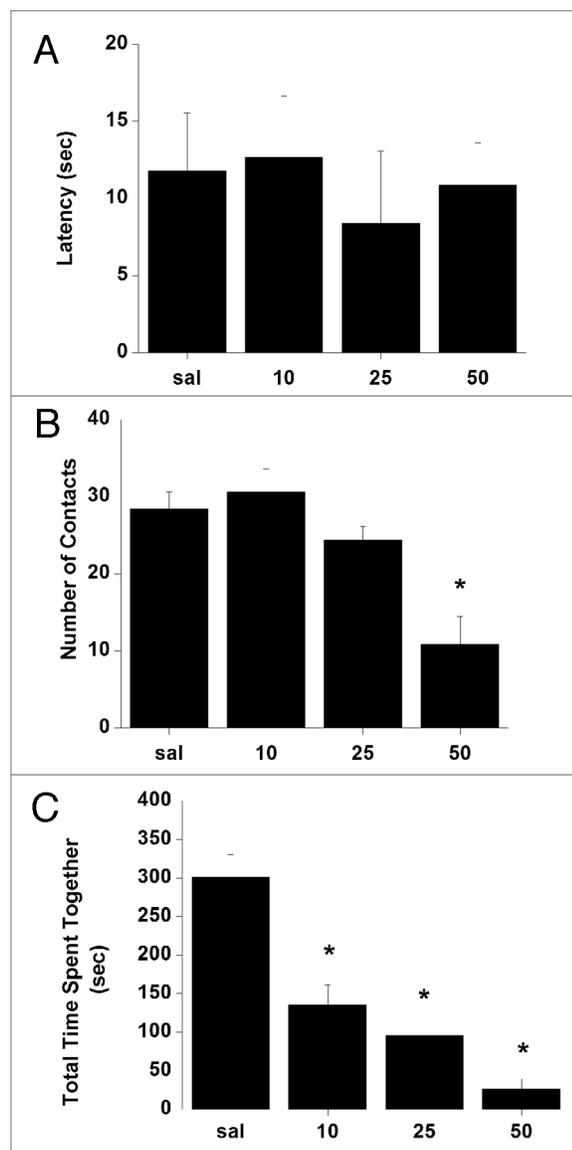


Figure 3. Impaired social behavioral is observed in ketamine treated animals. Social interaction was induced by different doses of ketamine and tested in unfamiliar conditions in an open field apparatus. Parameters analyzed were (A) Latency to start interacting; (B) number of contacts; (C) time spent engaged in social interaction 15 minutes after injection. Data were analyzed by one-way analysis of variance followed by Tukey test when F was significant. Values are expressed as mean \pm S.D., for ten animals in each group. *Different from control; $p < 0.01$.

other neurological disorders were already related with oxidative stress.^{43,44}

There are evidences relating hippocampal dysfunction in schizophrenia.⁴⁵⁻⁴⁸ Burbaeva et al.⁴⁹ observed decreased in CK-BB immunoreactivity levels in hippocampus of schizophrenic patients. Our results also showed a significant increase in the CK activity caused by the administration of sub-anesthetic dose of 50 mg/kg in habituated animals, suggesting a higher ATP demand probably due to hippocampal hyperactivity. Hippocampal hyperactivity might be an explanation for some symptoms found in schizophrenic patients, such as hallucinations and delusions.⁵⁰

An imbalance in energy metabolism can produce free radicals and an increase of CK activity may show an increase of ATP demand, so oxidative stress can also be related to the disease progression.⁵¹ Studies in *postmortem* brain tissues have also demonstrated a possible role for mitogen-activated protein kinases (MAPKs) in schizophrenia.⁵² Although the caspase molecule is associated to apoptosis in the CNS,⁵³ it has been shown in other studies that this molecule is upregulated in temporal cortex of subjects with schizophrenia,⁵⁴ and these events are associated to dysregulation of CK activity in brain of schizophrenic patients.⁴⁹

Animal models are attempted to mimic a human condition and many models have been used to simulate schizophrenic disorders. The mechanisms underlying the pathophysiology of schizophrenia are not completely understood. However we can state that 25 mg/kg of ketamine may be used as a model of schizophrenia. We also suggest that cerebral energy metabolism might be altered in the brain of schizophrenic patients, probably leading to drastic alterations that might be involved in the pathogenesis of schizophrenia.

Methods

Animals and reagents. Adult male Wistar rats (60 days-old) were obtained from Central Animal House of Universidade do Extremo Sul Catarinense. They were caged in group of five with free access to food and water and were maintained on a 12 h light-dark cycle (lights on 7:00 am), at a temperature of $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$. All experimental procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior (SBNc) recommendations for animal care, with the approval of Ethics Committee from Universidade do Extremo Sul Catarinense.

Animal model of schizophrenia. The animals were injected with saline or 10, 25, 50 mg/kg ketamine (CU Chemie Uetikon, Germany) intraperitoneally (i.p.) at a volume of 1 mL/100 g.

Locomotor activity. We used the open-field task to assess locomotor activity. The task was performed in a 40 x 60 cm open field surrounded by 50 cm-high walls made of brown plywood with a frontal glass wall. The floor of the open field was divided into 12 equal rectangles by black lines. The animals were gently placed on the left rear rectangle and were allowed to explore the arena. Covered distance was counted for assessed for 60 min in an automatic arena.

Locomotor activity is constantly monitored by a system installed in an arena containing six parallel bars, each bar containing sixteen infrared sensors that detect rat's exact position and movement, making possible a detailed analysis of animal's behavior. Information detected by the sensors is transmitted to a computer in which animal's activity is recorded each five minutes by a software (data base: Open Source version Interbase 6.01). Distance covered by the animal is considered by the sum of the changes in position monitored by the activity arena, the software calculates the distance between two locations, plus the previously traveled distances.

Both ketamine and saline groups were divided into habituated and non-habituated. Habituation is the interval in which animals are allowed to acknowledge and freely explore the testing environment. This is an important parameter for the animal to recognize the testing cage before data collection, reducing the anxiety during testing. Habituation time was considered 20 min before the injection, then, animals were injected with different doses of Ketamine and tested for 60 minutes after the injection. Non-habituated animals were injected with different doses of the compound and then gently placed in the activity arena.

Social interaction. Impaired social behavior is a key behavioral feature of rodent models of autism spectrum disorders and schizophrenia.⁵⁵⁻⁵⁸ Social interaction was tested in rats submitted to sub-anesthetic doses of ketamine (10, 25 and 50 mg/kg). For this task a total of 30 rats were tested under dim/light and unfamiliar conditions, in an open field apparatus. On the day of the experiment, the animals were socially isolated in plastic cages measuring 43 x 28 x 15 cm (l_w_h) for 3.5 h prior to the experiment. This isolation period has been shown to produce a half maximal increase in the amount of social play.⁵⁹ The task consisted in placing two animals from the same experimental group but from different cages into the test cage for 15 min. Pairs were tested in a randomized order per groups and animals did not differ by more than 15 g in body weight. The social behavior was assessed for a pair of animals, so behavior of individual animals was not analyzed.⁵⁸ Latency to start social behavior (following or approaching the test partner, mounting or crawling over the test partner, sniffing or grooming any part of the body of the test partner), the total time spent engaged in social behavior and the number of social contacts were measured.^{58,59}

Stereotypy. Stereotypy was defined as rapid, repetitive head and forelimb movements.^{60,61} This parameter was analyzed at the same time and place as hyperlocomotor activity. Stereotypy is considered by the software as an instable movement any time when repetitive movements are recorded in sequel readings, without alteration in animal's mass center. The possible units of measurement to be considered are mm (millimeters), cm (centimeters) and in (inches).

Tissue and homogenate preparation. Animals were killed by decapitation 60 minutes after behavioral analyses. Brains were removed and prefrontal cortex, cerebellum, hippocampus, striatum and cerebral cortex were homogenized (1:10, w/v) in SETH buffer, pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 IU/ml heparin).

Homogenates were centrifuged at 800x g for 10 min and the supernatants were kept at -70°C until used for enzyme activity determination. Protein content was determined by the method described by Lowry et al.⁶² using bovine serum albumin as standard.

Creatine kinase activity. Creatine kinase activity was measured in brain homogenates pre-treated with 0.625 μM lauryl

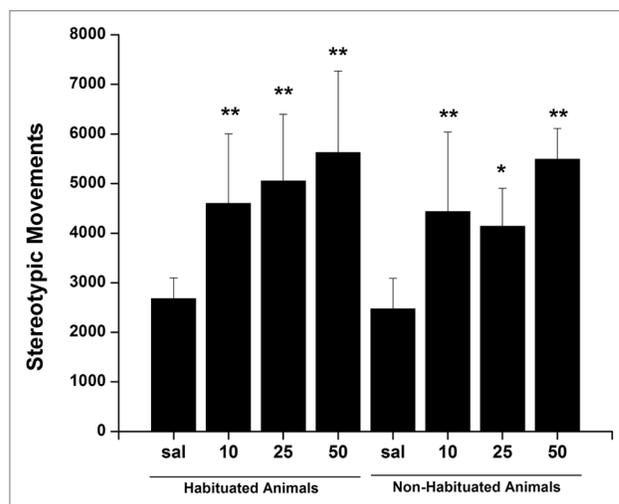


Figure 4. Stereotypic movements are considered as rapid, repetitive head and forelimb movements. It was induced by different doses of ketamine and analyzed 15 minutes after the injection. Data were analyzed by one-way analysis of variance followed by Tukey test when F was significant. Values are expressed as mean ± S.D. for ten animals in each group. *Different from control; $p < 0.01$. **Different to each other; $p < 0.01$. The units is millimeters (mm), centimeters (cm) and inches (in).

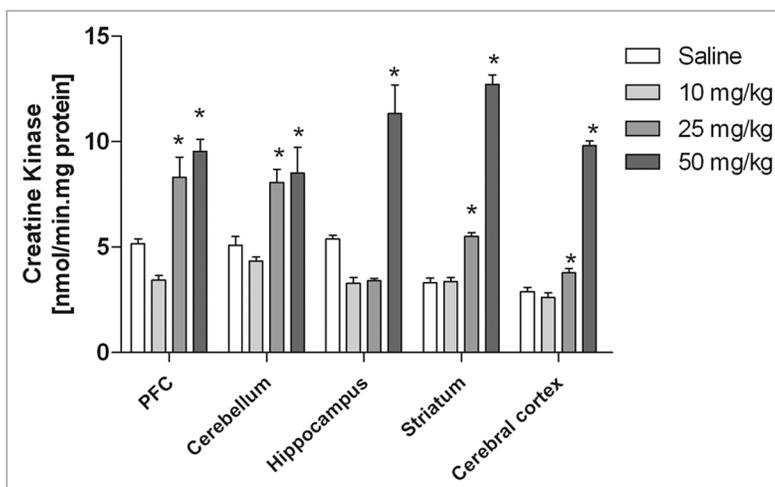


Figure 5. Creatine kinase activity. CK was measured by colorimetric method and the effect of sub-anesthetic doses of ketamine on this enzyme was verified in pre frontal cortex (PFC), cerebellum, hippocampus, striatum and total cortex. Control group is treated with saline 0.9%. Data were analyzed by ANOVA followed by Tuckey test for six independent experiments (animals) performed in duplicate. *A value of $p < 0.05$ was considered significant and different from control. Results were expressed as $(\text{min} \times \text{mg protein})^{-1}$.

maltoside. The reaction mixture consisted of 60 μM Tris-HCl, pH 7.5, containing 7 μM phosphocreatine, 9 mM MgSO₄ and approximately 0.4–1.2 mg protein in a final volume of 100 μL. After 15 min of pre-incubation at 37°C, the reaction was started by the addition of 0.3 mmol of ADP plus 0.08 μmol of reduced glutathione. The reaction was stopped after 10 min by the addition of 1 μmol of p-hydroxymercuribenzoic acid. The creatine formed was estimated according to the colorimetric method

of Hughes et al.⁶³ The color was developed by the addition of 100 μ L 2% μ -naphthol and 100 μ L 0.05% diacetyl in a final volume of 1 μ L and read spectrophotometrically after 20 min at 540 nm. Results were expressed as units (min x mg protein)⁻¹.

Statistical analysis. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey multiple range test when the F was significant. All analyses were performed using

the Statistical Package for the Social Sciences (SPSS). A value of $p < 0.05$ was considered to be significant.

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