Antidepressant Effects of Ketamine Are Not Related to 18F-FDG Metabolism or Tyrosine Hydroxylase Immunoreactivity in the Ventral Tegmental Area of Wistar Rats

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Received: 24 November 2014 / Revised: 16 March 2015 / Accepted: 6 April 2015 © Springer Science+Business Media New York 2015

Abstract Major depressive disorder (MDD) is an important health problem that is often associated to stress. One of the main brain regions related to MDD is the ventral tegmental area (VTA), a dopaminergic center, part of the reward and motivation circuitry. Recent studies show that changes to VTA dopaminergic neurons are associated with depression and treatment. Ketamine has recently shown a fast, potent antidepressant effect in acute, sub-anesthetic doses. Thus, our aims were to elucidate if ketamine would be able to revert depression-like behaviors induced by a chronic unpredictable stress (CUS) protocol and if it could cause alterations to metabolism and tyrosine hydroxylase (TH)-immunoreactivity in VTA. For this, 48 Wistar rats were divided into four groups: control + saline (CTRL + SAL), control + ketamine (CTRL + KET), CUS + saline (CUS + SAL), CUS + ketamine (CUS + KET). The CUS groups underwent 28 days of CUS protocol. Saline or ketamine (10 mg/kg) was administered intraperitoneally once on day 28. The behavior was assessed by the sucrose preference test, the open field test, and the forced swim test. Glucose brain metabolism was assessed and quantified with microPET. TH-immunoreactivity was assessed by estimating neuronal density and regional and cellular optical densities. A decrease in sucrose intake in the CUS groups and an increase in immobility was rapidly reverted by ketamine (p < 0.05). No difference was observed in the open field test. There was no alteration to VTA metabolism and TH-immunoreaction. These results suggest that the depressive-like behavior induced by CUS and the antidepressant effects of ketamine are unrelated to changes in neuronal metabolism or dopamine production in VTA.

Keywords Depression · Ketamine · MicroPET · Tyrosine hydroxylase · Ventral tegmental area

Introduction

Major depressive disorder (MDD) is a psychiatric illness affecting 300–350 million people worldwide that baffles doctors and researchers because of its complexity. Despite scientific efforts, the cause or origin of MDD remains unknown [17, 27, 64, 66]. As there is no apparent precise organic alteration, it is classified as a mood disorder characterized by chronic feelings of sadness, hopelessness, and self-worthlessness, often leading to suicidal thoughts and behaviors [3, 62]. In the attempt to understand the condition, researchers are currently investigating many brain regions, such as the prefrontal cortex, amygdala, and cingulate cortex. However, the core symptoms of MDD, anhedonia and lack of motivation, are typically associated with the reward and motivation circuitry, specifically, with an important dopaminergic center, the ventral tegmental area (VTA) [8, 48, 66].

The VTA, located in the ventral part of the mesencephalon, consists mainly of dopaminergic neurons (60–65 %) [48]. It is believed to be the initial region in the reward and motivation system and, interestingly enough, it
has projections to and from the same brain areas associated with MDD. The VTA can be divided in two distinct portions, lateral and medial. The neurons from the lateral portion (lVTA) project to the nucleus accumbens lateral shell, while the medial portion (mVTA) has dopaminergic neurons projecting to the nucleus accumbens medial shell, the nucleus accumbens core, the basolateral amygdala, and the medial prefrontal cortex [29]. A biochemical assessment of the dopaminergic system of preclinical models of MDD using the chronic unpredictable stress (CUS) protocol is well described [21]. In the frontal cortex and hippocampus, several studies have found a similar dopaminergic decrease produced by chronic stress [2, 13, 51], while in other areas, such as the striatum and amygdala, the dopaminergic levels remain unaltered or are decreased [26, 51]. Experimental evidence and the complex connections between the VTA and different brain regions affected by depression confirm that VTA plays a central role in the pathophysiology of MDD [19, 20, 61, 67].

Traditional treatments for MDD rely predominantly on selective serotonin reuptake inhibitors (SSRI) and on other monoaminergic-based drugs which increase the availability of serotonin and/or noradrenaline in the synaptic cleft in the same brain regions that receive the output from the dopaminergic VTA pathways [12, 29, 46, 66]. However, with traditional antidepressants, the therapeutic effects are usually observed 2–4 weeks after the start of the treatment [41, 55]. This scenario has begun to change with rapid-acting antidepressants, such as the N-Methyl-D-Aspartate (NMDA) receptor antagonist ketamine [28, 31, 45]. Several clinical trials have shown a rapid and robust effect in mood and suicidality of treatment-refractory depressed patients, although the mechanism of action is not fully understood [6, 14, 42, 49, 73]. One of the most significant characteristics of antidepressant ketamine is the time window of the therapeutic effect. In preclinical trials using rodents, the behavioral antidepressant effect can be seen to persist from 20 min to 48 h, to 4, 6, 8 days, up to 2 weeks post-injection [9, 10, 35, 37]. However, the reliability of the effects after 1 week is still under discussion. The molecular alterations include a plethora of analyses of synaptic-related proteins, phosphorylation, neurotrophic factors, among others, which vary from 30 min to 72 h after ketamine administration [9, 10, 31, 70, 71]. The discovery of ketamine as a fast-acting antidepressant is one of the most important advances in modern psychopharmacology for treating MDD.

Despite the clear involvement of the VTA and the reward circuit in MDD, few studies have documented dopaminergic changes of VTA neurons in depression and its treatments. Thus, the aim of this study was to evaluate the behavioral changes, glucose metabolism and immunoreactivity for tyrosine hydroxylase (TH) in the VTA of animals submitted to a depression-inducing protocol, CUS, treated or not with ketamine.

### Experimental Procedures

#### Animals

In this study 48 male Wistar rats, 3 months of age, were acquired from the Centro de Reprodução e Experimentação de Animais de Laboratório (CREAL)—UFRGS. The animals were housed in a rat vivarium, three per cage, on a ventilated rack with controlled temperature and humidity. They were kept in a 12-h dark-light cycle, with food and water ad libitum. The animals were divided into four groups of twelve: 1—Control + Saline (CTRL + SAL); 2—Control + Ketamine (CTRL + KET); 3—CUS + Saline (CUS + SAL); and 4—CUS + Ketamine (CUS + KET). All procedures were in accordance with the guide for the care and use of laboratory animals adopted by the National Institute of Health (USA), and with the university’s ethical committee, Comissão de Ética de Uso de Animais da Pontifícia Universidade Católica do Rio Grande do Sul (CEUA 12/00297-PUCRS).

#### CUS Protocol

The animals were exposed to a sequence of variable, mild stressors for 28 days continually. This procedure has been demonstrated to induce depressive-like behavioral changes [21, 50, 53, 65]. A total of eight different stressors were employed, twice daily, one during the light cycle and one in the dark cycle throughout the 28 day time period. The stressors included tail pinching, 45° inclination of the housing cage, 24 h food deprivation, 24 h water deprivation, 12 h food and water deprivation, social isolation, crowding, and dark during light cycle. The control groups did not undergo the CUS protocol (Fig. 1—Timeline).

#### Drug Administration

On the 28th day of the experiment, after the last stressor, the animals received a single, intraperitoneal (i.p.) injection of 10 mg/kg of ketamine (Cristália, Brazil) or 0.5 ml of 0.9 % saline solution. The ketamine dose chosen was based on previous studies that have shown behavioral and biochemical alterations befitting an antidepressant effect [28, 31].
Behavioral Analysis

Sucrose Preference Test (SPT)

To test for anhedonic behavior, two water bottles were placed in the housing cage, one with normal tap water and one with a 1% sucrose solution. The bottles were left for 12 h overnight, then the consumption in milliliters of both bottles was measured and the percentage of sucrose solution from total intake was calculated. Prior to this test, there were no stressors that would directly affect liquid consumption. The first trial, SPT 0, was used as baseline for the animals, at this point none of the experimental procedures had been carried out, then the following trials were performed on day 7 (SPT 1), day 14 (SPT 2), day 21 (SPT 3), and on day 28 (SPT 4) after the drug injections [9].

Open Field Test (OF)

For the evaluation of overall locomotion, each animal was individually placed in a circular chamber, 30 cm radius × 40 cm height, and its locomotion was recorded for 3 min, which is considered the “novelty” phase of the OF, when the depressed animal present locomotor alterations. The recordings were then run through the Anymaze software for analysis. Distanced traveled, distanced traveled by minute, average speed, time in center zone, and time in periphery zone were calculated for this study. This test was performed 12–15 h after the ketamine or saline injections [16, 24].

Forced Swim Test (FST)

The evaluation of “hopeless” behavior was conducted by individually placing each animal in a tank, 20 cm radius, with warm water. The tank was filled to the point where the animal was unable to reach the bottom or the border and was, therefore, forced to swim. The animal’s behavior was recorded for 3 min. The recordings were later analyzed and averaged for latency for first immobile episode, total immobile time, and climbing by two trained researchers. This test was performed on the afternoon following (16–20 h later) the injections [11, 24, 57].

18F-FDG MicroPET Scan

The rats (n = 4 for each experimental group) were transported to the preclinical imaging facility 12 h before the microPET scans. On day 30, the animals were individually anesthetized using a mixture of isoflurane and medical oxygen (3–4% induction and 2–3% maintenance dose) and 1 mCi of 18F-FDG was administered through the tail vein. Then, the animals were returned to the home cage for a 30 min period of conscious tracer uptake. After the uptake period, the rat was placed in a head-first prone position and scanned with the Triumph™ microPET (LabPET-4, TriFoil Imaging, Northridge, CA, USA) under inhalatory anesthesia. Throughout these procedures, the animals were kept on a pad heated to 36 °C. For radiotracer readings, 60-min list mode static acquisitions were acquired with the field of view (FOV; 3.75 cm) centered on the rat’s head. All data were reconstructed using a 3D ordered subsets expectation-maximization (3D-OSEM) algorithm with 20 iterations and no attenuation correction. Each reconstructed microPET image was spatially normalized into an 18F-FDG template using brain normalization in PMOD v3.5 and the Fusion Toolbox (PMOD Technologies, Zurich, Switzerland). An MRI rat brain VOI template was used to overlay the normalized images, previously coregistered to the microPET image database. The glucose metabolism in the VTA region was expressed as standard uptake values (SUVs) [58, 59, 68].

Euthanasia and Sample Extraction

The animals were deeply anaesthetized with sodium thiopental i.p. then perfused transcardially with 200 ml of 0.9% saline solution followed by 200 ml of 4% paraformaldehyde (PF) in PBS solution. The brains were dissected carefully from the animal’s skull then placed in a 4% PF solution for post-fixation for 4 h. To cryoprotect
the tissue, after the post-fixation, the brains were placed in a 30% sucrose solution and left until it sank (2–3 days). Once cryoprotected, the brains were flash frozen in isopentane cooled in liquid nitrogen, and then stored in −80 °C until immunohistochemistry.

**TH Immunohistochemistry**

Coronal sections (50 μm) from the VTA were obtained from each brain using a cryostat (Leica, Germany) at −20 °C, and one in every three sections was collected in PB saline (PBS), pH 7.4. The mesencephalic area that comprises the VTA was identified according to Paxinos and Watson’s Atlas (1998), between the following coordinates: interaural 4.2 mm, bregma −4.8 mm, and interaural 1.96 mm, bregma −7.04 mm. The free-floating sections were post-fixed in 4% PF for 15 min. To block endogenous peroxidase activity, the sections were pre-treated with 3% hydrogen peroxide in PBS for 30 min at room temperature. Thereafter, the sections were washed with PBS and treated with 2% bovine serum albumin (BSA) in PBS containing 0.4% Triton X-100 (PBS-Tx) for 30 min, and incubated with monoclonal TH antibody produced in mice (Sigma), diluted 1:3000 in PBS-Tx for 48 h at 4 °C. Sections were then washed in PBS-Tx and incubated with anti-mouse IgG produced in goat (Sigma), diluted 1:500 in PBS-Tx for 2 h at room temperature. Sections were washed in PBS, and incubated with peroxidase anti-peroxidase (PAP) mouse (Sigma) diluted 1:500 in PBS for 1 h 30 min at room temperature.

The immunohistochemical reaction was revealed in a medium containing 0.06% DAB dissolved in PBS for 10 min, and then 1 μL of 3% hydrogen peroxide per mL of DAB was added to the DAB medium for another 10 min. The sections were rinsed in PBS, dehydrated in series of increasing ethanol concentrations (70, 90 and 100%), cleared with xylene and covered with Entellan and coverslips.

**TH+ Neuronal Density Estimation**

The number of TH+ cells per mm² in the VTA was estimated using an Olympus BX 50 microscope coupled to an Opton camera and Image Pro-Plus software (Image Pro-Plus 6.1, Media Cybernetics. Silver Spring, USA).

For the analysis, five sections from each animal were analyzed. One digitalized image (4×) from each VTA section was acquired bilaterally. Altogether, ten images were analyzed per animal. Two virtual squares measuring 4219.1 μm² were overlaid the images, one on the lateral portion and one on the medial portion of the VTA, these are the areas of interest (AOI) (Fig. 6d). The TH+ cells located inside the AOI or intersected by the lower and/or left borders were counted, those cells outside or intersected by the upper and/or right borders were discarded.

This analysis revealed the densities of the lateral and medial portion of the VTA. For the full VTA, density values of the corresponding lateral and medial portions were averaged.

**TH Immunoreactivity Optical Density Evaluation**

The intensity of TH+ immunoreactivity was measured using semi-quantitative densitometric analysis [56, 69] with Image Pro-Plus software. The same images used for TH+ neuronal density estimation were used in the analysis of regional optical density (OD). The images were converted to an 8-bit gray scale (256 gray levels) and two AOIs (4219.1 μm²) were overlaid on each image, one in the lateral and one in medial portion of the VTA. For full VTA, the OD the values of the corresponding lateral and medial portions were averaged.

For the analysis of cellular OD, four digitalized images (20×) were obtained from each section bilaterally. Altogether 20 images were analyzed from each animal. Of the four digitalized images, two were from the lateral portion and two from the medial portion of the VTA. The images were converted to gray scale and one AOI measuring 10.7 μm² was placed over one TH+ cell soma in each image (Fig. 6h). The values for each lateral and medial portion were an average of the corresponding images. The OD value for the full VTA was obtained by averaging the corresponding lateral and medial values.

All lighting conditions and magnifications were kept constant during the process of capturing the images. Blood vessels and other artifacts were avoided and the background correction was performed according to a previously described formula [69].

**Statistical Analysis**

Data was assessed for normality using the Kolmogorov–Smirnov test and a parametric profile was observed. For the statistical analysis we used two-way repeated measures ANOVA with three independent factors: CUS (two levels), ketamine (two levels), and time (five levels) for the following dependent variables: 1—sucrose % intake, 2—total liquid intake, 3—sucrose intake, 4—distance traveled by minute (note: the time factor of this variable has three levels). Additionally, we used a two-way ANOVA with two independent factors (CUS and ketamine) with two levels each for the following dependent variables: 1—distance traveled, 2—average speed, 3—time in center, 4—time in periphery, 5—latency to first immobile episode, 6—time immobile, 7—climbing, 8—glucose metabolism, 9—neuronal density, 10—regional OD, and 11—cellular
OD. Statistical difference was considered significant when \( p < 0.05 \). All data are expressed as mean ± SEM. The statistical analysis was conducted using SPSS Statistics 17.0 software.

**Results**

**Behavior**

**Sucrose Preference Test (SPT)**

Figure 2a shows the relative intake per week, considering the CUS protocol was initiated at week 0 (SPT 0) and ketamine and saline treatment were performed at week 4 (SPT 4). All four groups have roughly the same average, 90–96% of relative sucrose intake, with no alterations to the total liquid intake (Fig. 2b). In SPT 2 both groups that underwent the CUS protocol decreased their intake (CUS \( \leq \) SAL: 83.57%, \( p < 0.01 \); CUS \( \leq \) KET: 77.40%, \( p < 0.01 \)), and the CTRL groups plateaued between 98 and 100%. In SPT 3 the results maintain the same pattern (CUS \( \leq \) SAL: 87.48%, \( p < 0.01 \); CUS \( \leq \) KET: 79.09%, \( p < 0.01 \)). In SPT 4, after the injection of ketamine, the CUS \( \leq \) KET group restored the relative intake to normal levels, 90.90%, and the CUS \( \leq \) SAL group showed a further decrease in relative sucrose intake (76.22%, \( p < 0.001 \)). Furthermore, in Fig. 2c we observe that the CUS-groups have a lower sucrose intake in SPT 2 (CUS \( \leq \) SAL: 0.51 g/kg, \( p < 0.05 \); CUS \( \leq \) KET: 0.33 g/kg, \( p < 0.05 \)) when compared to SPT 0 and both CTRL-groups. In SPT 3 the CUS-groups have a lower sucrose intake (CUS \( \leq \) SAL: 1.09 g/kg, \( p < 0.05 \); CUS \( \leq \) KET: 1.03 g/kg, \( p < 0.05 \)) when compared to the CTRL-groups, and the CTRL groups present a larger sucrose intake (CTRL \( \leq \) SAL: 1.77 g/kg, \( p < 0.05 \); CTRL \( \leq \) KET: 1.87 g/kg, \( p < 0.05 \)) when compared to SPT 0. Finally, after ketamine injection, in SPT 4, there is a general decrease in sucrose intake in all groups (CTRL \( \leq \) SAL: 0.63 g/kg, \( p < 0.05 \); CTRL \( \leq \) KET: 0.77 g/kg, \( p < 0.05 \); CUS \( \leq \) SAL: 0.40 g/kg, \( p < 0.05 \); CUS \( \leq \) KET: 0.36 g/kg, \( p < 0.05 \)). This data suggests that, after 2 weeks of CUS, the animals developed anhedonia and ketamine was able to rapidly reverse this behavior.

**Open Field Test (OF)**

In the open field test there was no statistical difference between the groups in any of the analyzed locomotor parameters (Fig. 3). The lack of difference in the time in the center and time in the periphery indicates that the animals that underwent the CUS protocol did not exhibit anxiety, which would have been seen if the animals had increased the time in periphery (Fig. 3c, d). Also, the animals which received ketamine showed no signs of sedation by the drug, as there was no alteration to the distance traveled and average speed (Fig. 3a, c) [38, 47]. No statistical difference was observed in the distance traveled by minute (Fig. 3b).

**Fig. 2 Sucrose preference test** Effect of CUS and ketamine on the consumption of sucrose solution in percentage throughout the experimental procedure. a Sucrose preference test, b total liquid intake, and c sucrose intake. \( ^{a} p < 0.05 \) when compared to SPT 0 time period in the same group; \( ^{b} p < 0.05 \) when compared to the CTRL \( \leq \) SAL and CTRL \( \leq \) KET groups in the same time period; and \( ^{c} p < 0.05 \) when compared to all other groups in the same time period.
**Forced Swim Test (FST)**

In the latency to first immobile episode (Fig. 4a), the CUS protocol was able to decrease the latency until the first immobile episode and that decrease was not reverted by ketamine (CTRL ? SAL: 67.58 s; CTRL ? KET: 70.41 s; CUS ? SAL: 41.66 s, p < 0.001; CUS ? KET: 43.25 s, p < 0.001). When examining the total time immobile (Fig. 4b), the CUS ? SAL group increased the amount of time immobile (p < 0.05) and that increase was promptly reverted by ketamine (CTRL ? SAL: 36.74 s; CTRL ? KET: 33.18 s; CUS ? SAL: 60.17 s; CUS ? KET: 38.99 s). In the assessment of climbing behavior (Fig. 4c) we see that the climbing time diminished in the CUS groups (CTRL + SAL: 60.5 s; CTRL + KET: 62.25 s; CUS + SAL: 37.41 s, p < 0.001; CUS + KET: 46 s, p < 0.001). This indicates that the CUS protocol evoked hopelessness in the animal models and that ketamine was partially able to rapidly reverse it (under 20 h).

**MicroPET**

In order to study changes in brain glucose metabolism induced by CUS, as well as the effect of ketamine on rats that underwent the CUS protocol, the animals were injected with \(^{18}\text{F-FDG}\) and scanned using a microPET system. The mean \(^{18}\text{F-FDG}\) SUVs for the VTA region in each group were calculated and compared. No statistically significant differences in \(^{18}\text{F-FDG}\) SUVs were observed between the groups for the VTA region (Fig. 5a).

**TH Immunohistochemistry**

In the qualitative analysis of TH immunohistochemistry, the neuronal somata presented a round to fusiform shape and the nucleus appeared as a clear, oval shaped form that occupies most of the somata space. In this parameter, no evident difference could be observed between the groups.

In order to confirm the qualitative analysis, quantitative (estimation of neuronal density) and semi-quantitative evaluations (regional and cellular optical density—OD) were employed. There was no statistical difference between the groups in neuronal density, regional OD, and cellular OD in the medial, lateral, or full VTA (Fig. 6a–c). This suggests that CUS failed to disrupt the normal immunohistochemical profile of TH in the VTA, and likewise, the basis of ketamine’s antidepressant effect is probably unrelated to changes in dopaminergic systems of VTA.
**Fig. 4** *Forced swim test* Effect of CUS and ketamine on the forced swim test. 

- **a** Latency until the first immobile episode,
- **b** total time immobile,
- **c** Climbing. 

***$p < 0.001$ when compared to CTRL + SAL and CTRL + KET groups; ###*$p < 0.001$ when compared to all other groups.

**Fig. 5** *MicroPET*. Effects of CUS and ketamine on glucose metabolism for the VTA region. 

- **a** $^{18}$F-FDG uptake in the VTA region and **b** $^{18}$F-FDG rat template in coronal (top-left), sagittal (mid-left), and transverse (bottom-left) views and sample individual scan normalized into a template in coronal (top-right), sagittal (mid-right), and transverse (bottom-right) views. The VTA, in red, was defined using a rat-ROI-template based on Paxinos coordinates in the PMOD software (Color figure online)
Discussion

Some technical considerations should be mentioned in our study. The first is related to the light period used in our study. Recent research has shown that the light cycle can influence the outcome of depressive-like behavior. During the dark period, CUS-depressed male rats are more prone to increase their locomotion in the OF and show no depressed behavior; while during the light period, the depressed behavior is observed and there are no alterations to locomotion during the OF [24]. For this reason our research was conducted during the light period.

The second is related to the stress/depression protocol used in our study. We used a well-established experimental model to induce depression, the CUS protocol [21, 50, 65]. However, despite being widely accepted, the CUS protocol suffers from variability regarding the length of exposure to stress. From our results with the repetitive SPT, we observed that anhedonia initiated after 14 days (Fig. 2). Corroborating with our result, a previous study tested several exposure times for depression onset and found it only occurred with a protocol of at least 15 days [5]. Also, two studies that performed repetitive SPT found depression-related alterations within 10 days of the CUS protocol, however, the SPT protocols in these experiments were different from the one we used [34, 63]. The evidence suggests that the onset of depression in a CUS animal model only occurs after at least 2 weeks of chronic stress.

The rapid antidepressant effect of the single dose of ketamine was clearly seen in our behavioral tests. In the SPT the intake of the sweet palatable solution returned to baseline levels during the same night in which ketamine was administered in the CUS + KET group (Fig. 2a–c), which is consistent with findings from other researchers [31, 32, 65]. The SPT showed the onset of depression started 2 weeks after the beginning of the CUS protocol [5, 63], and also showed the recovery from the anhedonic state within hours after ketamine injection [31]. This reinforces
the rapid antidepressant effect of ketamine that has been widely studied for the past decade [28, 44]. We can see in the sucrose preference test and in the sucrose intake graphs (Fig. 2a, c) that there is a losing interest in sucrose on the CUS groups while on the CTRL groups the intake of sucrose is stable, whereas in the total liquid intake graph (Fig. 2b) the data shows no alteration on liquid consumption. These suggest that the animals in the CUS groups are actively choosing tap water over the sucrose solution, and confirm the inexistence of any pre-existing spatial preference. A previous study, with similar results, has suggested this outcome [1].

Because of its traditional use as an anesthetic drug, we used the open field test to see whether this low dose of ketamine would have any effect on normal behavior in the animals. Our results showed no statistical difference between the groups in distance traveled or average speed (Fig. 3a, c), none of the groups presented anxious behavior (Fig. 3d, e), and no habituation to the test (Fig. 3b). Although we do not see any statistical differences between the groups in the distance traveled by minute, there is a gradual lowering of the mean of distance traveled in function of time within the groups. This effect is most likely to be the locomotor habituation to the test; however, 3 min is not enough to assess the habituation, at least 5–10 min is needed [16]. Our data are in accordance with previous studies that show no effect of CUS in the OF [9, 16, 34, 63].

In order to confirm the establishment of depression in an animal model and the antidepressant effect of a drug, it has been suggested that several behavioral tests should be performed to avoid false positive results [9, 57]. We performed all behavioral, metabolic, and immunochemical analyses from 12 to 72 h after ketamine injection because of ketamine’s broad time window of therapeutic effect [9]. In the forced swim test, the lowering of the latency to first immobile episode in the CUS groups confirmed the onset of a depressive state (Fig. 4a) and the fact the CUS + KET group maintained an average time immobile close to that of the CTRL groups confirmed the antidepressant effect of ketamine (Fig. 4b). As previously suggested, the climbing behavior observed in our study (Fig. 4c) is associated to noradrenergic mechanisms [11]. In our histological results, we saw no changes to TH immunoreactivity, a precursor to noradrenaline and adrenaline, as well as dopamine (Fig. 6). The VTA is a site with mainly dopaminergic activity, if any alteration to noradrenaline occurred it would be further down the biochemical pathway. Although TH is a precursor of dopamine and noradrenaline, our results from the FST suggest the CUS protocol may have had some effect on noradrenaline. However, there are no grounds in our research to predict any influence of ketamine on noradrenergic projections. Other researchers have shown that altered TH levels in the locus coeruleus play a major part in the physiopathology of depression [25, 75]. This is a brain region of great relevance to the understanding of depression and should be of interest in future studies. The results from the OF test discard the possibility of a motor dysfunction affecting the FST test. These data suggest that the animals in the CUS groups showed depression-like behavior and that ketamine exerted the expected rapid antidepressant effect without creating major behavioral alterations.

The VTA is connected to all brain regions associated to MDD [48]. Metabolic 18F-FDG analyses in previous studies are in accordance with our findings for the VTA, where no evident alteration in glucose metabolism is observed in the VTA after CUS protocol and curcumin treatment [23, 32]. Studies have shown that ketamine’s delayed behavioral antidepressant effect can last up to 7 days after a single dose, which is accompanied by biochemical, electrophysiological, and morphological alterations [9, 31, 35, 37]. We chose to perform the microPET 2 days later to avoid an acute effect of ketamine, and observe a possible sub-chronic metabolic effect during the biochemical, electrophysiological, and morphological adaptations.

Overall decrease in dopamine levels has been well described in the brain of depressed animals [21]. In some regions that receive axons projecting from the mVTA, there was a decrease in dopamine levels in the frontal cortex, and no dopamine alteration in the amygdala [13, 51]. In relation to the VTA hodology, the nucleus accumbens (NAc) is a region that deserves careful attention; it receives inputs from the mVTA and the IVTA, the mVTA projects to the NAc core and medial shell, while the IVTA projects only to the NAc lateral shell. Unfortunately, these portions of the NAc are rarely studied separately. In the literature there are conflicting results: some state that there is a decrease in dopamine levels in the NAc [2, 51, 52], whereas others show no alteration to dopamine levels in the NAc [26, 60] and only a few have shown an increase [72]. It is noteworthy that the studies showing dopamine reduction used a 7-day CUS protocol, which was shown by the data from ours and other studies to be insufficient to induce depression [5].

Another technical consideration concerns the immunohistochemistry. Previous studies from our laboratory have demonstrated that the quantification of immunoreactivity through optical density is a very reliable analysis. This procedure is sufficiently sensitive to evaluate large differences in TH expression in animal models of Parkinson’s disease [18, 54] and even subtle changes to TH levels in animals with experimental Diabetes mellitus [4, 43]. Also, the quantification of the levels of TH in immunohistochemistry accompanies alterations in dopamine metabolite.
levels measured by HPLC neurochemical assay [18]. Thus, our immunohistochemical results on neuronal density, and regional and cellular ODs demonstrate that there is no change in VTA dopamine levels after 4 weeks of CUS protocol. These data in association with the previously mentioned results point to a decrease in dopamine levels that can occur in the first stages of chronic mild stress (1 week), and that in the following 4 weeks of the same stress dopamine levels return to normal, indicating some kind of neurochemical adaptation to stress. Another study which proposes a model of cyclin-dependent kinase 5 (a regulator of TH phosphorylation) knock-out mice, that behave depressively, observed no alterations to total TH levels and a decrease in the levels of p-TH in the VTA [74]. However, comparisons between our study and the aforementioned study are of limited value because there are important differences between the animal models and analyses used. These data reinforced the concept that depression is more closely related to the release/binding of neurotransmitters than to neurotransmitter production [36].

A previous study suggested that ketamine was able to increase the dopaminergic release in mPFC in rats [39], but this was in the context of using ketamine as a psychotomimetic, which usually requires larger doses than those necessary to produce antidepressant effects. A later study reviewed this result and considered the findings to be controversial [33]. We observed that ketamine does not disrupt the TH immunohistochemical reaction in the VTA, which leads us to believe that the antidepressant effect is exerted on another region or neurochemical system. Because of ketamine’s pharmacology, it is reasonable to assume that the antidepressant effects are probably carried out by the glutamatergic interneurons of the VTA and other brain regions [48]. It is well documented that lesions to the VTA can elicit depressive-like behavior in rats [67], but recent optogenetic studies have shown that the firing rate of the VTA dopaminergic neurons plays a key role in the depressive-like and antidepressant behavior of rats [19, 20, 61]. One possibility is that ketamine could be influencing the firing rate of these dopaminergic neurons by altering the glutamatergic transmission. Current studies suggest that neurons can corelease different neurotransmitters [22]. Studies of the VTA have shown that a considerable amount of the dopaminergic cell population (12–37 %) co-expresses TH and VGluT2 (a vesicular glutamate transporter, which accumulates glutamate in synaptic vesicles) [40]. By blocking NMDAr, ketamine might increase the reuptake of glutamate by presynaptic and glial excitatory amino acid transporters (EAATs) [30], and thus interfere with the firing rate of these cells [19]. Our data shows that neither CUS nor ketamine influence upon TH content and production, which leads us to believe that the antidepressant mechanisms are more closely related to the synaptic transmission than dopamine production. The abovementioned approach above is our best hypothesis given our results and previous studies, and should be further studied.

Our study is, to the best of our knowledge, the first to show that the depressive-like behavior induced by a chronic stress protocol and the antidepressant effect of a low, single dose of ketamine are not related to alterations in glucose metabolism and changes in TH immunoreactivity in the VTA. This finding could be useful for futures studies in the same subject.

Acknowledgments The authors would like to thank the Brazilian funding agencies CNPq, CAPES, and FAPERGS for their support of this research. Dr. Diogo Lara, Dr. Jaderson Costa daCosta, and Dr. Léder Xavier are CNPq researchers.

Conflict of interest The authors declare they have no conflicts of interest.

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