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The monoaminergic pathways and inhibition of monoamine transporters interfere with the antidepressive-like behavior of ketamine

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ABSTRACT

Ketamine (KET), a NMDA receptor antagonist, has been studied for its rapid and efficacious antidepressant effect, even for the treatment-resistant depression. Although depression is a major cause of disability worldwide, the treatment can be feasible, affordable and cost-effective, decreasing the population health burden. We evaluated the antidepressive-like effects of KET and its actions on monoamine contents (DA and its metabolites, as well as 5-HT) and on tyrosine hydroxylase (TH). In addition DAT and SERT (DA and 5-HT transporters, respectively) were also assessed. Male Swiss mice were divided into Control and KET-treated groups. The animals were acutely treated with KET (2, 5 or 10 mg/kg, i.p.) and subjected to the forced swimming test, for evaluation of the antidepressive-like behavior. Imipramine and fluoxetine were used as references. The results showed that KET decreased dose-dependently the immobility time and shortly after the test, the animals were euthanized for striatal dissections and monoamine determinations. In addition, the brain (striata, hippocampi and prefrontal cortices) was immunohistochemically processed for TH, DAT and SERT. KET at its higher dose increased DA and its metabolites (DOPAC and HVA) and mainly 5-HT contents, in mice striata, effects associated with increases in TH and decreases in DAT immunoreactivities. Furthermore, reductions in SERT immunoreactivities were observed in the striatum and hippocampus. The results indicate that KET antidepressive-like effect probably involves, among other factors, monoaminergic pathways, as suggested by the increased striatal TH immunoreactivity and reduced brain DA (DAT) and 5-HT (SERT) transporters.

Introduction

The research group of Berman et al. (2000), was the first to establish the rapid and robust antidepressive effect of ketamine (KET) infusion (0.5 mg/kg, over 40 min), in patients with major depression. Subsequently, other clinical studies confirmed that finding (Zarate et al., 2006, 2012; Diaz-Granados et al., 2010; Valentine et al., 2011; Murrough et al., 2013). Furthermore, another work showed that infusions of three or six of this low KET dose to treatment-resistant patients could safely be given (Diamond et al., 2014).

Although the majority of clinical studies use intravenous KET infusions, its antidepressive action was also observed in major depressive patients, after intramuscular, intranasal, sublingual and oral administrations (Irwin and Iglewicz, 2010; Irwin et al., 2013; Lara et al., 2013; Chilukuri et al., 2014; Lapidus et al., 2014). However, the clinical effects of KET are transient and the studies were not entirely conclusive, after its chronic administration (see Browne and Lucki, 2013, for a review).

Another important issue refers to the possible involvement of monoaminergic neurotransmission with KET antidepressive effects, since most pre-clinical studies focused on the glutamatergic system. After previous works (Irifune et al., 1991; Lannes et al., 1991) showing a link between KET and the dopaminergic system,

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several other studies suggested an involvement of dopamine and serotonin neurotransmission with the antidepressive-like actions of ketamine (Tan et al., 2012; Gigliucci et al., 2013; Yoshizawa et al., 2013; Nishitani et al., 2014; Iskandrani et al., 2015; Li et al., 2015; Pham et al., 2017; Du Jardin et al., 2016; Fukumoto et al., 2016). However, most of these studies focused on dopaminergic and serotonergic receptors and not on brain monoamine concentrations or monoamine transporters.

Furthermore, only the racemate ketamine, (*R,S*)-ketamine, is generally used in clinic. The antidepressive effect of KET is due to its *S*-ketamine stereoisomer component. However the racemate form is the responsible for the drug psychotomimetic actions (Gao et al., 2016). Evidences (Yang et al., 2015) indicate that (*R*)-ketamine, besides being a potent, long-lasting and safe antidepressive, is free from psychotomimetic effects and abuse liability. Such findings were recently (Fukumoto et al., 2017) confirmed and these authors demonstrated that (*R*)-ketamine exerted a sustained antidepressive effect in refractory models of depression.

In the present work, we studied the (*R,S*)-ketamine antidepressive-like effects in mice, by the forced swimming test, which is the most frequent behavioral test for measuring depressive-like behavior in rodents. The focus was on the antidepressive-like effects of KET and measurements of the striatal monoamine concentrations, after its acute intraperitoneal administration to mice. KET effects on tyrosine hydroxylase (TH) and dopamine (DAT) and serotonin (SERT) transporters, in the mice brain, were also evaluated by immunohistochemical assays.

Material and methods

Drugs

Ketamine free base (hydrochloride) was from König (Santana de Parnaíba, São Paulo, Brazil). Antibodies for immunohistochemistry assays were from Santa Cruz Biotechnology (Dallas, TX, USA) or Abcam (Cambridge, UK). All other reagents were of analytical grade and the drugs diluted in distilled water, before use.

Animals

Male Swiss mice (30 g) from the Animal House of the Faculty of Medicine Estácio of Juazeiro do Norte (Estácio/FMJ), Ceará, Brazil, were maintained at a 24 ± 2 °C temperature, in a 12 h dark/12 h light cycle, with standard food and water ad libitum. The study was approved (number 2014-004) by the Estácio/FMJ Ethics Committee for Animal Experimentation. All experiments followed the ethical principles established in the Guide for the Care and Use of Laboratory Animals, USA, 2011.

Forced swimming test

This is a rodent behavioral test used for evaluation of antidepressive efficacy of new compounds in experiments aimed at rendering or preventing depressive-like states (Petit-Demouliere et al., 2005; Can et al., 2012). The test is based on the observation that, when the animals are subjected to a stressful situation with no possibility for escaping, they adopt a posture of immobility after an initial period of agitation. The reduction of this immobility time is suggestive of an antidepressive-like action. A glass cylinder (30 cm height × 20 cm diameter) is filled with water (15 cm from the bottom, at 25 °C). The animals (n = 13–24) were administered with ketamine (KET 2, 5 and 10 mg/kg, i.p.). Imipramine (IMI: 30 mg/kg, p.o.) and fluoxetine (FLUOX, 2 mg/kg, p.o.) were used as references. The control group was administered with distilled water (0.1 mL/100 g). After 30 min (KET groups) or 1 h (IMI and FLUOX groups), each mouse was placed individually in the cylinder and, 2 min later, the immobilization time was recorded for 5 min. After that, the apparatus and testing area were cleaned with 70% ethanol, before starting the next test. All animals were euthanized by decapitation, around 30 min after the behavioral test, and the brains removed for neurochemical and immunohistochemical measurements.

Neurochemical determinations of DA, its metabolites and 5-HT, in mice striata

Although the monoamine hypothesis has dominated the pathophysiology and pharmacotherapy of depression for several decades, it has been questioned in several aspects. Despite of that, the main target of the current generation of antidepressive drugs is SERT (Hinz et al., 2012; Goldberg et al., 2014; Jeon and Kim, 2016). Then, in the present study, we decided to determine striatal monoamine contents after KET administration (5 to 16 animals/group). The striatal contents of DA, DOPAC, HVA and 5-HT were determined by HPLC. Homogenates were prepared in 10% $HClO_4$ and centrifuged at 4 °C (15,000 rpm, 15 min). The supernatants were filtered and 20 μL injected into the column (Shim-Pak CLC-ODS, 25 cm) coupled to an electrochemical detector (model L-ECD-6A from Shimadzu, Japan), at a flow of 0.6 mL/min. A mobile phase was prepared with monohydrated citric acid (150 mM), sodium octil sulfate (67 mM), 2% tetrahydrofuran and 4% acetonitrile, in deionized water. The pH of the mobile phase was adjusted to 3.0 with NAOH (10 mM). Monoamines were quantified by comparison with standards which were processed at the same manner as the samples. The results are expressed as ng/g tissue. The DOPAC/DA and HVA/DA ratios for the KET-treated groups were also determined.

Immunohistochemistry assays for tyrosine hydroxylase (TH), dopamine transporter (DAT) and serotonin transporter (SERT), in mice brain

Monoamine transporters were examined after the acute intraperitonial administration of KET. Brain slices from 3 animals were cut, according to the following Bregma levels (striatum: −1.34 to −2.92 mm; hippocampus: −1.58 to −2.12 mm; prefrontal cortex: +1.94 to +3.14 mm), after being fixed in 10% buffered formaldehyde, for 24 h, followed by a 70% ethanol solution. Afterwards, 5 μm sections were embedded in paraffin wax for slices processing on appropriate glass slides. These slices were placed in the oven at 58 °C, for 10 min, followed by deparaffinization in xylol, rehydration in alcohol at decreasing concentrations, washing in distilled water and PBS (0.1 M sodium phosphate buffer, pH 7.2), for 10 min. The endogenous peroxidase was blocked with a 3% hydrogen peroxide solution, followed by incubation with the appropriate primary anti-antibody for TH (52B83, sc-52746, mouse monoclonal antibody, Santa Cruz, USA, 1:200 dilution), DAT (H-174, sc-8310, rabbit polyclonal antibody, Santa Cruz, USA, 1:100 dilution) and SERT (M-19, sc-1747, goat polyclonal antibody, Santa Cruz, USA, 1:100 dilution). After 2 h, at room temperature in a moist chamber, the slices were washed with PBS (3 times, 5 min each) and incubated with the biotinylated secondary antibody, for 1 h, at room temperature. Then, they were washed again with PBS and incubated with streptavidin-peroxidase, for 30 min, at room temperature. After another wash in PBS, they were incubated in a 0.1% DAB solution (in 3% hydrogen peroxide). Finally, the slices were washed in distilled water and counterstained with Mayers hematoxilyn, washed in tap water, dehydrated in alcohol (at increasing concentrations), diaphonized in xylol and mounted on Entelan® for optic microscopy examination, with the Nikon Eclipse Trinocular Microscope (Nis) and x100 or x400 magnifications. The immunos-

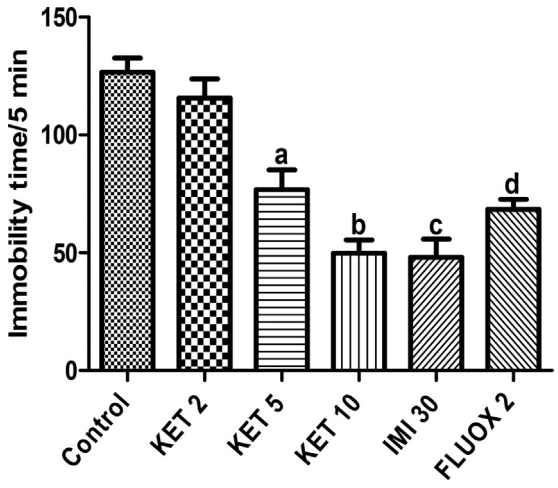

Fig. 1. Ketamine (KET, 2, 5 and 10 mg/kg, i.p.) dose-dependently decreases the immobility time/5 min, in the forced swimming test in mice. Imipramine (IMI, 30 mg/kg) and fluoxetine (FLUOX, 2 mg/kg) were used as references. The results are expressed as means ± SEM (from 13 to 24 animals). **a**. vs. Control, q = 7.241, p < 0.001; **b**. vs. Control, q = 11.17, p < 0.001; **c**. vs. Control, q = 10.87, p < 0.001; **d**. vs. Control, q = 7.554, p < 0.001 (One-way Anova and Tukey as the post hoc test).

taining was quantified (as relative optical density) by the Image J Software (NIH, USA).

Statistical analyses

For statistical analyses, One-way Anova followed by Tukey as the post hoc test for multiple comparisons were used. The data were analyzed by the GraphPad Prism software, version 6, and presented as means ± SEM. Besides, the results also show the q-values, as well as p-values. The q-values are the adjusted *p*-values found, using an optimized false discovery rate (FDR). The photomicrograph data were quantified by the Image J software, from the National Institutes of Health (NIH, Bethesda, Maryland, USA). Differences were considered significant at $p < 0.05$.

Results

Forced swimming test, after ketamine (KET) acute administrations

The acute intraperitoneal (i.p.) administrations of KET, at the doses of 5 and 10 mg/kg, reduced by 39 and 61%, respectively, the immobility time/5 min, as evaluated by the forced swimming test in mice. No significant effect was observed at the dose of 2 mg/kg. Imipramine (IMI, 30 mg/kg) and fluoxetine (FLUOX, 2 mg/kg) decreased the immobility time by 62 and 46%, respectively in relation to the Control group (Fig. 1).

Monoamine determinations in mice striata, after the acute KET administration

Measurements of striatal monoamines (ng/g tissue) were performed after acute intraperitoneal KET administrations, with the doses of 5 and 10 mg/kg, since the lower dose (2 mg/kg) showed no effect on the forced swimming test. A significant increase in DA contents (1.4 times) was observed after the dose of 10 mg/kg, while imipramine (IMI, 30 mg/kg) caused a 1.7 times increase, relatively to the Control group. KET10 also increased by 1.5 and 1.9 times DA metabolites (DOPAC and HVA), respectively (Fig. 2). However, the most important finding was the high increase in serotonin contents (2.8 times) after KET administration, at the dose of 10 mg/kg. Fluoxetine (FLUOX, 2 mg/kg) also induced a 2.7 times increase in serotonin levels, compared with the Control group (Fig. 3). The

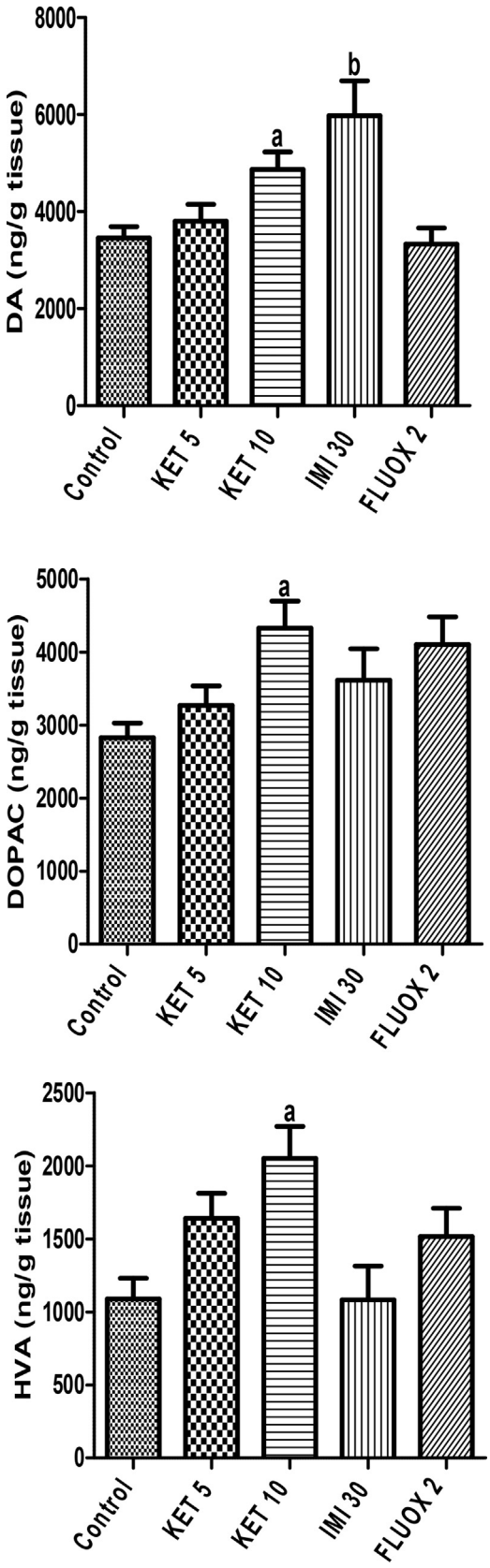

Fig. 2. Ketamine (KET, 10 mg/kg) significantly increased striatal dopamine (DA) and its metabolites (DOPAC and HVA), in mice striata. Imipramine (IMI, 30 mg/kg) and fluoxetine (FLUOX, 2 mg/kg) were used as references. The results are expressed as means ± SEM (from 5 to 13 animals). **DA: a.** vs. Control, q = 4.153, p < 0.05; **b.** vs. Control, q = 5.727, p < 0.01. **DOPAC: a.** vs. Control, q = 5.040, p < 0.01. **HVA: a.** vs. Control, q = 4.887, p < 0.01 (One-way Anova and Tukey as the post hoc test).

Table 1
Monoamines and metabolites concentrations (ng/g tissue) in mice brains after the forced swimming test.

Group	DA	DOPAC	HVA	DOPAC/DA	HVA/DA
Control	3460 ± 230.8 (13)	2825 ± 200.6 (12)	1088 ± 142.9 (12)	0.82	0.31
KET 5	3800 ± 349.4 (13)	3267 ± 269.4 (12)	1640 ± 171.8 (14)	0.86	0.43
KET 10	4871 ± 360.5 (12)	4328 ± 372.0 (16)	2052 ± 217.6 (10)	0.89	0.42

The monoamine ratios were calculated from the monoaminergic concentrations determined by HPLC in the striata. The data are means ± SEM from the number of animals in parentheses.

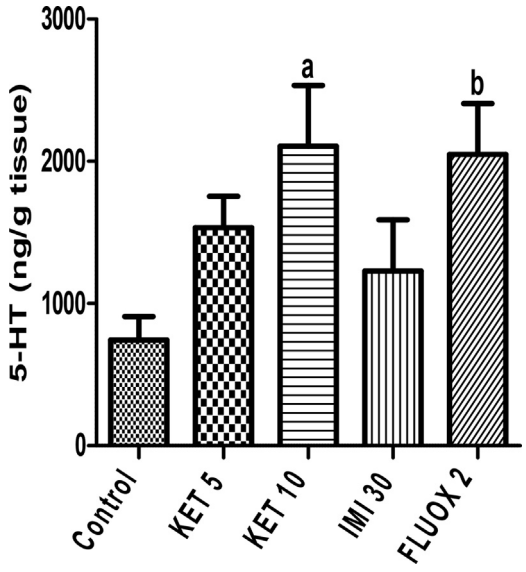

Fig. 3. Ketamine (KET, 10 mg/kg) significantly increased serotonin (5-HT), in mice striata. Imipramine (IMI, 30 mg/kg) and fluoxetine (FLUOX, 2 mg/kg) were used as references. The results are expressed as means ± SEM (from 5 to 15 animals). **a.** vs. Control, $q = 4.384$, $p < 0.05$; **b.** vs. Control, $q = 4.139$, $p < 0.05$ (One-way Anova and Tukey as the post hoc test).

DOPAC/DA ratios increased slightly in the KET groups (KET5: 0.86; KET10: 0.89), relatively to the Control group (0.82). The HVA/DA ratios also increased after KET treatments, in comparison with the Control group (Control: 0.31; KET5: 0.43; KET10: 0.42) (Table 1).

Immunohistochemical assays for tyrosine hydroxylase (TH) and dopamine transporter (DAT), in mice striata

Tyrosine hydroxylase (TH) is the rate limiting enzyme of catecholamine biosynthesis and is inhibited, in a feedback fashion, by catecholamine neurotransmitters (Daubner et al., 2011). The dopamine transporter (DAT) controls the dopamine (DA) neurotransmission, by driving the reuptake of this extracellular transmitter into presynaptic neurons (Vaughan and Foster, 2013). We showed increases around 42 and 59% in striatal TH immunoreactivities, relatively to the Control group, after the acute KET treatments with the doses of 5 and 10 mg/kg, respectively. A small (12%) but significant increase in TH immunostaining was also noticed in the KET10 group, compared with the KET5 group (Fig. 4A). On the contrary, significant decreases of 24 and 88% were observed in the striatal immunoreactivity to DAT, after KET treatments with the doses of 5 and 10 mg/kg, respectively (Fig. 4B).

Immunohistochemical assays for the serotonin transporter (SERT), in KET-treated mice brain

The serotonin transporter (SERT) regulates the serotonergic system and its receptors, via modulation of extracellular serotonin concentrations. Thus, SERT has the function of taking up serotonin released during neurotransmission and, in this sense, it is an important target for psychostimulant drugs (Murphy et al., 2004; Rudnick, 2006). We showed 19 and 78% decreases of striatal SERT immunostaining, in KET5 and KET10 groups, compared with Controls (Fig. 5A). In the CA1 hippocampal subfield, 24 and 88% reductions were observed, after KET treatments with the doses of 5 and 10 mg/kg, respectively (Fig. 5B). Similar decreases (23 and 67%) in SERT immunostaining were also observed in the dentate gyrus (DG), in KET5 and KET10 groups, respectively, in relation to Controls (Fig. 5C). In addition, decreases of 23 and 63% were also demonstrated in the prefrontal cortex (PFC), after KET treatments with the doses of 5 and 10 mg/kg, respectively (Fig. 5D).

Discussion

Ketamine (KET), as an anesthetic, has been used in clinical practice for over 50 years. Since the clinical study by Berman et al. (2000), showing a rapid decrease in depressive symptoms, in patients refractory to the conventional treatment, there are growing numbers of clinical as well as pre-clinical studies on this subject. Although many reports from pre-clinical studies indicate that the acute administration of KET produces anti-depressive-like effects in rodents and that there is a protracted effect after a single dose, the results are still not entirely conclusive (Yilmaz et al., 2002; Maeng et al., 2008).

The forced swimming test is frequently used for measuring depressive-like behavior in rodents and to study KET effects. The test has strong predictive validity, and short-term administration of antidepressant compounds have been shown to reduce the animal's immobility time (Cryan et al., 2005). The majority of studies, using the forced swimming test, reports the acute effect of KET after intraperitoneal administration at doses ranging from 10 to 50 mg/kg (see Browne and Lucki, 2013, for a review). In the present study, we used lower doses of KET (up to 10 mg/kg) acutely administered by the intraperitoneal route.

By using the forced swimming test, we demonstrated decreases in the immobility time and significant and dose-dependent effects. However, the fact that we did not use mice showing a depressive-like behavior, as that induced by repeated doses of corticosterone, could be a limitation of our study. Recently (Vale et al., 2016), we showed that KET presents antidepressive-like effects, even with a dose of 2 mg/kg. Other findings (Garcia et al., 2008) observed no antidepressive-like effects of KET, after the acute administration of 5 mg/kg, i.p., considered an inactive dose that, according to the authors, becomes active after the chronic treatment for 14 days.

The literature on the possible effects of KET on dopaminergic neurotransmission is contradictory. Thus, KET has been reported to increase (Irifune et al., 1991; Verma and Moghaddam, 1996; Witkin et al., 2016), to have no effect (Lannes et al., 1991; Micheletti et al., 1992), or to decrease striatal DA turnover or extracellular DA levels (Rao et al., 1989). Others (Li et al., 2015) indicated that DA, D2/D3, but not D1 receptors are involved in the rapid antidepressive-like effects of KET.

In the present work, we showed a significant increase in the striatal DA and its metabolites (DOPAC and HVA) contents, at the higher dose (10 mg/kg, i.p.) of KET acutely administered. Interestingly, this effect seems to be correlated to alterations in the tyrosine

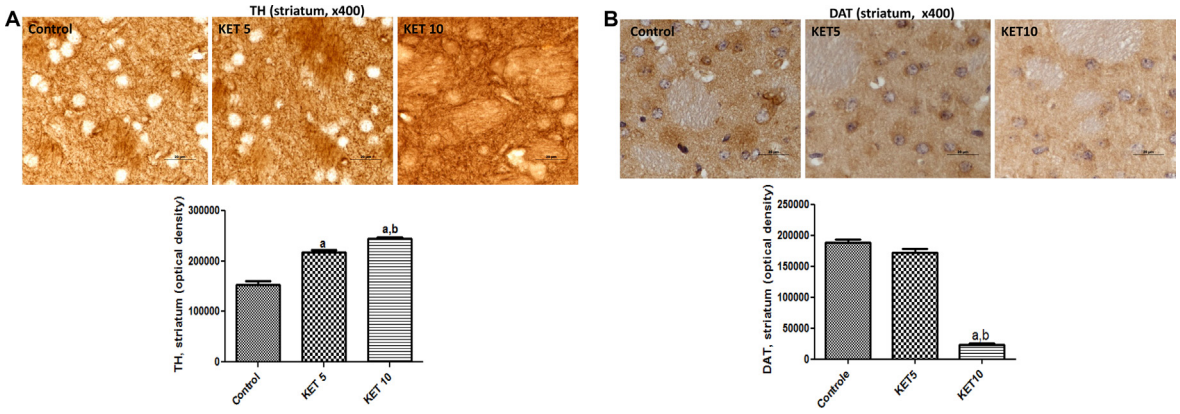

Fig. 4. Representative photomicrographs (x400) showing that ketamine (KET, 5 and 10 mg/kg) significantly increased tyrosine hydroxylase (TH) and decreased dopamine transporter (DAT), in mice striata. **TH: a.** vs. Control, $q = 8.917$, $p < 0.01$; **b.** vs. KET10, $q = 6.512$, $p < 0.01$; **c.** vs. Control, $q = 15.43$, $p < 0.001$. **DAT: a.** vs. Control, $q = 8.000$, $p < 0.001$; **b.**vs. KET10, $q = 18.77$, $p < 0.001$; **c.** vs. Control, $q = 25.70$, $p < 0.001$. The data were quantified by the Image J software (NIH, USA), by using the entire image areas from 3 different animals (One-way Anova and Tukey as the post hoc test).

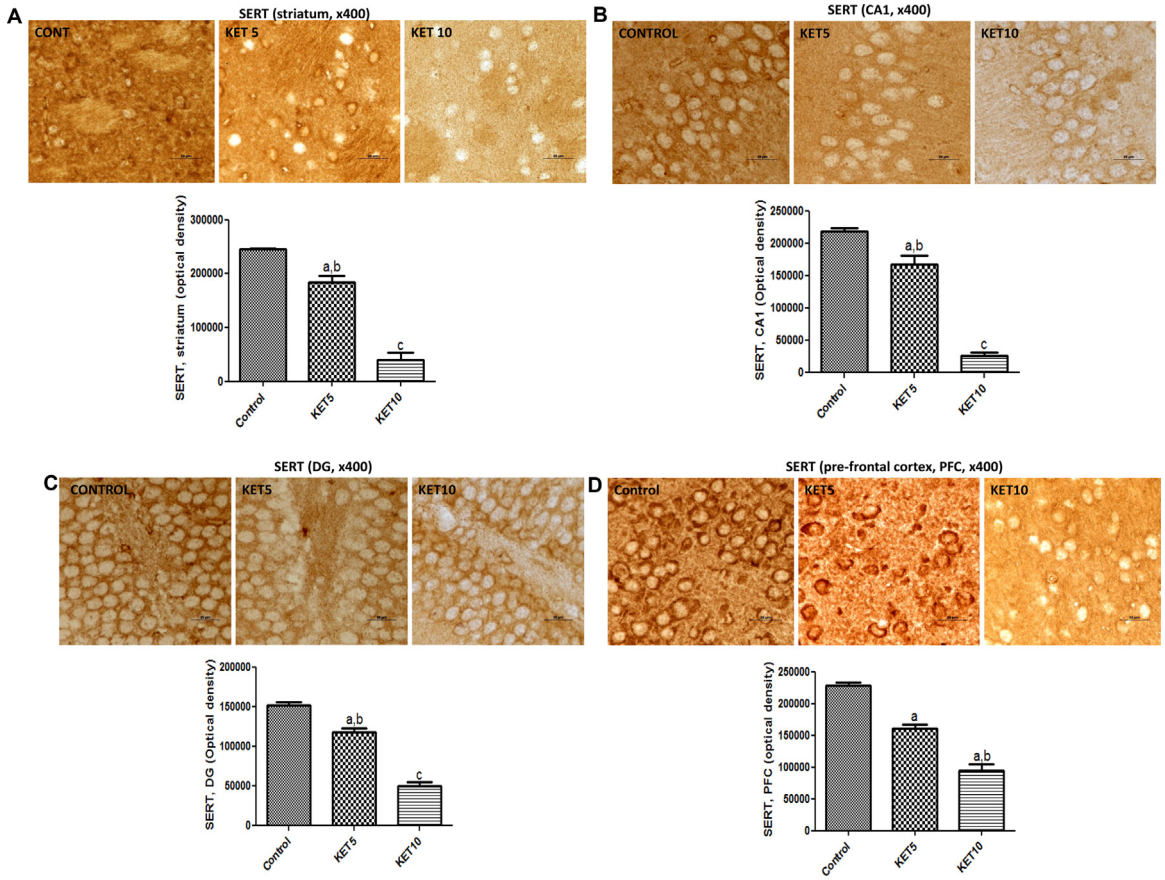

Fig. 5. Representative photomicrographs (x400) showing that ketamine (KET, 5 and 10 mg/kg) significantly decreased serotonin transporter (SERT), in mice brain. **Striata: a.** vs. Control, $q = 4.884$, $p < 0.05$; **b.** vs. KET10, $q = 15.25$, $p < 0.001$; **c.** vs. Control, $q = 20.14$, $p < 0.001$. **Hippocampal CA1: a.** vs. Control, $q = 5.681$, $p < 0.01$; **b.** vs. KET10, $q = 15.53$, $p < 0.001$; c. vs. Control, $q = 21.22$, $p < 0.001$. **Dentate gyrus, DG: a.** vs. Control, $q = 7.545$, $p < 0.01$; **b.** vs. KET10, $q = 14.95$, $p < 0.001$; **c.** vs. Control, $q = 22.49$, $p < 0.001$. **Prefrontal cortex, PFC: a.** vs. Control, $q = 21.05$, $p < 0.001$; **b.** vs. KET10, $q = 56.90$, $p < 0.001$; **c.** vs. Control, $q = 35.85$, $p < 0.001$. The data were quantified by the Image J software (NIH, USA), by using the entire image areas from 3 different animals (One-way Anova and Tukey as the post hoc test).

hydroxylase (TH) and dopamine transporter (DAT) immunostainings, in mice striata. In these assays, small but significant increases in TH were observed after the two doses of KET, while significant decreases were seen in DAT immunoreactivity. Interestingly, systemic TH treatment has been shown to improve the behavioral dispair, as evaluated by the forced swimming and tail suspension tests. A previous finding (Ferro et al., 2007) observed a lower percentage of reduction in nigral TH-immunostained cells, in a Parkinson's disease model, pointing out to a neuroprotective KET effect. Others demonstrated that KET inhibits DAT stereoselectively, what may contribute to its antidepressive-like effect

and potentiation of monoaminergic neurotransmission (Nishimura et al., 1998; Nishimura and Sato, 1999).

Furthermore, an in *vitro* study (Tan et al., 2012) showed that KET decreased the viability of PC12 cells and increased DA concentrations, in a dose-dependent manner. In addition, in the long-term (3 months), KET treated mice presented significant increases in DA contents in the midbrain. These findings were supported by upregulation of TH, as observed by us. However, in our studies, changes in DA contents were observed shortly after the acute treatment. Evidences (Iskandrani et al., 2015) indicate that the acute KET administration produces a rapid increase of cathecolaminergic neurons firing, through an amplification of AMPA transmission that could be crucial to KET effects.

More important than the changes in striatal DA and its metabolites, as demonstrated in the present work, were the higher increases in striatal 5-HT levels, observed after KET administration. A growing number of evidences (Yoshizawa et al., 2013; Gigliucci et al., 2013; Nishitani et al., 2014; Du Jardin et al., 2016; Fukumoto et al., 2016) has shown the involvement of serotonergic neurotransmission with the antidepressive-like effect of KET. In cell cultures, KET was shown to inhibit SERT in a dose-dependent manner and to increase serotonin contents in rodent brain (Zhao and Sun, 2008; Chatterjee et al., 2012). In addition, a PET study with nonhuman primates (Yamanaka et al., 2014) revealed that KET significantly increased 5-HT1B receptor binding, in the *nucleus accumbens* and *ventral pallidum*, and significantly reduced SERT binding.

Interestingly, we also showed a slight decrease in DOPAC/DA as well as in HVA/DA ratios, in mice striata after KET treatments. These results indicate a change in DA turnover and release. Previously (Murphy et al., 1996), an anxiogenic beta-carboline was shown to elevate dopamine turnover in the prefrontal cortex.The authors concluded that the higher dopamine activity is detrimental to cognitive functions. In our case, the decrease in DOPAC/DA turnover could suggest an anxiolytic-like effect of KET, also shown by others (Engin et al., 2009). However, this is still not conclusive, since a recent work (Pham et al., 2017) indicates that KET has no anxiolytic-like effect.

In line with these findings, we showed that KET (at the doses of 5 and 10 mg/kg) significantly decreased the serotonin transporter (SERT), in the striata. SERT is responsible for the serotonin reuptake into neurons, after its release, and represents the primary target for 2nd generation antidepressants, as fluoxetine (Tavoulari et al., 2009). Interestingly, these findings were also demonstrated in the hippocampus and prefrontal cortex.Evidences (Yamamoto et al., 2013) indicate that subanesthetic ketamine selectively enhances serotonergic transmission, by inhibition of SERT activity and, according to these authors, this KET action coexists with the rapid antidepressive-like effect of the drug. The transcriptional profile of KET reflects its multi-target pharmacological nature and similarities between its effects and monoaminergic antidepressives (Ficek et al., 2016).

In conclusion, our findings point out to the monoaminergic neurotransmission involvement, at the onset of KET antidepressive-like effect. This result is in disagreement with a recent work (Can et al., 2016) showing that neither KET enantiomers nor its metabolites have affinity for DA receptors or to DA, NE and 5-HT transporters. However, those authors used a different method for measuring extracellular DA concentrations (fast-scan cyclic voltametry) and for monoamine transporters (HTLA cells culture). In our work, we used *ex-vivo* assays for measuring monoamines by HPLC. In addition, TH, DAT and SERT immunohistochemistry assays were analyzed, shortly after the acute treatment. Most importantly, our work points out to serotonin as a possible target for KET actions, at least at the onset of its antidepressive-like effects, enhancing the serotonergic transmission by inhibiting SERT activity.

Authors contributions

CCX, EMV, JPL and VJA carried out the experiments and were responsible for the animals' treatments. ROC and KRTV carried out the HPLC experiments and immunohistochemistry assays. GSBV was responsible for the design and writing of the manuscript first draft. All authors contributed to the revision and approved the final version of the manuscript.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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