



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.

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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 $24 \pm 2^\circ\text{C}$ 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 \times 20 cm diameter) is filled with water (15 cm from the bottom, at 25°C). The animals ($n = 13\text{--}24$) 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 octyl 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 intraperitoneal 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 Mayer's hematoxylin, washed in tap water, dehydrated in alcohol (at increasing concentrations), diaphonized in xylol and mounted on Entellan[®] for optic microscopy examination, with the Nikon Eclipse Trinocular Microscope (Nis) and $\times 100$ or $\times 400$ magnifications. The immunos-

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