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Expression of heat shock protein HSP-70 in the retrosplenial cortex of rat brain after administration of (*R,S*)-ketamine and (*S*)-ketamine, but not (*R*)-ketamine

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ABSTRACT

The *N*-methyl-D-aspartate receptor (NMDAR) antagonist (*R,S*)-ketamine has robust antidepressant effects in depressed patients although it has detrimental side effects such as psychotomimetic and dissociative symptoms. (*R,S*)-Ketamine is known to cause the expression of heat shock protein HSP-70 (a marker for neuronal injury) in the retrosplenial cortex of rat brain, suggesting that the neuropathological changes may play a role in the detrimental side effects of (*R,S*)-ketamine. This study was undertaken to examine whether (*R,S*)-ketamine and its two enantiomers, (*R*)-ketamine and (*S*)-ketamine, causes the expression of HSP-70 in the rat retrosplenial cortex after a single administration. The HSP-70 immunohistochemistry in the rat brain was performed 24 h after intraperitoneal administration of saline (1 ml/kg), (+)-MK-801 (or dizocilpine: 1.0 mg/kg), (*R,S*)-ketamine (100 mg/kg), (*S*)-ketamine (25, 50, or 75, mg/kg), or (*R*)-ketamine (25, 50, or 75 mg/kg). Marked expression of HSP-70 immunoreactivity in the retrosplenial cortex was detected after administration of dizocilpine or (*R,S*)-ketamine (100 mg/kg). Higher does (50 and 75 mg/kg) of (*S*)-ketamine, but not low dose (25 mg/kg), caused expression of HSP-70 in this region. In contrast, all doses of (*R*)-ketamine did not induce the expression of HSP-70 in this region. These findings suggest that marked expression of HSP-70 in the retrosplenial cortex after a single dose of (*R,S*)-ketamine or (*S*)-ketamine may have detrimental side effects in the rat brain. Therefore, it is likely that (*R*)-ketamine is a safer compound in humans than (*R,S*)-ketamine and (*S*)-ketamine.

1. Introduction

The *N*-methyl-D-aspartate receptor (NMDAR) antagonist (*R,S*)-ketamine exhibits rapid-acting and long-lasting antidepressant effects in treatment-resistant patients with major depressive disorder or bipolar disorder (Kishimoto et al., 2016; Newport et al., 2015; Xu et al., 2016). Although there remain concerns about the safety of repeated (*R,S*)-ketamine infusions, (*R,S*)-ketamine has been used widely as off-label treatment for mood disorders (Sanacora et al., 2017; Wilkinson et al., 2017; Zhang et al., 2016).

The non-competitive NMDAR antagonists such as (*R,S*)-ketamine (Ki = 0.53 μM for NMDAR), phencyclidine (PCP) (Ki = 0.060 μM for NMDAR), and (+)-MK-801 (or dizocilpine) (Ki = 0.0019 μM for NMDAR) (Ebert et al., 1997) are known to cause neuropathological changes in the posterior cingulate cortex and retrosplenial cortex of rat brain (Olney et al., 1989, 1991). The order of potencies of neuropathological changes by the NMDAR antagonists in these regions is associated with the binding affinity of these compounds at NMDAR (Olney et al., 1989). Furthermore, the NMDAR antagonists, including (*R,S*)-ketamine, PCP and dizocilpine, were reported to cause heat shock protein HSP-70 (a marker of neuronal injury) in the same regions (Hashimoto et al., 1996, 1997; Nakki et al., 1996; Sharp et al., 1991, 1992; Tomitaka et al., 1996). Taken together, these findings raise the questions regarding the safety of (*R,S*)-ketamine in clinical and off-label use for mood disorders.

(*R,S*)-Ketamine (Ki = 0.53 μM at NMDAR) is a racemic mixture containing equal parts of (*R*)-ketamine (arketamine) (Ki = 1.4 μM at NMDAR) and (*S*)-ketamine (esketamine) (Ki = 0.3 μM at NMDAR) (Ebert et al., 1997). (*S*)-ketamine shows approximately 3–4-fold greater anesthetic potency and greater undesirable psychotomimetic side effects than (*R*)-ketamine (Domino, 2010). Meanwhile, (*R*)-ketamine shows greater potency and longer-lasting antidepressant effects than (*S*)-ketamine in animal models of depression (Fukumoto et al., 2017; Yang et al., 2015, 2017a, 2017b, 2018; Zhang et al., 2014). Unlike (*S*)-ketamine, (*R*)-ketamine does not induce behavioral side effects or exhibit abuse potential in rodents (Yang et al., 2015, 2016). Furthermore,

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we reported a marked reduction of dopamine $D_{2/3}$ receptor binding in conscious monkey striatum after a single infusion of (*S*)-ketamine but not that of (*R*)-ketamine, suggesting that (*S*)-ketamine-induced dopamine release might be associated with acute psychotomimetic and dissociative side effects in humans (Hashimoto et al., 2017). Therefore, (*R*)-ketamine could be a safer antidepressant in humans than (*S*)-ketamine (Hashimoto, 2016a, 2016b, 2016c, 2017). At present, the neurotoxic effects of two ketamine enantiomers, (*S*)-ketamine and (*R*)-ketamine, in these brain regions have not been explored.

This study was undertaken to examine whether (*R,S*)-ketamine and its two enantiomers, (*S*)-ketamine and (*R*)-ketamine, could cause the expression of HSP-70 in the retrosplenial cortex of rat brain after a single administration. Furthermore, we used the potent NMDAR antagonist dizocilpine as a positive control since a single dose of dizocilpine (1.0 mg/kg) caused the marked expression of HSP-70 in these regions (Hashimoto et al., 1996, 1997; Okamura et al., 2003, 2004; Sharp et al., 1991, 1992).

2. Methods

2.1. Animals

Female Sprague-Dawley rats (n = 27), aged 12 weeks (body weight 260–310 g, Charles River Laboratories Japan, Inc., Tokyo, Japan). Animals were housed under controlled temperatures and 12 h light/dark cycles (lights on between 07:00–19:00 h), with ad libitum food (CE-2; CLEA Japan, Inc., Tokyo, Japan) and water. In this study, we used female rats since female rats are known to be more sensitive than male rats to the neurotoxic effects of the NMDAR antagonists (Farber et al., 1995; Fix et al., 1995; Matsuki et al., 2001). The protocol was approved by the Chiba University Institutional Animal Care and Use Committee (Permission number: 30–324). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, USA. All efforts were made to minimize suffering.

2.2. Materials

(*R*)-Ketamine hydrochloride and (*S*)-ketamine hydrochloride were prepared by recrystallization of (*R,S*)-ketamine (Ketalar®, ketamine hydrochloride, Daiichi Sankyo Pharmaceutical Ltd., Tokyo, Japan) and D-(−)-tartaric acid and L-(+)-tartaric acid, as described previously (Zhang et al., 2014). Previously, we reported that the dose (20 mg/kg) of (*R*)-ketamine showed rapid-acting and long-lasting antidepressant effects in the rat learned helplessness model of depression (Yang et al., 2015; Shirayama and Hashimoto, 2018). In addition, the dose (50 and 75 mg/kg as hydrochloride) of (*R,S*)-ketamine caused the expression of HSP-70 in the retrosplenial cortex although the dose (25 mg/kg) did not cause the expression of HSP-72 in the same regions (Tomitaka et al., 1996). Therefore, the doses (25, 50 and 75 mg/kg) of (*R*)-ketamine and (*S*)-ketamine were used in this study. Dizocilpine (or (+)-MK-801 hydrogen maleate, 1.0 mg/kg, Sigma-Aldrich Co., Ltd., St Louis, MO, USA) and (*R,S*)-ketamine (100 mg/kg) were used as positive control. All compounds were dissolved in the physiological saline. Other reagents were purchased commercially.

2.3. HSP-70 immunohistochemistry

Twenty four hours after intraperitoneal administration of saline (1 ml/kg), dizocilpine (1.0 mg/kg), (*R,S*)-ketamine (100 mg/kg), (*S*)-ketamine (25, 50 or 75 mg/kg), or (*R*)-ketamine (25, 50 or 75 mg/kg), rats were deeply anaesthetized with isoflurane and sodium pentobarbital. The time allows maximal HSP-70 induction after administration of NMDAR antagonists (Hashimoto et al., 1996, 1997; Okamura et al., 2003, 2004; Sharp et al., 1991; Tomitaka et al., 1996).

Rats were transcardially perfused with 100 ml of isotonic saline followed by 250 ml of ice-cold 4% para-formaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed, postfixed for 90 min at 4 °C, and 100 μm thick coronal sections were cut from each brain with a vibrating blade microtome (Leika VT1000S, Leika Microsystems KK, Tokyo, Japan) on a through the retrosplenial cortex just posterior to splenium of the corpus callosum, ~5.3–6.0 mm posterior to bregma (Paxinos and Watson, 1998). Although the retrosplenial cortex is referred by some authors as the posterior cingulate cortex, it will be called the retrosplenial cortex in this study, according to Paxinos and Watson (1998).

HSP-70 immunohistochemistry was performed as described previously (Hashimoto et al., 1996, 1997; Okamura et al., 2003, 2004; Tomitaka et al., 1996) using the Vestastain® Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). Sections were places in PBS containing 2% horse serum, 0.2% Triton X-100, 0.1% bovine serum albumin (HS-PBST) and 2 h at room temperature. They were then incubated overnight at 4 °C in the primary antibody to mouse anti-human HSP-70 (cat #: SMC-100B, StressMarq Biosciences, Victoria, BC, Canada) diluted 1:2000 in HS-PBST. Sections were then washed twice in PBS, incubated for 2 h in the second antibody (biotinylated gorse anti-mouse IgG adsorbed against rat serum) and incubated in avidin-horseradish peroxidase solution, prepared from the kit, for 2 h at room temperature. Sections were washed twice in ice-cold PBS and the antibody reaction was developed with 3,3′-diaminobenzidine (0.015%) and 0.001% hydrogen peroxidase in PBS. Sections were washed for 1 h and mounted on gelatinized slides, dehydrated through an ethanol gradient and cleared in xylene, and coverslipped with Permount® (Fisher Scientific, Fair Lawn, NJ, USA). For quantitative analysis, the number of HSP-70-immunoraective neurons within a 0.036 mm^2 area of the granular retrosplenial cortex and the granular retrosplenial cortex (5.8 mm posterior to bregma) was counted. The numbers of HSP-70-immunoreactive neurons per three sections were determined by direct visual counting at high magnification with the use of a light microscope. The number of HSP-70-immunoreactive neurons per slide per four sections was averaged for each rat. Anatomical identification of sections was made according to the rat brain atlas of Paxinos and Watson (1998). The representative image of HSP-70 immunoreactivity in the rat retrosplenial cortex was obtained using Keyence BZ-9000 Generation microscope (Keyence Co., Ltd., Osaka, Japan).

2.4. Statistical analysis

The data show as the mean ± standard error of the mean (S.E.M.). Analysis was performed using PASW Statistics 20 (formerly SPSS Statistics; SPSS, Tokyo, Japan). The data were analyzed using the one-way analysis of variance (ANOVA), followed by *post-hoc* Dunnet test. The P-values of < 0.05 were considered statistically significant.

3. Results

3.1. Behavioral observations

Behavioral responses occurred within 5 min after a single administration of (*R,S*)-ketamine, or (*S*)-ketamine. Administration of these compounds produced head weaving, biting in a stationary posture, and increased locomotion within 15 min of injection. Although we did not quantify the behavioral abnormalities, the order of potencies of behavioral abnormalities was (*S*)-ketamine > (*R,S*)-ketamine > (*R*)-ketamine, indicating that these behavioral abnormalities are associated with binding affinities of the compounds at NMDAR (Ebert et al., 1997). Rats treated with dizocipline (1.0 mg/kg) showed abnormal behaviors, including head weaving, biting in a stationary posture, as previously reported (Hashimoto et al., 1997).