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Behavioral and biochemical sensitivity to low doses of ketamine: Influence of estrous cycle in C57BL/6 mice

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ABSTRACT

Rationale: Low-dose ketamine is a rapid-acting antidepressant, to which female rodents are more sensitive as compared to males. However, the mechanism mediating this sex difference in ketamine sensitivity remains elusive.
Objectives: We sought to determine whether male and female mice differ in their behavioral sensitivity to low doses of ketamine, and uncover how ovarian hormones influence females' ketamine sensitivity. We also aimed to uncover some of the molecular mechanism(s) in mood-related brain regions that mediate sex differences in ketamine antidepressant effects.
Methods: Male and female mice (freely-cycling, diestrus 1 [D1], proestrus [Pro], or D1 treated with an estrogen receptor (ER) α, ERβ, or progesterone receptor (PR) agonist) received ketamine (0, 1.5, or 3 mg/kg, intraperitoneally) and were tested in the forced swim test (FST) 30 min later. Ketamine's influence over synaptic plasticity markers in the prefrontal cortex (PFC) and hippocampus (HPC) of males, D1, and Pro females was quantified by Western blot 1 h post-treatment.
Results: Males, freely cycling females, D1 and Pro females exhibited antidepressant-like responses to 3 mg/kg ketamine. Pro females were the only group where ketamine exhibited an antidepressant effect at 1.5 mg/kg. D1 females treated with an agonist for ERα or ERβ exhibited an antidepressant-like response to 1.5 mg/kg ketamine. Ketamine (3 mg/kg) increased synaptic plasticity-related proteins in the PFC and HPC of males, D1, and Pro females. Yet, Pro females exhibited an increase in p-Akt and p-CaMKIIα in response to 1.5 and 3 mg/kg ketamine.
Conclusion: Our results indicate that females' enhanced sensitivity to ketamine during Pro is likely mediated through estradiol acting on ERα and ERβ, leading to greater activation of synaptic plasticity-related kinases within the PFC and HPC.

1. Introduction

The N-methyl-D-aspartate (NMDA) receptor antagonist, ketamine, has shown great promise as a rapid-acting antidepressant in humans and rodents (Abdallah et al., 2015; Kavalali and Monteggia, 2015). In clinical studies, a single infusion of low-dose of ketamine alleviates symptoms of depression within hours (Berman et al., 2000; Zarate et al., 2006). This rapid effect is superior to other antidepressants (e.g., selective serotonin-reuptake inhibitors), which can take weeks to months to provide therapeutic benefit (Nutt, 2002; Rush et al., 2006). These exciting findings have burgeoned interest in uncovering the neural loci and molecular mechanisms of ketamine's rapid antidepressant effects. Low doses of ketamine promote the expression of downstream signaling of synaptic plasticity-related pathways within mood-related brain regions, such as the prefrontal cortex (PFC) and hippocampus (HPC) (Björkholm and Monteggia, 2016; Duman et al., 2016). While there have been great advances in our understanding of some molecular mechanisms of ketamine's rapid effects, the majority of these investigations have been conducted in males; creating a serious gap in our knowledge, since females experience a two-fold increased risk of depression as compared to males (Kessler, 2003; Van de Velde et al., 2010). In addition the disparity in risk of depression, there is evidence that pharmacodynamics and pharmacokinetics of antidepressant compounds may differ between the sexes and can

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be influenced by a variety of factors including levels of ovarian hormones (Bigos et al., 2009). Ovarian hormones, estrogen (E_2) and progesterone (P_4), influence many common biochemical targets to promote synaptic plasticity and increase spine density in mood-related brain regions (Frick, 2015; Woolley and McEwen, 1992; Woolley et al., 1997). Thus, it is possible for peripherally- or centrally-mediated factors to influence females' sensitivity to the antidepressant-like effects of ketamine.

Preclinical studies from our lab and others have shown female rodents exhibit behavioral sensitivity to doses of ketamine that are subthreshold for an antidepressant-like effect in males (Carrier and Kabbaj, 2013; Franceschelli et al., 2015; Sarkar and Kabbaj, 2016; Zanos et al., 2016). While most of these studies did not control for the stage of estrous, there is evidence that females' sensitivity may be mediated by the cyclic fluctuations of the ovarian hormones, E_2 and P_4 (Carrier and Kabbaj, 2013; Sarkar and Kabbaj, 2016). This is based upon evidence that surgical removal of the ovaries blocked females' sensitivity and pharmacological replacement of E_2/P_4 restored females' sensitivity to a male-subthreshold dose of ketamine (Carrier and Kabbaj, 2013). However, the molecular mechanism(s) mediating these sex differences remain elusive. In the first part of this work, we examined sensitivity to low doses of ketamine in male and female mice in which we did not control for the stage of estrous cycle (i.e., freely cycling). Then, to investigate the impact of estrous cycle on sensitivity to ketamine, we then evaluated sensitivity to ketamine in females experiencing a natural nadir (diestrus 1, D1) or a peak (proestrus, Pro) in ovarian hormone levels. To further determine which hormone receptor subtypes might be mediating ketamine sensitivity in females, D1 females were treated with an estrogen receptor (ER) α, ERβ, or progesterone receptor (PR) agonist and tested 24 h later in the FST. Finally, we examined sex/estrous stage differences and ketamine-induced activation of neurotrophic signaling pathways within the prefrontal cortex (PFC) and hippocampus (HPC) of males, D1, and Pro females to explore which one(s) may mediate sex differences in ketamine sensitivity.

2. Materials and methods

2.1. Animals

One-hundred and eighty five adult male and female C57BL6/J mice (46 males and 139 females, mean weight at time of testing 23.7 and 18.8 g, respectively) from The Jackson Laboratory (Bar Harbor, Maine) aged 7 weeks at time of arrival were used in these experiments. Upon arrival, mice were pair-housed in same-sex pairs and maintained on a 12 h light:12 h dark cycle in a temperature-controlled vivarium in standard Plexiglass cages. Food and water were available *ad libitum*. Males and females were habituated to animal facilities and handling for one week prior to the start of behavioral testing. All experimental procedures were approved by the Florida State University Institutional Animal Care and Use Committee and conform to the NIH Guide for Care and Use of Laboratory Animals.

2.2. Drug treatments

Mice received a single intraperitoneal injection of sterile saline (Veh), 1.5, or 3 mg/kg of racemic ketamine hydrochloride (Ketasthesia®, Henry Schein) and behavioral tests were conducted 30 min later. Females in D1 received a subcutaneous injection of either 4,4′,4″-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT, 0.5 mg/kg, Tocris Bioscience; ERα agonist), Diarylpropionitrile (DPN, 0.5 mg/kg, Tocris Bioscience; ERβ agonist) or progesterone (P_4, 0.5 mg/kg, Sigma-Aldrich), 24 h prior to behavioral testing. This estrous stage was chosen because of the low levels of E2 and P4 during D1. The idea tested here is that during D1 ketamine at 1.5 mg/kg will have no antidepressant effects, however if we stimulate ERs or progesterone receptors during D1 (as to mimic what happens during Pro) ketamine may have an antidepressant-like effect. This experiment will also allow us to determine which receptors are critical in enhancing females' sensitivity to ketamine antidepressant effects.

2.3. Vaginal cytology

Female mice were gently handled and lavaged daily with 25 μl sterile 0.9% saline as described elsewhere (McLean et al., 2012) within the first 3 h of the light cycle. For daily analysis of estrous stage, vaginal epithelial cells were deposited onto glass slides and immediately viewed with a light microscope. Degenerated, cornified epithelial cells and few stained leukocytes characterized the D1 estrous stage. The Pro stage was characterized by an abundance of rounded, nucleated epithelial cells. For crystal violet staining, slides were dried for 6 h at room temperature, stained with 0.1% crystal violet (Sigma-Aldrich) for 1 min, rinsed with DDH20 for 1 min x2, sealed with 30 μl Permount (Sigma-Aldrich), and coverslipped. If a female remained in single estrous stage for more than 4 days, she was excluded from the study.

2.4. Forced swim test (FST)

The FST is a sensitive measure of behavioral effects of antidepressant treatments in rodents (Porsolt et al., 1977). Mice were placed into a 4-L Pyrex glass beaker containing 3 L of water at 24 ± 1 °C and forced to swim for 6 min. After the FST, mice were returned to their home cage and placed in a holding room until sacrifice. The FST was recorded by a video camera and videos were saved for later analysis. Beakers were cleaned with 70% EtOH and filled with fresh water between each FST. Treatments were delivered in a completely randomized design and counterbalanced such that cage mates received different treatments, and were examined in the FST in parallel. An observer who was blind to experimental assignments when the FST was conducted scored immobility time during the final 4 min of the FST. The final 4 min are commonly scored because mice are active during the onset of this test and this activity may mask the effect of the treatment (Can et al., 2012). Immobility time was defined as the duration of time in which the mouse maintained this stationary posture and only made movements necessary to keep its head above water.

2.5. Tissue collection and processing

Mice were sacrificed via rapid decapitation 24 min after the conclusion of the FST, which corresponds to 1 h post-vehicle or post-ketamine treatment. This time point was chosen due to reports that ketamine can rapidly, yet transiently influence protein expression and phosphorylation of molecules implicated in plasticity (Autry et al., 2011; Li et al., 2010). Trunk blood was collected into tubes containing chilled 0.5 M EDTA (Sigma-Aldrich) and stored on ice. Plasma was extracted via refrigerated centrifugation (2800 rpm for 20 min, at 4 °C), transferred to sterile pre-chilled microcentrifuge tubes, and stored at −80 °C. Brains were rapidly removed, flash frozen in methyl butane (Sigma-Aldrich) on dry ice at −20 °C and stored at −80 °C. Brains underwent coronal sectioning at 100 μM at −20 °C in a cryostat, tissue punches containing the PFC (prelimbic and infralimbic cortices) and HPC (CA1, CA3, and dentate gyrus) were collected in sterile microcentrifuge tubes and stored at −80 °C. Total protein was extracted from these punches using the Tri Reagent protocol (Molecular Research