**SEX DIFFERENCES IN THE RAPID AND THE SUSTAINED
ANTIDEPRESSANT-LIKE EFFECTS OF KETAMINE IN STRESS-NAÏVE
AND “DEPRESSED” MICE EXPOSED TO CHRONIC MILD STRESS**

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Anthony Albert Franceschelli

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Name: Franceschelli, Anthony Albert

APPROVED BY:

Pothitos M. Pitychoutis, Ph.D.
M.Sc. Advisor
Assistant Professor
Department of Biology
University of Dayton

Carissa Krane, Ph.D.
Committee Member
Associate Professor
Department of Biology
University of Dayton

Amit Singh, Ph.D.
Committee Member
Associate Professor
Department of Biology
University of Dayton

ABSTRACT

SEX DIFFERENCES IN THE RAPID AND THE SUSTAINED ANTIDEPRESSANT-LIKE EFFECTS OF KETAMINE IN STRESS-NAÏVE AND “DEPRESSED” MICE EXPOSED TO CHRONIC MILD STRESS

Name: Franceschelli, Anthony Albert
University of Dayton

Advisor: Dr. Pothitos M. Pitychoutis

During the past decade, one of the most striking discoveries in the treatment of major depression was the clinical finding that a single infusion of a sub-anesthetic dose of the N-methyl-D-aspartate receptor antagonist ketamine produces a rapid (i.e. within a few hours) and long-lasting (i.e. up to two weeks) antidepressant effect in both treatment-resistant depressed patients and in animal models of depression. Notably, converging clinical and preclinical evidence support that responsiveness to antidepressant drugs is sex-differentiated. Strikingly, research regarding the antidepressant-like effects of ketamine has focused almost exclusively on the male sex. Herein we report that female C57BL/6J stress-naïve mice are more sensitive to the rapid and the sustained antidepressant-like effects of ketamine in the forced swim test (FST). In particular, female mice responded to lower doses of ketamine (i.e. 3 mg/kg at 30 min and 5 mg/kg at 24h post-injection), doses that were not effective in their male counterparts. Moreover, tissue levels of the excitatory amino acids

glutamate and aspartate, as well as serotonergic activity, were affected in a sex-dependent manner in the prefrontal cortex and the hippocampus, at the same time-points. Most importantly, a single injection of ketamine (10 mg/kg) induced sex-dependent behavioral effects in mice subjected to the chronic mild stress (CMS) model of depression. Intriguingly, female mice were more reactive to the earlier effects of ketamine, as assessed in the open field and the FST (at 30 min and 24 h post-treatment, respectively) but the antidepressant potential of the drug proved to be longer-lasting in males, as assessed in the splash test and the FST (days 5 and 7 post-treatment, respectively). Taken together, present data revealed that ketamine treatment induces sex-dependent rapid and sustained neurochemical and behavioral antidepressant-like effects in stress-naïve and CMS-exposed C57BL/6J mice.

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LIST OF ABBREVIATIONS

WHO: world health organization

NMDAR: N-methyl-D-aspartate receptor

CMS: chronic mild stress

FST: forced swim test

HIPP: hippocampus

PFC: prefrontal cortex

VEH: vehicle

HPLC: high performance liquid chromatography

5-HT: serotonin, 5-hydroxytryptamine

5-HIAA: 5-hydroxy-indoleacetic acid

ANOVA: analysis of variance

GABA: γ -aminobutyric acid

SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptor

INTRODUCTION

According to the World Health Organization (WHO), by 2020 major depression is expected to rise to become the number two contributor to the global burden of disease (Collins et al., 2011). Current treatments for depressive disorders are only partly effective and patients who suffer from treatment-resistant depression require innovative pharmacological agents with novel mechanisms of action. Of note, women experience major depression at roughly twice the rate of men (Holden, 2005, Marcus et al., 2005, Grigoriadis and Robinson, 2007) and respond differentially to different types of antidepressant treatment (Young et al., 2009). The neurobiological mechanisms underlying these differences remain a largely neglected area of research and current treatments are based almost exclusively on research in male subjects (Beery and Zucker, 2011).

During the past decade, one of the most striking discoveries in the treatment of major depression was the clinical finding that a single infusion of a sub-anesthetic dose of the N-methyl-D-aspartate receptor (NMDAR) antagonist ketamine produces a rapid (i.e. within a few hours) and sustained (i.e. lasting from one day up to two weeks) antidepressant effect in both treatment-resistant depressed patients and in animal models of depression (Skolnick et al., 2009). In this context, administration of a single dose of ketamine in rodents has been shown to induce effects in antidepressant-predictive behavioral tasks including the forced swim test (FST) and the chronic mild stress (CMS) model of depression

(Garcia et al., 2008, Maeng et al., 2008, Engin et al., 2009, Li et al., 2010, Autry et al., 2011, Bechtholt-Gompf et al., 2011, Li et al., 2011). Of note, CMS is an ideal paradigm with which to screen the antidepressant-like effects of novel pharmacotherapeutic agents in rodents and possibly the most valid method for the preclinical investigation of the antidepressant-like effects of ketamine (Browne and Lucki, 2013, Franceschelli et al., 2014). Indeed, the discovery of ketamine's antidepressant-like effects has reshaped and revolutionized antidepressant drug discovery (Browne and Lucki, 2013, Duman, 2014) and the behavioral and neurobiological mechanisms underlying its rapid and sustained antidepressant-like effects are under intensive investigation.

Converging clinical and experimental evidence from our group and others support that responsiveness to antidepressant drugs is sex-differentiated (Sloan and Kornstein, 2003, Marcus et al., 2005, Pitychoutis et al., 2010, Dalla et al., 2011, Pitychoutis et al., 2011, Pitychoutis et al., 2012). Strikingly, research regarding the antidepressant-like effects of ketamine has focused almost exclusively on the male sex. Recently, Carrier and Kabbaj (2013) provided the first evidence that female Sprague-Dawley rats are more sensitive to the rapid antidepressant-like effects of a single injection of ketamine in the FST, as they respond to a lower dose (i.e. 2.5 mg/kg), a dose that does not induce antidepressant-like effects in male rats. C57BL/6J is the most widely used inbred mouse strain and has been widely used in preclinical ketamine research (Autry et al., 2011, Browne and Lucki, 2013). To our knowledge, sex differences in response to ketamine have never been reported in this strain.

Despite the fact that both the glutamatergic and the serotonergic systems have been implicated in ketamine's mechanism of action (Gigliucci et al., 2013, Nishitani et al., 2014, Yamanaka et al., 2014), the intricate neurochemical mechanisms

underlying its effects remain elusive. For instance, microdialysis studies report that antidepressant-relevant doses of ketamine increase extracellular levels of the excitatory amino acid glutamate in the prefrontal cortex (PFC) due to a possible local disinhibition of γ -aminobutyric acid (GABA)-ergic interneurons (Lorrain et al., 2003, Duman, 2014). However, whether this effect is restricted to certain brain regions is not known. Notably, chronic treatment with conventional antidepressants has been shown to affect the serotonergic system and the levels of excitatory amino acids, glutamate and aspartate, in the prefrontal cortex (PFC) and the hippocampus (HIPP) in a sex-dependent manner (Kokras et al., 2009, Pitychoutis et al., 2011, Pitychoutis et al., 2012). However, putative sex differences in the neurochemical effects of ketamine have never been investigated.

In the present study we sought to investigate whether administration of a single dose of ketamine would induce sex-dependent behavioral and neurochemical effects in C57BL/6J mice. Our results show that female stress-naïve mice are more sensitive to both the rapid (i.e. at 30 min) and the sustained (i.e. at 24 h) antidepressant-like effects of ketamine in the FST. Moreover, ketamine treatment (10 mg/kg) induced region-dependent and sex-differentiated neurochemical effects in the PFC and the HIPP. Most importantly, a single injection of ketamine (10 mg/kg) induced intriguing sex-dependent behavioral effects in mice subjected to CMS for five weeks. Specifically, female CMS-exposed mice were more reactive to the earlier effects of ketamine, but the antidepressant potential of the drug proved to last longer in males.

METHODS

Animals

Adult male and female C57BL/6J mice used in this study were bred at the Vivarium of the University of Dayton from a mouse colony originally obtained from the Jackson Laboratory (ME, USA). Experimental procedures and animal husbandry were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23; revised 1996) and approved by the University of Dayton Animal Care and Use Committee (IACUC; N°: 014-01). Mice were kept on a 12 h/12 h light/dark cycle, in groups of four per cage, under standard laboratory conditions and were given access to food and water *ad libitum*, unless otherwise specified. All efforts were made to minimize the number of animals used and their suffering.

Drug treatment

Mice received a single injection of 3, 5 or 10 mg/kg body weight (BW) of ketamine hydrochloride (Henry-Schein; NY; USA) or vehicle (VEH; 0.9% NaCl). Drugs were injected intraperitoneally (i.p.) in a volume of 10 mL/kg of body weight. The sub-anesthetic doses of ketamine implemented, times and route of administration selected are commonly used to screen for the antidepressant-like effects of ketamine in preclinical rodent models (Browne and Lucki, 2013).

Experimental Design

In the current study we investigated whether behavioral and neurochemical responses of male and female C57BL/6J mice are sex-differentiated in three independent experiments. In an initial dose-dependent study (*Experiment #1*) we questioned whether naïve male and female mice would show differential sensitivity to increasing doses of ketamine in the FST, an antidepressant-predictive animal model. The 10 mg/kg ketamine dose that reliably induced both rapid and sustained antidepressant-like effects in the FST in both sexes was further implemented for neurochemical assessments in naïve mice (*Experiment #2*). Lastly, in Experiment #3, we investigated the antidepressant-like behavioral effects of a single 10 mg/kg dose of ketamine in “depressed” mice subjected to the chronic mild stress (CMS) model of depression. Given our aim to determine whether there is a basic sex difference in the way males and females respond to ketamine, the female reproductive state was not taken into account (McCarthy et al., 2012). Since it is generally agreed that the first stage of any sex-difference study should be a comparison of gonadally intact adult females and males (Greenspan et al., 2007), a two-group design (males and females) was implemented for testing for sex-differentiated traits, as in previous studies (Pitychoutis et al., 2012, Carrier and Kabbaj, 2013).

Experiment #1: Sex differences in the antidepressant-like effects of ketamine in stress-naïve mice subjected to the FST

Male (N=6-8/group) and female mice (N=7-8/group) received a single injection of ketamine (3, 5, or 10 mg/kg) or VEH and were subjected to the FST at two different time-points: at 30 min or 24h to screen for sex differences in the rapid

and the sustained antidepressant-like effects of this drug, respectively. Each mouse received a single ketamine injection and was tested in the FST only once.

Experiment #2: Sex differences in the neurochemical effects of ketamine in stress-naïve mice

Male and female mice (N=7-8/group) were treated with 10 mg/kg ketamine or saline (VEH) and were then sacrificed at 30 min or 24 h post-injection. Serotonergic neurochemical responses and glutamate and aspartate tissue concentrations were assayed *ex vivo* in the PFC and the HIPPO, two brain regions implicated in the pathophysiology of major depression and the antidepressant response.

Experiment #3: Sex differences in the effects of ketamine in mice exposed to chronic mild stress (CMS)

This experiment assessed the effects of ketamine in singly housed mice subjected to the CMS model of depression for 5 weeks. Singly housed mice not subjected to CMS served as controls (N=7-8/group). Mice received a single injection of ketamine (10 mg/kg) or saline (VEH), and during the following week they were subjected to a behavioral test battery. Tests were performed in the following order over 7 days post-injection, as shown in **Fig. 1**: open field (at 30 min), FST (at 24 h; day 1), marble burying (day 4), splash test (day 5), sucrose test (day 6), FST (day 7). Based on the dose-response FST study conducted in *Experiment #1*, the 10 mg/kg dose of ketamine was administered in the CMS experiment, as this dose was proven to induce rapid and sustained antidepressant-like effects in both sexes.

Behavioral testing

Forced swim test (FST)

The FST was conducted as previously described (Adachi et al., 2008, Autry et al., 2011, Stratinaki et al., 2013). Specifically, swim sessions were conducted by placing mice individually in a glass 4 L beaker filled with 3 L of water (23-25°C). Mice were gently placed in water and the duration of immobility (i.e. the time that mice spent making only the required movements to keep their head above water) was measured during the last 4 min of a 6-min trial. Mice were then removed from water, dried and placed in their home cage. In the CMS experiment, mice were subjected to the FST twice, based on the recent observation that C57BL/6 mice display stable behavior upon repeated FST exposure (Pozzi et al., 2014).

Chronic mild stress (CMS) model of depression

Male and female C57BL/6J mice (8 weeks of age) were housed in individual cages and allowed for 2 weeks to habituate before beginning experimentation. Mice were subjected to 5 weeks of CMS after the protocol described by Elizalde et al. (2008), with minor modifications. Briefly, the following stressors (two–three in any 24 h period) were applied: stroboscopic illumination (in dark 8 h), white noise (an untuned radio, 4 h), cage tilt 45° (8 h), soiled bedding (200 mL of water per cage; 6 h), paired housing (with new partner 2 h), food and water deprivation (8 h, not prior to sucrose consumption test), and overnight illumination and removal of nesting material (12 h) (Elizalde et al., 2008). Hedonic *status* was evaluated by weekly monitoring of sucrose intake, as indicated in the respective section. Following 5 weeks of CMS exposure and anhedonia establishment, both CMS and control mice were matched

into VEH and ketamine groups (N = 6-8 mice/group) according to their sucrose intake.

Spontaneous locomotor activity

Mice received a single dose of ketamine, and 30 min later they were placed in the center of an open-field arena (45 x 45 x 45 cm). Horizontal locomotor activity (distance traveled in cm) and time spent in the center of the open-field arena were recorded for 60 min using a video tracking system (SMART v3.0; PanLab; Barcelona, Spain) in a softly illuminated experimental room.

Sucrose consumption

Hedonic status was evaluated by weekly monitoring of sucrose intake, as previously described (Elizalde et al., 2008, Elizalde et al., 2010). After 2 weeks of initial habituation to single housing, mice were trained to consume a 2.5 % sucrose solution every other night (from 7:00 PM to 10:00 AM). Following the establishment of sucrose intake baseline, all mice were divided into CMS and control groups matched for their relative sucrose consumption, and the CMS application started. Once per week, mice were given a 15-h exposure to the sucrose solution in their home cage as described above, without prior food or water deprivation. Body weight measurements were taken weekly in both CMS and control mice and the relative sucrose intake was calculated as absolute intake (g) per mouse body weight.

Marble Burying

This is a test suggested to measure anxiety/compulsive behavior in mice. Marble burying behavior is attenuated by low doses of benzodiazepines, and on the basis of this pharmacological evidence, it is suggested that mice bury marbles because marbles evoke anxiety (Deacon, 2006). Mice were placed in a clean cage and 20 glass marbles of 15mm in diameter were placed in 5 rows, in a regular pattern evenly spaced from one another on the surface of approximately 5 cm-thick standard wood bedding. The test duration was 30 min and the number of marbles buried (>50% marble covered by bedding material) was recorded (Thomas et al., 2009).

Splash test

The splash test was performed during the dark period under red lighting, as previously described (Nollet et al., 2013). In this test, a 10% sucrose solution was sprayed on the dorsal coat of mice. The sprayer allowed delivery of a fixed volume (about 0.7 ml) of sucrose solution (each mouse received two sprays). The duration of grooming was recorded during five minutes after the vaporization of sucrose solution. Exposure to CMS has been shown to decrease grooming duration in the splash test, and this is reversed by chronic antidepressant treatment (Nollet et al., 2013).

Neurochemical analysis with high performance liquid chromatography (HPLC)

Following decapitation, the brain was carefully removed from the skull and the brain regions of interest were rapidly isolated on ice. After weighing, the tissue was homogenized and deproteinized in 0.2 N perchloric acid solution, as previously described (Pitychoutis et al., 2011). The homogenate was centrifuged at 15,000 rpm for 30 min at 4°C, and the supernatant was stored at -80 °C. Neurotransmitter

analysis was performed by HPLC with a coulometric detector. Briefly, samples were injected into an HPLC system that consisted of a pump (Dionex Ultimate 3000, Fisher Scientific, PA, USA), a reverse-phase column (Acclaim Polar Advantage II, 2.2 μ m, 3.0x100 mm or Accucore C18 2.6 μ m, 3.0x100mm) and coulometric detector (Dionex Ultimate 3000, Fisher Scientific, PA, USA) with a 6011RS ultra analytical cell to quantify tissue levels of 5-hydroxytryptamine (serotonin; 5-HT) and its metabolite, 5-hydroxy-indoleacetic acid (5-HIAA). The composition of the monoamine mobile phase was 75 mM Na₂HPO₄·H₂O, 1.7 mM 1-octanesulfonic acid sodium salt, 100 μ L/L triethylamine, 25 μ M EDTA and 10% (v/v) acetonitrile (pH 3.0). The first electrode was set at +50 mV and the second electrode at +350 mV, column temperature was set at 32°C and the flow rate was constant at 0.5 mL/min. The excitatory amino acids glutamate and aspartate were measured as their OPA/ β ME derivatives (Donzanti and Yamamoto, 1988). The composition of the amino acid mobile phase was 100mM Na₂HPO₄·2H₂O, 20% (v/v) methanol and 3.5% (v/v) acetonitrile, pH 6.7. The first electrode was set at +150 mV and the second electrode at +550 mV, column temperature was set at 40°C and the flow rate at 0.6 mL/min. The sensitivity of both assays was tested for each series of samples using external standards. The signal of the second electrode was used to quantify all compounds by comparison of the area under the peaks with the area of reference standards with specific HPLC software (Chromeleon 7; Fisher Scientific, PA, USA). The 5-HT turnover ratio (5-HIAA/5-HT) was calculated as index of the activity of the cells that integrate the synthesis, release, reuptake, and/or metabolism of serotonin (Commissiong, 1985, Elhwuegi, 2004).

Statistical analysis

All data are presented as means $\pm$ SEM. Statistical analyses were performed using the SPSS v.21 statistical software (SPSS Inc., Chicago, IL, USA). Data were analyzed by one- or two-way analysis of variance (ANOVA) for each sex, with stress (CMS vs. control) and drug treatment (Ketamine vs. VEH) as independent factors or by repeated-measures ANOVA followed by pair-wise comparisons. Homogeneity of covariance was assessed with the Mauchly's test of sphericity. In cases where sphericity was violated, the ANOVA F was modified according to the Greenhouse-Geisser correction to make it more conservative. The level of statistical significance was set at 0.05.

RESULTS

Experiment #1

Females are more sensitive to the rapid and the sustained antidepressant-like effects of ketamine in the FST

Male and female C57BL/6J mice displayed differential sensitivity to the rapid (i.e. at 30 min) antidepressant-like effects of a single dose of ketamine in the FST [$F_{(3,28)} = 10.548, p < .001$ and $F_{(3,28)} = 4.769, p = .009$, respectively]. Post-hoc analysis revealed that male mice responded to the higher doses of ketamine implemented (i.e. 5 and 10 mg/kg; $p = .045$ and $p < .001$, respectively) whereas females responded to the full dose-range (i.e. 3, 5 and 10 mg/kg; $p = .05$, $p = .037$ and $p < .001$, respectively; **Fig. 2a**). Similarly, the sustained antidepressant-like effects of ketamine (at 24 h post-injection) were differentially expressed between male and female mice [$F_{(3,24)} = 3.150, p = .046$ and $F_{(3,27)} = 4.198, p = .016$, respectively]. Post-hoc analysis revealed that male mice responded only to the highest dose of ketamine (i.e. 10 mg/kg; $p = .014$), whereas females responded to both the higher doses of ketamine administered (i.e. 5 and 10 mg/kg; $p = .012$, $p = .026$, respectively; **Fig 2b**).

Experiment #2

Ketamine induced rapid sex-dependent alterations in excitatory amino acids in the HIPP and the PFC

Ketamine treatment induced sex-dependent rapid (30 min) and sustained (24 h) neurochemical alterations in the HIPP and the PFC. In particular, at 30 min, ketamine induced a rapid decrease of glutamate concentrations in the HIPP of male mice [$F_{(1,13)} = 7.816$, $p = .016$; **Fig. 3a**] and an increase of aspartate levels in the PFC of female mice [$F_{(1,13)} = 4.798$, $p = .049$; **Fig. 3b**]. At 24 h post-ketamine administration, 5-HIAA/5-HT turnover ratios in the PFC and the HIPP were found decreased in female mice in a sex-specific manner [$F_{(1,15)} = 7.181$, $p = .018$; $F_{(1,15)} = 5.968$, $p = .028$, respectively] (**Table 1**).

Experiment #3

CMS exposure induced anhedonia in both sexes

Following CMS exposure, anhedonia was established in both sexes (**Fig. 4a, b**). Repeated measures ANOVAs revealed significant main effects of stress in both male and female mice [$F_{(1,26)} = 14.470$, $p = .001$ and $F_{(1,26)} = 13.036$, $p = .003$, respectively], as well as a time x stress interaction in females [$F_{(4,104)} = 7.057$, $p < .001$]. Further analysis revealed that CMS-exposed mice had lower sucrose intake scores, as compared to their control counterparts (males: $p = .02$, $p = .017$, $p = .007$, $p = .016$ for weeks 2-5 respectively; females: $p < .001$ for weeks 2-4 and $p = .014$ for week 5).

CMS-induced anhedonia and anxiety were not reversed by ketamine treatment

Ketamine treatment did not reverse CMS-induced anhedonia in either sex. In particular, two-way repeated measures ANOVAs (baseline vs. day 6) revealed significant main effects of stress in both male and female mice [$F_{(1,24)} = 8.283$, $p =$

.008; $F_{(1,24)} = 13.537, p < .001$] (**Fig. 4c,d**). Additional 2-way ANOVAs revealed that sucrose consumption at baseline was lower in male and female CMS-exposed mice, as compared to their control counterparts, irrespective of ketamine treatment [$F_{(1,28)} = 5.288, p = .030$; $F_{(1,28)} = 12.969, p < .001$]. Similarly, the same analysis revealed that sucrose consumption 6 days post-ketamine administration was still lower in male and female CMS-exposed mice, as compared to their control counterparts, regardless of drug treatment [$F_{(1,28)} = 4.684, p = .041$; $F_{(1,28)} = 10.818, p = .003$]. In the marble burying test, CMS exposure increased the number of marbles buried by both male and female mice, as indicated by significant main effects of stress [$F_{(1,27)} = 13.598, p < .001$ and $F_{(1,29)} = 7.439, p = .012$, respectively] but this was not reversed by ketamine treatment in either sex (**Fig. 4e**).

Ketamine affected locomotor activity of females in a sex-specific manner

CMS exposure and ketamine induced a female-specific effect in open-field behavior. Concerning distance traveled in female mice, a two-way repeated-measures ANOVA revealed a significant main effect of time [$F_{(3,281, 82.019)} = 21.181, p < .001$], a main effect of stress [$F_{(1,29)} = 20.756, p < .001$] and a main effect of drug treatment [$F_{(1,29)} = 4.215, p = .05$]. In particular, CMS increased total distance traveled in females, regardless of drug treatment, and ketamine decreased locomotor activity in female controls ($p = .002$) (**Fig. 5b**). Indeed, further analysis revealed that ketamine-treated female control mice spent less time moving in the open field during the 2nd ($p = .004$), 3rd ($p = .004$), 4th ($p = .001$) and 6th ($p = .026$) 10-min intervals, as compared to their VEH-treated counterparts. Concerning the total time spent in the center of the open-field arena, the same analysis revealed significant main effects of time [$F_{(3,454, 86.358)} = 3.918, p = .008$] and drug treatment [$F_{(1,29)} = 16.310, p < .001$]. Further

analysis revealed that CMS-exposed ketamine-treated female mice spent significantly less time in the center of the open-field arena during the 2nd ($p = .007$), 4th ($p = .015$), 5th ($p = .003$) and 6th ($p = .018$) 10-min intervals, as compared to CMS-exposed VEH-treated counterparts (**Fig. 5d**). Ketamine treatment did not affect open-field behavior in male mice (**Fig. 5a,c**).

Ketamine reduced immobility in the FST at 24 h in both sexes

Two-way ANOVAs revealed significant main effects of ketamine treatment for both male and female mice [$F_{(1,27)} = 11.428$, $p = .003$ and $F_{(1,29)} = 18.274$, $p < .001$, respectively] (**Fig. 6a,b; day 1**). Further analysis revealed that ketamine decreased immobility in both control and CMS male mice ($p = .05$ and $p = .023$) and in control and CMS female mice ($p = .042$ and $p < .001$, respectively).

Long-lasting antidepressant-like effects of ketamine in the FST and the splash test were evident only in male CMS-exposed mice

Mice were subjected to a second FST at 7 days following ketamine treatment in order to screen for putative long-lasting effects in this test. A two way-ANOVA revealed a significant stress x drug interaction only in male mice [$F_{(1,27)} = 6.254$, $p = .020$]. Further analysis revealed that the long-lasting antidepressant-like effects of ketamine were still evident only in male CMS-exposed mice ($p = .033$; **Fig. 6a,b; day 7**). Additionally, ketamine treatment induced sex-dependent effects in the splash test (**Fig. 6c**). In particular, in male mice a two-way ANOVA revealed a significant stress x drug interaction [$F_{(1,29)} = 7.279$, $p = .012$]. Further analysis revealed that CMS induced a decrease in grooming duration in male mice ($p = .044$) that was reversed upon ketamine treatment ($p = .006$). Female mice subjected to CMS displayed a

deficit in grooming behavior, as revealed by a significant main effect of stress [$F_{(1,30)} = 4.590, p = .042$] but this was not reversed by ketamine administration.

DISCUSSION

Herein, we report that male and female C57BL/6J stress-naïve mice present differential sensitivity to the rapid (at 30 min) and the sustained (at 24 h) antidepressant-like effects of ketamine in the FST. Moreover, at the same time-points tissue levels of the excitatory amino acids glutamate and aspartate, as well as serotonergic activity, were affected in a sex-dependent manner in the PFC and the HIPPOCAMPUS upon ketamine administration. Most importantly, a single injection of ketamine (10 mg/kg) induced sex-dependent behavioral effects in mice subjected to the CMS model of depression for five weeks. Intriguingly, female mice were more reactive to the earlier effects of ketamine but the antidepressant potential of the drug proved to be longer-lasting in males.

Female mice are more sensitive to the antidepressant-like effects of ketamine in the FST

The FST is the most frequently used behavioral test for assessing the antidepressant-like effects of pharmacological agents in rodents (Cryan et al., 2002) and has been vastly used in the characterization of the antidepressant effects of ketamine in rodents (Browne and Lucki, 2013). In our experimental setup, ketamine induced sex-dependent rapid (at 30 min) and sustained (at 24 h) antidepressant-like effects in the FST. In particular, female mice responded to lower doses of ketamine (i.e. 3 mg/kg at 30 min and 5 mg/kg at 24h), doses that were not effective in their male counterparts. Present results essentially replicate Carrier and Kabbaj's (2013)

findings in another species. This higher female sensitivity to lower doses of ketamine in rodents is most likely gonadal hormone-dependent. Specifically, these authors reported that the rapid antidepressant-like effects of a low dose of ketamine (i.e. 2.5 mg/kg), that was effective only in female rats, were completely abolished in ovariectomized rats subjected to FST but emerged again upon restoration of physiological levels of estrogen and progesterone (Carrier and Kabbaj, 2013). These data suggested an important role for sex hormones in enhancing the antidepressant-like effects of lower doses of ketamine in the female sex, but the innate mechanisms pertaining to these effects are still elusive.

Ketamine induces sex-dependent neurochemical effects in the HIPP and the PFC

Administration of an antidepressant-relevant dose of ketamine (i.e. 10 mg/kg) induced sex-differentiated and region-selective rapid alterations in the levels of excitatory amino acids glutamate and aspartate in the HIPP and the PFC, at 30 min post-injection. Herein we found that ketamine treatment reduced hippocampal glutamate concentrations in male mice in a sex-selective manner. Interestingly, administration of a high dose of ketamine (i.e. 100 mg/kg) has been previously reported to induce a rapid selective decrease of glutamate levels in the hippocampus of male Swiss albino mice at 30 min post-administration (Chatterjee et al., 2012). Importantly, the observed decrease in glutamate release could be associated with a hippocampus-selective ketamine-induced inhibition of presynaptic release machinery. Upon this, it was recently shown that administration of a single sub-anesthetic dose of ketamine (15 mg/kg) in male Sprague-Dawley rats resulted in a rapid reduction in the accumulation of SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complexes in hippocampal synaptic membranes at 1 h post-ketamine

administration, that is suggestive of a reduction of neurotransmitter release in the hippocampus (Muller et al., 2013). Interestingly, our data in male mice is in agreement with Kavalali and Monteggia (2012) who suggested that ketamine-induced decrease in hippocampal glutamatergic activity may underlie the rapid antidepressant actions of ketamine. Indeed, these authors also suggest that screening for compounds that block the tonic and spontaneous glutamate presynaptic release could prove valuable in developing fast-acting antidepressants (Kavalali and Monteggia, 2012). Given that hippocampal glutamate concentrations were not altered in female mice it could be hypothesized that ketamine may impact hippocampal presynaptic release machinery and the expression of presynaptic proteins, in a sex-dependent manner.

Aspartate is the other major excitatory neurotransmitter of the brain that can excite neurons through selective activation of NMDARs (Curran and Dingledine, 1992). In our study, ketamine induced a sex-specific increase of aspartate concentrations in the PFC of female but not male mice. Earlier studies have shown that both acute and chronic citalopram treatment resulted in diminished aspartate release in the rat PFC (Golembiowska and Dziubina, 2000), while in a recent study chronic clomipramine and mirtazapine treatment reduced aspartate levels in the PFC of male but not female Sprague-Dawley rats (Kokras et al., 2009). Given that the neurochemical effects of antidepressants on the brain's aspartate levels are poorly understood, the sex-differentiated pattern in response to ketamine exposed herein warrants further investigation.

At 24 h post-ketamine administration serotonergic activity in the PFC and the HIPPO was found decreased in female mice. Interestingly, a battery of evidence strongly implicates the serotonergic system in the expression of sex-differentiated responsiveness to classical antidepressant drugs (Young et al., 2009, Dalla et al.,

2011, Pitychoutis et al., 2012), but it remains unclear as to whether the serotonergic system is involved in the rapid and/or sustained antidepressant-like effects of ketamine (Gigliucci et al., 2013). Microdialysis studies have shown that ketamine administration induces a rapid increase of extracellular 5-HT in the medial PFC of male rodents (Nishitani et al., 2014), and recently, Gigliucci et al. (2013) supported a role for serotonin in mediating the sustained antidepressant activity of ketamine in male rats subjected to FST. Still, the putative serotonergic mechanisms underlying the rapid and/or sustained antidepressant effects of ketamine remain to be elucidated (Duman et al., 2012, Gigliucci et al., 2013).

Taken together, our *ex vivo* neurochemical findings provide a “snapshot” of brain’s chemistry at specific time-points and suggest that ketamine induces sex-dependent and region-selective effects in the PFC and the HIPV. Indeed, these brain regions have been strongly implicated in sex-related neurobehavioral responses to stress and antidepressant treatments (Anderson et al., Carballedo et al., Groenewegen and Uylings, 2000, Celada et al., 2004, Drevets, 2007, Pitychoutis et al., 2011, Pitychoutis et al., 2012). Notably, this neurochemical profile could also reflect sex-dependent kinetics in the regulation of excitatory amino acid release in the HIPV and the PFC. Future *in vivo* microdialysis studies in male and female cycling and/or gonadectomized mice are expected to shed light into the mechanisms implicated in this sex-differentiated neurochemical response. Despite the fact that a direct association between neurochemical alterations and behavioral data cannot be made herein, present findings are suggestive of ketamine-induced sex-dependent synaptic molecular events; whether these are directly or indirectly related to its antidepressant profile remains to be elucidated in future studies.

Sex-differentiated responsiveness to ketamine's antidepressant-like effects in mice exposed to the CMS model of depression

CMS produces a constellation of behavioral alterations in rodents thought to resemble core features of depressed patients, such as anhedonia or loss of grooming (Willner, 1997, 2005) and thus, is considered an ideal paradigm with which to screen the antidepressant-like effects of novel therapeutics like ketamine (Browne and Lucki, 2013). Importantly, the CMS paradigm, despite its limitations, has been successfully employed to assess a constellation of interdisciplinary research questions in the sex differences field and has served as a '*silver bullet*' in assessing the sexually dimorphic neurobiology of major depression and antidepressant response (for review see Franceschelli et al., 2014).

In our study, ketamine treatment (10 mg/kg) induced sex-dependent effects in open field and FST behavior at 30 min and at 24 h post-treatment, respectively. In particular, ketamine induced a decrease in locomotor activity in female control mice, as well as an anxiogenic-like effect, as evidenced by the reduced time female mice spent in the center of the arena regardless of CMS application. However, ketamine administration did not affect open field behavior in male mice. Indeed, most studies in rats or mice typically report either no change or reduction of locomotor activity shortly following administration of antidepressant-relevant doses of ketamine (Engin et al., 2009, Lindholm et al., 2012, Browne and Lucki, 2013, Gigliucci et al., 2013, Ma et al., 2013, Pozzi et al., 2014). Interestingly in an earlier study conducted in male individually-housed Wistar rats, a low dose of ketamine (i.e. 7 mg/kg) was shown to induce anxiogenic-like effects in the elevated plus maze (Silvestre et al., 1997). In our CMS study, both stressed and unstressed mice were also individually housed, thus an interaction between sex and social *status* pertaining to the actions of ketamine could

be considered (Carrier and Kabbaj, 2013). Surprisingly, ketamine induced a greater % decrease in FST immobility scores in CMS-exposed female *versus* male mice at 24 h post administration (47% vs 32%, as compared to their respective controls). Thus, these findings suggest that CMS-exposed females are more reactive to ketamine treatment at 30 min and at 24 h post-administration.

Intriguingly, the long-lasting antidepressant potential of ketamine was evidenced only in male CMS-exposed mice. Ketamine increased the time these mice spent grooming in the splash test (day 5) and reduced immobility duration in the FST (day 7) but did not exert relevant effects in females. Herein, the anti-anhedonic effects of ketamine were not observed at 6 days post-administration. Ketamine has been previously shown to induce a long-lasting (up to 7 days) reversal of CMS-induced anhedonia in rats and mice (Li et al., 2011, Ma et al., 2013), but this effect was not observed in other studies (Garcia et al., 2009, Rezin et al., 2009). Interestingly, methodological differences regarding the assessment of anhedonia could explain the discrepancies reported herein such as testing duration (e.g. 1 h *versus* 15 h), prior food and water deprivation, assessment of sucrose intake versus sweet food consumption or even % of sucrose solution implemented. Moreover, ketamine was not effective in reversing the CMS-induced anxiogenic-like phenotype, as assessed in the marble burying test. It is likely that the time-points selected for screening for marble burying and sucrose intake behavior were not optimal. However, the antidepressant-like effects of ketamine in male mice were still evident in the splash test (day 5) and the FST (day 7). Overall, these data support the notion that the antidepressant-like effects of ketamine in stressed mice should not be considered as an “all-or-none” phenomenon but rather depend on the behavioral output measured (e.g. anhedonia in sucrose consumption test *versus* grooming in splash test).

Taken together, our behavioral findings suggest that CMS-exposed females are more reactive to the earlier effects of ketamine (i.e. at 30 min and at 24 h) but the antidepressant properties of this drug are longer-lasting in males and “wear-off” sooner in females. Given the short half-life of ketamine, the protracted behavioral effects measured days after administration result from rapid and sustained molecular alterations induced following a single ketamine injection (Browne and Lucki, 2013). In their landmark paper, Li et al. (2010) showed that the rapid antidepressant effects of a single dose of ketamine in male rats is mediated through the induction of synaptogenesis in the PFC, a brain region implicated in the pathophysiology of major depression (Li et al., 2010). These new synapses are unstable and lost after several days, which coincide with relapse in depressed patients (Duman, 2014). Thus, it is tempting to speculate that the sex-dependent reactivity and antidepressant time course of ketamine observed in CMS-exposed mice could reflect a sex-dependent time course of induction and maintenance of spine formation, a hypothesis that is currently under investigation.

Conclusions

In the present study we report that female C57BL/6J stress-naïve mice are more sensitive to the rapid and the sustained antidepressant-like effects of ketamine in the FST. Moreover, excitatory amino acid levels and serotonergic activity were affected in a sex-dependent manner in the HIPV and the PFC at 30 min or 24 h post-ketamine administration. Most importantly, ketamine induced sex-dependent behavioral effects in mice subjected to CMS. Intriguingly, female mice appeared to be more reactive to the earlier effects of ketamine (i.e. at 30 min and 24 h) but its antidepressant-like properties were longer-lasting in males (up to day 7). Further

research in both naïve mice and in mice subjected to various animal models of depression is imperative in order for the intricate behavioral and neurobiological mechanisms underlying these sex differences to be elucidated.

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Figure 1: Experimental timeline of CMS paradigm: CMS procedures were performed for 5-weeks and then ketamine (10 mg/kg) or VEH were administered in male and female mice exposed to CMS or their respective controls. All mice were subsequently subjected to the behavioral tests as shown in the figure.

Figure 2: Sex differences in the rapid and the sustained antidepressant-like effects of ketamine in the FST: Male and female naïve mice were treated with different doses of ketamine (3, 5, or 10 mg/kg) or VEH and were subjected to the FST at **a)** 30 min (rapid effects) or **b)** 24 h post-administration (sustained effects). Bars represent immobility means $\pm$ SEM (N = 6-8 per group). * p < .05; *** p < .001 as compared to VEH-treated mice of the same sex.

Figure 3: Rapid neurochemical effects of ketamine in male and female mice:

Male and female naïve mice were treated with ketamine (10 mg/kg) or VEH and were sacrificed 30 min later. Levels of excitatory amino acids glutamate and aspartate ($\mu\text{g/g}$ tissue) were assessed in the prefrontal cortex (PFC) and the hippocampus (HIP) by means of HPLC. Bars represent means $\pm$ SEM (N = 6-8 per group). * $p < .05$; as compared to VEH-treated mice of the same sex.

Figure 4: Effects of CMS and ketamine on sucrose consumption and marble burying: Exposure to CMS reduced sucrose consumption (g/ g body weight; BW) in both **a)** male and **b)** female mice. Ketamine treatment did not reverse CMS-induced anhedonia on day 6 (**c,d**) or anxiety as assessed in the marble burying test on day 4

(e). Data represent means $\pm$ SEM (N=7-8/group). $^{\&}p < .05$; $^{\&\&}p < .01$; $^{\&\&\&}p < .001$, as compared to control mice; $^{\#}p < .05$; $^{\#\#}p < .01$; $^{\#\#\#}p < .001$, main effect of stress in two-way ANOVAs, compared to control mice.

Figure 5: Ketamine treatment affects open-field behavior in a sex-dependent manner: a) CMS and ketamine treatment did not affect locomotor activity in male mice; b) locomotor activity of female mice was reduced upon ketamine treatment; c) ketamine did not affect the time male mice spent in the center of the open field, but d) reduced the time female mice spent in the center of the open field irrespectively of CMS application. Data are expressed as means $\pm$ SEM (N = 6-8 per group). * $p < .05$; ** $p < .01$ as compared to VEH-treated mice of the same sex; ### $p < .001$ main effect

of stress in two-way ANOVAs, compared to unstressed control mice; ⁺ $p < .001$ main effect of ketamine treatment in two-way ANOVAs, compared to VEH-treated mice.

Figure 6: The long-lasting antidepressant-like effects of ketamine in the splash test and the FST are evident in male but not female CMS-exposed mice: Ketamine treatment reduced immobility in the FST in both control and CMS-exposed mice of both sexes on day 1. However, when these mice were re-tested in the FST on day 7 the antidepressant-like effects of ketamine were evidenced only in CMS-exposed male mice (a,b). CMS reduced grooming duration in the splash test in both sexes but this was reversed by ketamine treatment only in male CMS-exposed mice

(c). Data are expressed as means $\pm$ SEM (N=7-8/group). * $p < .05$; ** $p < .01$; *** $p < .001$, as compared to VEH-treated mice of the same sex; # $p < .05$ main effect of stress in two-way ANOVAs, compared to unstressed control mice; & $p < .05$ as compared to control VEH-treated mice.

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Males			Females	
PFC-30 min	VEH	Ketamine 10 mg/kg	VEH	Ketamine 10 mg/kg
5-HT	320.15 ± 16.82	331.39 ± 25.12	342.88 ± 40.39	336.27 ± 19.42
5-HIAA	263.20 ± 28.56	231.77 ± 26.50	276.50 ± 39.81	251.94 ± 16.21
5-HIAA/5-HT ratio	0.82 ± 0.06	0.70 ± 0.05	0.80 ± 0.07	0.75 ± 0.07
Glutamate	2092.7 ± 142.5	1885.8 ± 105.4	2341.7 ± 102.0	2323.7 ± 350.2
Males			Females	
PFC-24 h	VEH	Ketamine 10 mg/kg	VEH	Ketamine 10 mg/kg
5-HT	510.54 ± 24.56	570.16 ± 28.08	521.3 ± 51.89	527.2 ± 35.99
5-HIAA	188.38 ± 16.51	202.29 ± 23.51	246.5 ± 42.63	183.93 ± 11.42
5-HIAA/5-HT ratio	0.37 ± 0.03	0.35 ± 0.04	0.46 ± 0.03	0.35 ± 0.02 (*)
Glutamate	1567.0 ± 98.5	1619.9 ± 64.7	1511.7 ± 89.0	1543.1 ± 65.8
Aspartate	279.4 ± 22.9	312.7 ± 16.7	262.8 ± 16.7	287.2 ± 21.8
Males			Females	
HIPP-30 min	VEH	Ketamine 10 mg/kg	VEH	Ketamine 10 mg/kg
5-HT	451.24 ± 14.33	456.08 ± 20.98	524.23 ± 31.98	542.44 ± 61.72

5-HIAA	469.75 ± 25.78	426.25 ± 45.43	531.52 ± 36.79	565.11 ± 68.86
5-HIAA/5-HT ratio	1.04 ± 0.06	0.93 ± 0.08	1.02 ± 0.05	1.05 ± 0.07
Aspartate	396.2 ± 26.9	343.4 ± 28.3	448.5 ± 95.5	419.2 ± 51.4
	Males		Females	
HIPP-24 h	VEH	Ketamine 10 mg/kg	VEH	Ketamine 10 mg/kg
5-HT	555.12 ± 45.43	487.54 ± 41.41	566.8 ± 59.39	534.51 ± 28.79
5-HIAA	453.25 ± 60.94	346.33 ± 49.81	525.1 ± 45.41	421.48 ± 33.83
5-HIAA/5-HT ratio	0.81 ± 0.09	0.70 ± 0.07	0.94 ± 0.04	0.79 ± 0.05 (*)
Glutamate	1593.1 ± 77.5	1464.2 ± 73.6	1523.1 ± 54.2	1521.1 ± 80.2
Aspartate	241.9 ± 13.2	238.6 ± 13.3	239.2 ± 9.1	246.3 ± 14.0
* <i>p</i> < 0.05 difference between VEH and ketamine-treated female mice appears in bold				

Table 1: Neurochemical effects of a single ketamine injection (10 mg/kg) or VEH in stress-naïve mice at 30 min and at 24 h post-administration: 5-HT, 5-HIAA (ng/g tissue) and glutamate and aspartate tissue concentrations (µg/g tissue) and 5-HIAA/5-HT turnover rate values in the prefrontal cortex (PFC) and the hippocampus (HIPP). Data are presented as means±SEM.

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