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Title Page

Title:

Sex differences in the pharmacokinetics of low-dose ketamine in plasma and brain of male and female rats.

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Running Title Page

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Sex differences in ketamine pharmacokinetics in rats

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Abstract

Recent work from our group and others has revealed a higher sensitivity of female rodents to the antidepressant-like effects of N-methyl d-aspartate receptor (NMDAR) antagonist, ketamine, strongly influenced by circulating estrogen (E2) and progesterone (P4) levels. However, in the absence of any preclinical studies of pharmacokinetic sex differences using low-dose ketamine in rats, it is unclear whether the effects of sex and hormonal milieu on ketamine's behavioral actions are influenced by differences in ketamine metabolism between male and female rats. Therefore, this work examined whether or not sex and hormonal status effect ketamine metabolism and distribution in male and female rats using a low antidepressant-like dose

selectively-effective in females. Intact male rats and female rats in either diestrus (low E2, P4) or proestrus (high E2, P4) were administered low-dose ketamine, and their plasma and brains collected to analyze levels of ketamine and its metabolites, norketamine (NK) and dehydronorketamine (DHNK). Females exhibited greater concentrations of ketamine and NK over the first 30 minutes following treatment in both the brain and plasma, largely accounted for by slower clearance rates and longer half-lives. Interestingly, despite the impact of ovarian hormones on behavioral sensitivity to ketamine, no appreciable differences in pharmacokinetic parameters existed between proestrus and diestrus female rats. This work is the first to demonstrate sex differences in ketamine pharmacokinetics in rats, and suggests that while sex differences in metabolism may influence the amount of ketamine and NK reaching target areas in the brain, the impact of circulating hormone levels here is negligible.

1. Introduction

The pressing need for more effective and faster-acting treatments for depression is underscored by the increasing global burden of depression as a leading cause of disability worldwide (Patel et al., 2016), owed in part to ignorance of complex disease mechanisms and stagnant progress in novel pharmacotherapeutic development. In light of this urgency, renewed hope was recently generated by the discovery that the noncompetitive N-methyl d-aspartate receptor (NMDAR) antagonist, ketamine, can rapidly relieve depressive symptoms and suicidal ideation in many patients—notably amongst those with treatment-resistant depression (Abdallah et al., 2016). These findings have since been corroborated numerous times in clinical and preclinical settings, spurring significant efforts into understanding ketamine's underlying mechanisms with the goal of identifying new targets for rapid-acting treatments with sustained efficacy in a broader range of patients (Abdallah et al., 2016).

In the era of personalized medicine, greater emphasis on identifying biomarkers or predictors of rapid antidepressant response to ketamine has emerged in an attempt to address the large heterogeneity observed in patient response to currently-available treatments (Zarate et al., 2013). Yet despite a well-established female preponderance in depressive disorders (Patel et al., 2016) and variable sex differences in antidepressant response (Keers and Aitchison, 2010), sex is a variable yet to be thoroughly investigated as a potential moderator of response to ketamine. Akin to genetic and environmental factors, sex is a naturally-occurring disease and treatment modifier of particular interest (Becker et al., 2016; Keers and Aitchison, 2010), in that protective or treatment-enhancing factors in one sex may reveal prevention or treatment strategies in the other (de Vries and Forger, 2015).

Importantly, sex is a variable that influences nearly all pharmacokinetic processes—absorption, distribution, metabolism, and elimination—which might ultimately influence treatment response (de Vries and Forger, 2015). As a weak and highly lipophilic base, ketamine rapidly distributes to the brain upon administration primarily via passive diffusion across the blood-brain

barrier (BBB). This parent drug is predominantly N-demethylated into norketamine (NK), and further transformed into dehydronorketamine (DHNK) and six diastereomeric hydroxynorketamine (HNK) metabolites (Mion and Villevieille, 2013)—all of which are BBB-penetrant and pharmacologically active within the brain. On a basic level, the therapeutic efficacy and tolerability of ketamine is limited by the availability of the drug and/or its active metabolites in unbound form at relevant target sites within the brain and periphery, making pharmacokinetic processes fundamental to the understanding of variability in treatment response across individuals both within and between sexes (Saland et al., 2017). Unfortunately, the lack of studies investigating sex differences in ketamine pharmacokinetics has left very little evidence regarding such effects in humans. The few that do exist suggest that observations of sex effects in ketamine metabolism and clearance are likely dose-dependent (Sigtermans et al., 2009; Zarate et al., 2012).

As drug/metabolite levels available at the site(s) of action within the brain do not necessarily correlate with those in the periphery, preclinical studies can provide essential information not able to be directly obtained in humans. Indeed, recent work found HNK, but not ketamine or NK, levels to be greater in the brain of female mice following acute systemic administration of 10 mg/kg ketamine (i.p.), in addition to greater female behavioral sensitivity to ketamine's antidepressant-like effects when compared to males (Zanos et al., 2016). However, as in humans, circulating hormone levels were not controlled for in this study. It is clear that species- and dose-specific differences impede a clear understanding of how sex may influence ketamine pharmacokinetics and treatment response at low doses used in clinic.

Notably absent are related studies in rats, which may help bridge this gap in knowledge to paint a clearer picture of whether and how sex differences observed preclinically may apply to the human condition. As well, there is an absence of any information regarding how fluctuating hormone levels may influence ketamine pharmacokinetics, as they are known to affect antidepressant-like behavioral responses to this drug at the preclinical level (Carrier and Kabbaj,

2013; Saland et al., 2016). Therefore, herein we investigated ketamine, NK and DHNK exposure levels in the plasma and brain of proestrus (high hormone) and diestrus (low hormone) female versus male rats following 2.5 mg/kg ketamine—a dose behaviorally effective in females but not in males. In addition, potential regional differences in the medial prefrontal cortex (mPFC) and hippocampus, depression- and ketamine-relevant brain regions, were examined independently.

2. Methods

2.1. Animals

Adult male (250-270g) and female (200-225g) Sprague-Dawley rats (Charles River, Wilmington, MA) were pair-housed in 43x21.5x25.5cm plastic cages. Animals were maintained on a 12h:12h light:dark cycle (lights on at 0700 hours) in a temperature- and humidity-controlled room, and food and water were available ad libitum throughout the duration of the study. All animal protocols were carried out in accordance with the NIH Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Florida State University.

2.2. Estrous cycle monitoring

Animals were habituated to handling procedures daily for one week upon arrival. Following habituation, daily estrous cycle monitoring and stage assignment of intact female rats was performed via vaginal lavage and characterization of cytological smears as previously detailed (Hollis et al., 2011; Saland et al., 2016). Only rats exhibiting at least 2 consecutive 4-day cycles were used in the present work. Four female rats not meeting this criterion were excluded prior to group assignment and analysis. During this time, male rats received a similar brief daily handling treatment to minimize potential for stress and handling confounds between sexes.

2.3. Pharmacokinetics experimental procedures

2.3.1. Ketamine treatment and sample collection

The complete experimental design and sample preparation workflow are illustrated in Figure 1. Separate adult male rats (n=4/time point) and female rats in either diestrus (low E2P4, n=4/time point) or proestrus (high E2P4, n=4/time point) received a single intraperitoneal (i.p.) injection of ketamine hydrochloride (Butler Schein Animal Health, Inc.) at 2.5 mg/kg, and were sacrificed under non-stressful conditions after 5, 10, 30, 60, 90 or 180 min. Brains were immediately removed, snap-frozen in 2-methylbutane and stored at -80°C, along with plasma separated from trunk blood, until further processing. Drug was administered at a volume of 1

mL/kg. Ketamine was administered between 1400 h and 1500 h, at an approximate time period when circulating levels of estradiol and progesterone have been shown to be elevated in proestrus and low in diestrus female rats (Becker et al., 2005). In accordance with our previous work (Carrier and Kabbaj, 2013; Saland et al., 2016), this time period was chosen in order to determine whether or not the relative levels of these hormones in females at the time of ketamine administration would influence its metabolism and/or distribution, in addition to analysis of sex effects on these parameters.

2.3.2. Sample preparation

260 uL of plasma was transferred to a pre-chilled 1.5 mL microcentrifuge tube and centrifuged for 15 min at 2000xg at 4°C to pellet any contaminants. Supernatant was transferred to a clean tube and an appropriate volume of ketamine-d4 (Cerilliant, Round Rock, TX) was added as an internal standard (IS) at 100 ng/mL for a 300 uL final volume. Samples were pulse-vortexed for 1 min then mixed on a shaker plate for 10 min at room temperature (RT) to equilibrate the IS. 250 uL spiked plasma was then transferred to a clean, pre-chilled tube, diluted 1:1 with acidified water (0.4N HCl), vortexed and stored at 4°C overnight to disrupt plasma protein binding and precipitate proteins. The following day, samples were thawed on ice, vortexed and centrifuged for 20 min at 8000xg at RT to clarify prior to solid phase extraction (SPE). For brain tissue samples, ketamine-d4 was added at 300 ng/g to frozen tissue punches (1.0mm) collected from 200µm sections of the dorsal hippocampus (HPC) and medial prefrontal cortex (mPFC). Spiked tissue samples were homogenized via sonication in 200 uL MilliQ water. 800 uL 100% methanol (MeOH) was immediately added and samples were vortexed for 1 min to mix. Following homogenization, samples were sonicated in an ice bath for 15 min and stored at 4°C overnight. The following day, samples were briefly vortexed and centrifuged for 10 min at 3000xg at 4°C. Supernatant was transferred to a clean tube, dried via SpeedVac and resuspended in 500 uL 0.2N HCl.

For SPE, acidified samples (250 uL for plasma, 500 uL for brain) were loaded onto Oasis MCX (1cc/30mgm) cartridges (Water, Milford, MA) preconditioned with 1mL MeOH and 1 mL

MilliQ water, and allowed to elute via gravity. Columns were washed with 1 mL 0.1N HCl, followed by 1 mL MeOH. K, NK and DHNK analytes were then eluted twice with 500 uL 5% NH4OH in MeOH via gravity, and dried via SpeedVac. Pellets were stored at -20°C until further processing.

2.4. Quantification of ketamine and metabolites in biological matrices

2.4.1. HPLC for plasma samples

Dried plasma extracts after SPE were re-dissolved in 0.5% acetonitrile aqueous solution with 0.1% formic acid (1:2 v:v plasma:solvent). Two uL of the above solutions were loaded to the NanoAquity nanoLC system (Waters, Milford, MA) for liquid chromatography (LC) separations with a single pump trapping fluidic configuration. A Symmetry C18 5um 180 um x 20 mm trap column was followed by a HSS T3 1.8 um 75 um x 150 mm analytical column (both by Waters, Milford, MA) in a vented configuration. Buffer A was aqueous solution with 0.1% formic acid; buffer B was acetonitrile with 0.1% formic acid. The LC gradient profile was as follows with a flow rate of 400 nL/min: 1% B at 0 minute, 85% B at 15-20 minutes and 1% B at 25-35 minutes.

2.4.2. HPLC for brain tissue samples

Dried brain extracts after SPE were re-dissolved in 2% acetonitrile aqueous solution with 0.1% formic acid (1:12 mg:uL tissue:solvent). Two uL of the above solutions were loaded to the NanoAquity nanoLC system (Waters, Milford, MA) for LC separations with a single pump trapping fluidic configuration. A Symmetry C18 5um 180 um x 20 mm trap column was followed by a HSS T3 1.8 um 150 um x 100 mm analytical column (both by Waters, Milford, MA) in a vented configuration. Buffer A was aqueous solution with 0.1% formic acid; buffer B was acetonitrile with 0.1% formic acid. The LC gradient profile was as follows with a flow rate of 2 uL/min: 1% B at 0 minute, 85% B at 5-5.5 minutes and 1% B at 6-7 minutes.

2.4.3. Mass spectrometry

LC eluents were ionized in positive ion mode by nano electrospray ionization (nESI). Thus, generated analyte ions were detected on-line with a Xevo TQ-S Triple Quadrupole Mass Spectrometer (Waters, Milford, MA). We optimized the conditions for nanoelectrospray source as

follows: +3.3 kV capillary voltage, 43 V cone voltage, 50 V source offset, 100°C source temperature, and 0.20 bar spray gas. Standard solutions were directly infused (without LC separation) to optimize the multiple reaction monitoring (MRM) transitions for quantification: ketamine (m/z 238 → 125), norketamine (m/z 224 → 125), dehydronorketamine (m/z 222 → 142) and ketamine-d4 (internal standard, IS, m/z 242 → 129) with 3 ms dwell time and 25 V collision energy. For plasma samples, seven calibration standards were prepared by spiking standards to "blank" plasma followed by SPE extraction. Concentration of those spiked standards were as follows: ketamine-d4 (IS) 100 ng/mL; ketamine, norketamine and dehydronorketamine 1, 5, 25, 100, 500, 800 & 1000 ng/mL. The calibration linearity was observed for 1-500 ng/mL range. For brain tissue samples, five calibration standards were prepared by spiking standards to "blank" tissues followed by SPE extraction. Concentration of those spiked standards were as follows: ketamine-d4 (IS) 300 ng/g; ketamine, norketamine and dehydronorketamine 5, 25, 100, 500, & 1000 ng/g. The calibration linearity was observed for 5-1000 ng/g range. Standard curves are presented in Fig. 2 for plasma (Fig. 2a) and brain tissue (Fig. 2b).

All samples and calibrants LC-MS/MS experiments were run in triplicates. TargetLynx software (Waters, Milford, MA) was used to automatically quantify ketamine, norketamine and dehydronorketamine in plasma and brain tissues. TargetLynx extracted the area under curve (AUC) of those targets of interest in the MRM chromatograms with a restraint of retention time. TargetLynx then normalized the response by comparing the AUC to that of IS. Calibrants response were used to generate calibration curves which were used to calculate the concentration in the unknown samples.

2.5. Pharmacokinetics data analysis

Pharmacokinetic parameters following i.p. ketamnine administration in male and female rats were determined from mean plasma concentration-time data via noncompartmental analysis (NCA) using PKSolver 2.0 (Zhang et al., 2010). The maximum plasma and tissue concentrations (Cmax) and time to reach Cmax (Tmax) were directly observed from data. The area under the

concentration-time curves from 0-180 min after ketamine administration (AUC0-t) and extrapolated to infinity (AUC0-∞) were calculated for K, NK and DHNK using the linear-up/log-down trapezoidal method. As well, the mean residence time (MRT), or the amount of time a molecule remains in the body, was calculated for ketamine and both metabolites by dividing AUC by the area under the first moment concentration-time curve (AUMC). For mean K and NK concentration-time profiles, NCA was used to calculate terminal elimination half-life (t1/2) and rate constant (kel) values in both plasma and brain tissue, as well as apparent volume of distribution at the terminal phase (Vz/F) and the total systemic clearance (CL/F) for plasma samples. The terminal phase of DHNK for female rats was unidentifiable due to the unsuitability of our timeframe in accommodating for the later peak observed in this sex; therefore, only Cmax, Tmax, and AUC0-t were calculated.

2.6. Statistical analyses

All data were first subjected to the Anderson-Darling Normality test, and determined to follow a normal distribution. Plasma and brain concentrations of ketamine and metabolites across time were analyzed by two-way analysis of variance (ANOVA), with sex/estrous cycle and time as independent factors. Due to the sparse sampling protocol required herein for parallel analysis of plasma and brain tissue, repeated-measures analysis across timepoints collected was not possible. Where appropriate, significant main or interaction effects were followed by Bonferroni's multiple comparisons test to determine between-group differences across time. Multiplicity-adjusted p-values are reported. GraphPad Prism 6.0 software (GraphPad Software Inc., San Diego, CA) was used for all statistical analyses and production of graphs. Alpha was set to 0.05 for all statistical analyses.

3. Results

3.1. Plasma concentrations of ketamine and metabolites in male and female rats

Following systemic administration of the same low dose of ketamine to male and female rats, plasma concentrations of the parent drug were significantly higher in female rats 5 ($p=0.0367$) and 10 ($p=0.0385$) min post-treatment as compared to male rats (Fig. 3a; Sex: $F(1,59)=18.72$, $p<0.0001$; Time: $F(5,59)=48.53$, $p<0.0001$; Interaction: $F(5,59)=1.337$, $p=0.2615$). Accordingly, greater NK levels in females versus males were observed at subsequent 30- ($p=0.0013$) and 90-minute ($p=0.0111$) time points (Fig. 3c; Sex: $F(1,57)=26.20$, $p<0.0001$; Time: $F(5,57)=24.41$, $p<0.0001$; Interaction: $F(5,57)=1.708$, $p=0.1474$). That DHNK levels were greater in male than female rats 10 ($p=0.0328$) and 30 ($p=0.0031$) minutes after ketamine administration suggests greater rate of transformation of DHNK from NK in males (Fig. 3e; Sex: $F(1,60)=3.108$, $p=0.0830$; Time: $F(5,60)=3.744$, $p=0.0051$; Interaction: $F(5,60)=4.784$, $p=0.0010$).

Interestingly, no meaningful differences were observed between diestrus and proestrus females in concentrations of ketamine (Fig. 3b; Estrous Cycle: $F(1,36)=0.003740$, $p=0.9516$; Time: $F(5,36)=38.61$, $p<0.0001$; Interaction: $F(5,36)=0.8209$, $p=0.5430$), norketamine (Fig. 3d; Estrous Cycle: $F(1,36)=0.08903$, $p=0.7672$; Time: $F(5,36)=18.65$, $p<0.0001$; Interaction: $F(5,36)=0.5766$, $p=0.7175$), or dehydronorketamine (Fig. 3f; Estrous Cycle: $F(1,36)=0.2833$, $p=0.5978$; Time: $F(5,36)=5.370$, $p=0.0009$; Interaction: $F(5,36)=1.806$, $p=0.1365$), suggesting that ovarian hormones did not significantly influence ketamine pharmacokinetics at this low dose and route of administration during these cycle stages (see Table 1 and Table 2 for pharmacokinetic parameter values).

3.2. Brain tissue concentrations of ketamine and metabolites in male and female rats

We next sought to measure the active-site concentrations of ketamine and norketamine within key brain regions relevant to ketamine's antidepressant-like action. Ketamine levels in both the HPC (Fig. 4a) and mPFC (Fig. 4e) paralleled sex differences seen in plasma, with significantly greater concentrations of the parent drug in female rats 5 and 10 min post-treatment (p's < 0.0001)

relative to male rats (HPC, Sex: $F(1,59)=134.0$, $p<0.0001$; Time: $F(5,59)=572.4$, $p<0.0001$; Interaction: $F(5,59)=78.74$, $p<0.0001$; mPFC, Sex: $F(1,58)=6415$, $p<0.0001$; Time: $F(5,58)=22557$, $p<0.0001$; Interaction: $F(5,58)=3690$, $p<0.0001$). Females also exhibited higher norketamine levels than males in both brain regions (Fig. 4c,g) rapidly beginning at 5 min up to 60 min after administration (p's < 0.0001). (HPC, Sex: $F(1,58)=179.4$, $p<0.0001$; Time: $F(5,58)=112.1$, $p<0.0001$; Interaction: $F(5,58)=12.94$, $p<0.0001$; mPFC, Sex: $F(1,59)=567.4$, $p<0.0001$; Time: $F(5,59)=251.8$, $p<0.0001$; Interaction: $F(5,59)=42.59$, $p<0.0001$). DHNK could not reliably detected in all samples and was below the lower limit of quantitation (LLOQ) at all timepoints in both brain regions, and was therefore omitted from analysis.

As in plasma, while HPC NK was slightly greater in diestrus versus proestrus female rats, measured concentrations of K and NK were markedly similar between these groups in the HPC (K, Fig. 4b; Estrous Cycle: $F(1,35)=0.004494$, $p=0.9469$; Time: $F(5,35)=468.2$, $p<0.0001$; Interaction: $F(5,35)=0.1070$, $p=0.9900$; NK, Fig. 4d: Estrous Cycle: $F(1,34)=5.179$, $p=0.0293$; Time: $F(5,34)=113.6$, $p<0.0001$; Interaction: $F(5,34)=0.9861$, $p=0.4406$) and mPFC (K, Fig. 4f; Estrous Cycle: $F(1,36)=0.07046$, $p=0.7992$; Time: $F(5,36)=25491$, $p<0.0001$; Interaction: $F(5,36)=1.326$, $p=0.2754$; NK, Fig. 4h: Estrous Cycle: $F(1,36)=1.179$, $p=0.2848$; Time: $F(5,36)=277.4$, $p<0.0001$; Interaction: $F(5,36)=0.2875$, $p=0.9168$). The only exception was a modestly greater initial NK level in diestrus females 5 min ($p=0.0413$) post-dose restricted to the HPC (Fig. 4d) relative to that of proestrus female rats (see Table 2 for pharmacokinetic parameter values).

3.3. Sex differences in metabolism and brain distribution of ketamine and norketamine

Concentration-time relationships between ketamine and its metabolites in plasma and brain tissues were examined to identify whether sex and/or hormonal milieu influence metabolism and CNS distribution of low-dose ketamine in behaviorally-relevant brain regions. As DHNK was not reliably detected at or above quantifiable levels in brain tissue, primary emphasis was on ketamine and norketamine in the present analyses. In order to directly compare ketamine and

metabolite exposure levels, their plasma (ng/mL) and brain tissue (ng/g) concentrations—assuming a specific mass of 1 g/mL—were first converted into micromolar (μM) to correct for molecular weight differences between molecules which contribute to overall AUC (exposure) values. The results of these comparisons are depicted graphically in Figure 5 for visual comparison of exposure levels between sexes across time—numerical values presented therein (Fig. 5) are provided in Table 3, along with female-to-male ratios for comparison at each parameter.

4. Discussion

Comparative evaluation of pharmacokinetic profiles and brain tissue distribution of low-dose ketamine and its metabolites in male and female rats is necessary for improved understanding of their differential behavioral sensitivity to the drug observed preclinically, and translation of such information across species. In this work, we provide the first characterization of ketamine pharmacokinetics across sex and hormonal status in rats following a low-dose administration of 2.5 mg/kg—a dose selectively effective in female but not male rats in eliciting antidepressant-like and pro-hedonic behaviors. Here, females exhibited greater peak ketamine and norketamine (NK) levels rapidly upon systemic administration in both plasma and two depression-relevant brain regions, the medial prefrontal cortex (mPFC) and hippocampus (HPC). Longer half-lives and slower clearance rates in females contributed to their greater exposure levels of ketamine and its primary metabolite over the three-hour time course. Notably, while our previous work demonstrated an important role for ovarian hormones in the enhanced female behavioral sensitivity to low-dose ketamine (Carrier and Kabbaj, 2013; Saland et al., 2016), proestrus and diestrus female rats exhibited remarkably similar pharmacokinetic profiles, suggesting a more prominent influence of sex hormones on pharmacodynamic rather than pharmacokinetic systems in conferring sex-dependent behavioral sensitivity to ketamine.

Absent from preclinical work are pharmacokinetic analyses of ketamine and metabolite concentrations across a broad range of doses within which behaviorally-effective doses of ketamine reside. Therefore, it was of particular interest here to determine concentrations of ketamine and its metabolites for a low dose of ketamine that exerts antidepressant-like effects in female rats, but not in males, in order to obtain results that directly parallel behavioral and molecular findings presented in our previous studies utilizing the same low dose (Carrier and Kabbaj, 2013; Saland et al., 2016; Sarkar and Kabbaj, 2016). Following systemic administration of 2.5 mg/kg ketamine, ketamine concentrations peaked rapidly 5 minutes after dosing the plasma of male and female rats. Notably, peak concentrations of ketamine in female rats were

significantly greater than those in males for the first 10 minutes after the drug was administered. Concentrations of its primary metabolite NK peaked slightly later at 30 minutes in both sexes as ketamine declined, exhibiting greater levels in female rats from its peak time through 90 minutes following treatment when compared to males. Interestingly, DHNK (derived from NK) showed an opposite trend, in which males displayed greater and earlier peak concentrations at 30 minutes than females, whose levels peaked much later around 90 minutes. Over the time points examined, AUC values indicated that total exposure levels to both ketamine and NK in females were nearly double those observed in males, whereas DHNK exposure was similar between sexes. Longer half-lives and slower clearance rates of both ketamine and NK in females contributed to their overall greater exposure levels compared to males. Given that the same trends in these parameters were observed for both ketamine and its primary metabolite, NK, greater peak concentrations of ketamine in the plasma of females cannot be explained by more extensive or rapid metabolism to NK in males. Interestingly, male rats did exhibit a larger apparent volume of distribution than females for ketamine, and to a lesser extent NK, according to values obtained via non-compartmental analysis. It is therefore possible that this drug was more extensively distributed to other tissue and fluid compartments in male rats, which could help to explain its lower levels observed in plasma compared to females across the time period measured.

To this end, we examined ketamine and metabolite concentrations within the brain in two regions relevant to depressive-like behaviors and ketamine's related mechanism of action in rodents—the mPFC and HPC. Ketamine and NK were detected rapidly in the brains of male and female rats, both peaking 5-10 minutes after systemic administration of the parent compound. These findings agree with previous reports in mice and rats performed using similar routes of administration, and confirm what is already known regarding distribution of ketamine after systemic and intravenous administration of the drug across species (Mion and Villevieille, 2013). As in plasma, ketamine levels were significantly higher in female versus male rats 5-10 minutes after injection. Norketamine peaked sooner in the brain than in plasma in both sexes, but was

also detected at much greater levels in the mPFC and HPC of females compared to males over the first 90 minutes after administration. Unfortunately, DHNK levels detected were below the limit of quantification in both brain regions, and were therefore not reported. Failure to reliably detect DHNK in the brain at a dose this low is unsurprising based on similar reports in rats administered low-dose ketamine systemically. Given that females displayed greater ketamine and NK concentrations than males in both the brain and plasma, greater distribution of ketamine to brain tissue in males cannot explain lower levels of ketamine and NK in males. Determination of drug levels in other tissues and compartments are needed to help explain the greater apparent volume of distribution observed in males.

In order to better understand the observed sex differences in ketamine concentrations over time, direct comparisons of pharmacokinetic parameters and concentration-time curves between the plasma and brain were made within each sex, then plotted against each other for further insight. First, to determine whether differences in metabolism of ketamine to downstream metabolites could help explain the greater female exposure to ketamine in the plasma and brain, cumulative ratios of total exposure levels (depicted by AUC values) for norketamine to ketamine were plotted over time at each measurement taken in the plasma and brain. Interestingly, despite greater concentrations of NK in the plasma of females compared to males, the NK:ketamine ratios were remarkably similar over the 180-minute time period examined, with roughly 5 times greater norketamine exposure compared to ketamine in both sexes. This similarity was true at each timepoint examined, suggesting that metabolism of ketamine into NK does not contribute to differing plasma concentrations between male and female rats. In the brain, however, NK exposure was roughly twice that of ketamine in females, whereas males displayed an NK:ketamine ratio in equilibrium. Given that ketamine is not known to undergo local metabolism within the brain, this suggests that either distribution or permeability of NK into the brain is greater in females than in males independent from the rate or extent of hepatic metabolism of the parent drug.

To examine this further, AUC values of ketamine and NK were compared between the brain and plasma in each sex, then plotted against each other for evaluation. In agreement with previous studies, ketamine levels were roughly 3-4 times greater in the brain than in plasma of both males and females; however, the rate of brain penetration of the parent compound differed between sexes. Over the first 10 minutes following ketamine administration, females displayed brain:plasma ketamine ratios of ~3 compared to ~2 in males. This trend shifted to favor males by 60 minutes, who displayed nearly 4 times greater ketamine concentrations in the brain than in plasma, compared to ~3 times greater levels in females at this timepoint. Despite greater brain concentrations of ketamine in females, it is possible that males exhibit either slower elimination or greater retention of ketamine within the brain than females. Indeed, the mean residence time (MRT) of ketamine was nearly 20% greater in males than in females. Differences in the rate of ketamine's elimination from the brain over time could have a significant impact on the time course of molecular consequences of ketamine action within the brain, and therefore behavior, at the low dose used in the present work.

Because NK is also known to be a pharmacologically active metabolite, sex differences in its concentrations within the brain also have the potential to influence ketamine's rapid behavioral effects. Owing to its reduced lipophilicity compared to ketamine, brain penetrance of NK was reduced relative to ketamine in both sexes. Norketamine brain:plasma ratios were near 1 in males, suggesting near equilibrium between the 2 compartments. This ratio was roughly doubled in females for the first 10 minutes following ketamine administration, but lowered to equilibrium by 60-90 minutes post-dose. These data suggest similar elimination from and penetrance of NK in the brain, with slight differences in elimination rates for ketamine, between male and female rats, suggesting a low probability that these parameters contribute to the sex-dependent behavioral sensitivity to low-dose ketamine in rats. However, it is important to note that ketamine and NK displayed regional differences in concentrations in females, where concentrations of both were significantly greater in the mPFC than the HPC for the first 10 minutes after ketamine was

administered. No difference was apparent in males, however, whose concentrations were similar in both regions examined. This sex discrepancy could help to explain the molecular differences between male and female rats shown to occur in both the mPFC and HPC rapidly following low-dose ketamine administration (Carrier and Kabbaj, 2013; Saland et al., 2016; Sarkar and Kabbaj, 2016). As this is the first report of regional differences in ketamine distribution within the brain between males and females, it is unknown the extent to which these differences occur throughout other regions of the brain which may have behaviorally-relevant implications. It is an exciting finding, nonetheless, and warrants further investigation.

While the pharmacokinetic differences between male and female rats are certainly of interest, the lack of difference between proestrus and diestrus females are equally important to highlight when considering potential relevance of pharmacokinetic differences to those reported in depression-relevant behavioral assays. Despite differences in peripheral and central levels of estradiol and progesterone in proestrus and diestrus stages of the estrous cycle, levels of ketamine and NK in the plasma and brain tissue of these females were remarkably similar. Alone, this is not necessarily surprising, as no studies prior to the present work have investigated hormonal influence on pharmacokinetic parameters of ketamine in either rodents or humans. However, these findings hold great import when considered in the context of behavioral data reporting hormone-dependent effects of ketamine on depressive-like behavior in female rats. Whereas circulating estradiol and progesterone appear essential to the heightened behavioral sensitivity of female rats to low-dose ketamine, differences in their circulating levels do not appear to significantly affect metabolism or brain distribution of the same behaviorally-relevant dose of ketamine in intact females (at least in the regions investigated herein). Therefore, it is more plausible that estrous cycle-dependent behavioral effects of a single low-dose of ketamine in rats are a consequence of pharmacodynamic, rather than pharmacokinetic, differences that occur with cyclic hormonal fluctuations. It should be noted that secondary metabolites, including HNK, were

not examined in the present work. As such, estrous cycle-dependent differences in metabolism of NK to HNK and its distribution are unknown, and warrant further investigation.

Given the single dose administration used in this study, these findings should not be generalized to repeated administration regimens. It is possible that estrous cycle could influence pharmacokinetic parameters of ketamine over repeated administration of a low dose. For example, estrous cycle appears to influence both maintenance of intravenous ketamine self-administration, as well as reinstatement to ketamine-paired cues in rats (Wright et al., 2017). While pharmacokinetic differences between proestrus and diestrus rats are not apparent after a single systemic injection of low-dose ketamine, one cannot exclude the possibility that cycle-dependent reinforcing properties of ketamine following repeated administration (intravenous or otherwise) are influenced by pharmacokinetics. Future pharmacokinetic analyses of ketamine using different routes of administrations, doses and treatment regimens (single vs. repeated) in females across the estrous cycle and males would greatly benefit comprehension of sex and cycle-dependent behavioral and molecular differences reported across studies.

While this is the first investigation to examine and report pharmacokinetic sex differences of ketamine in brain and plasma of rats, conflicting findings have recently been reported in mice (Zanos et al., 2016). Here, Zanos and colleagues found that higher HNK, but not ketamine or NK, levels are observed in the brain of female mice following acute administration of 10 mg/kg ketamine (*i.p.*), in addition to greater female behavioral sensitivity to ketamine's antidepressant-like effects when compared to males (2016). Further experiments showed that systemically administered HNK is able to cross the blood-brain barrier and elicit antidepressant-like activity in mice without inducing ketamine-like side effects. However, sex differences were either not examined or not reported in this case, so it is unclear whether behavioral sensitivity to HNK differs between males and females. Here, it should be noted that females, but not males, exhibited an antidepressant-like response to 3 mg/kg ketamine, whereas both sexes responded to the 10 mg/kg dose used for pharmacokinetic analysis. Therefore, a direct association between greater

HNK levels and enhanced female antidepressant-like response to ketamine cannot be conclusively inferred. In contrast, the present findings demonstrate greater ketamine and NK exposure in the plasma and brain of cycling female versus male rats following 2.5 mg/kg ketamine—a dose behaviorally effective in females but not males. In addition, the regional differences observed herein when the mPFC and hippocampus were examined independently may not be directly comparable to the whole brain analysis performed by Zanos et al. (2016). These findings further suggest species differences in not only behavioral, but also pharmacokinetic parameters following low-dose ketamine exposure, and highlight the need for pharmacokinetic analysis across multiple behaviorally-relevant doses across species in both sexes.

Unfortunately, the lack of studies investigating sex differences in ketamine pharmacokinetics has left very little evidence regarding such effects in humans. The few that do exist suggest that observations of sex effects in ketamine metabolism and clearance are likely dose-dependent. For example, 20% greater ketamine and NK clearance and lower drug/metabolite concentrations have been observed in healthy women when compared to men following ketamine infusion at a higher dose >10 mg/kg, i.v. (Sigtermans et al., 2009). These sex differences were reflected at the behavioral level, with greater effects on cardiac output and heat pain-related indices in men than in women (Sigtermans et al., 2010). Conversely, Zarate and colleagues (2012) identified smaller sex differences in metabolism of low-dose ketamine in MDD and bipolar patients, where females displayed greater plasma levels of DHNK and HNK4a/c metabolites compared to males. Importantly, however, no sex differences in antidepressant response were apparent, and the differences in metabolite levels—notably that of HNK4a—had no significant association with treatment response. In fact, independent of sex, HNK5 was negatively associated with treatment response in bipolar depression patients (Zarate et al., 2012), suggesting that pharmacokinetic sex differences may not actually impact treatment response in clinical depression. Of note, hormone levels were not controlled for in these studies, which may

have obscured potential differences in clinical response between sexes—particularly given the overlap in brain regions whose activity is modulated by both circulating ovarian hormone levels in women and ketamine itself (Arélin et al., 2015).

While underlying factors responsible for these varying differences in ketamine metabolism observed between males and females remain unknown, sex differences in hepatic expression and activity of ketamine-metabolizing cytochrome P450 enzymes are well-known (Waxman and Holloway, 2009)—and subject to hormonal regulation by estrogen, progesterone and testosterone, which also happen to be substrates of several P450 enzymes responsible for ketamine metabolism (de Vries and Forger, 2015; Waxman and Holloway, 2009). As well, physiological differences influencing xenobiotic distribution, metabolism and clearance (i.e., body weight, adipose tissue levels and distribution) are present between males and females of a variety of species (de Vries and Forger, 2015). Ultimately, whether sex- and/or hormone-dependent pharmacokinetic processes contribute to differences between males and females in ketamine's antidepressant response is unclear, but the evidence strongly supports their consideration both preclinically and clinically. Likewise, non-negligible pharmacokinetic-related species differences have been highlighted herein, encouraging further examination to better translate findings between rodents and humans.

Authorship Contributions

Participated in research design: Saland and Kabbaj

Conducted experiments: Saland

Performed data analysis: Saland

Wrote or contributed to the writing of the manuscript: Saland and Kabbaj

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Footnotes

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Legends for Figures

Figure 1. Pharmacokinetics experimental design and sample preparation workflow. Abbreviations: AUC, area under the curve; DHNK, dehydronorketamine; HCl, hydrochloric acid; HPC, hippocampus; K, ketamine; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MeOH, methanol; mPFC, medial prefrontal cortex; NK, norketamine; PK, pharmacokinetic.

Figure 2. Calibration curves for ketamine and metabolite standards in blank plasma and brain matrices. Linearity (R2 > 0.9995) was observed for ketamine (K), norketamine (NK) and dehydronorketamine (DHNK) calibrants (Cal) within a range of 1-500 ng/mL for plasma (a), and 5-1000 ng/g for brain (b). Response depicted as the ratio of the peak area (AUC) of each analyte to that of the internal standard (IS). Data expressed as mean of 3 technical replicates ± SEM.

Figure 3. Plasma concentration-time profiles of ketamine and its metabolites in male and cycling female rats. (a,b) Females displayed greater levels of ketamine (K) rapidly upon i.p. administration at 5 ($p=0.0367$) and 10 ($p=0.0385$) min post-dose, relative to their male counterparts, regardless of hormonal status. (c,d) Higher concentrations of the norketamine (NK) metabolite were also subsequently observed in female rats at 30- ($p=0.0013$) and 90-min ($p=0.0111$) timepoints, when compared to males, in an estrous-cycle independent manner. (e) However, dehydronorketamine (DHNK) was significantly elevated in male compared to female rats 10 ($p=0.0328$) and 30 ($p=0.0031$) min after ketamine administration. (f) Estrous cycle had minimal influence on DHNK levels in females. (g-j) Superimposed K, NK and DHNK concentrations reveal greater metabolite-to-parent concentrations in males than in females through the first 30 min post-treatment, despite their overall lower levels of K and NK during this time. $*p < 0.05$ vs. Male, $**p < 0.01$ vs. Male.

Figure 4. Brain concentration-time profiles of ketamine and norketamine in male and cycling female rats. (a,e) Concentrations of ketamine (K) were ~2-fold greater in female compared to male rats 5-10 min ($p < 0.0001$) following administration in the hippocampus (HPC) and medial prefrontal cortex (mPFC), regardless of estrous cycle stage (b,f). (c,g) These differences were more pronounced for its metabolite norketamine (NK), whose levels in females were significantly higher 5-60 min post-treatment in both regions ($p < 0.0001$) when compared to those in males. (d) While NK levels in the HPC were higher in diestrus than in proestrus females 5-min after ketamine treatment ($p = 0.0413$), hormonal status had minimal effect on distribution of the parent drug and its metabolite within the HPC and mPFC. Data are expressed as mean ± SEM (n=3-4/group/timepoint). $**p < 0.0001$ vs. Male, $*p < 0.05$ vs. Proestrus.

Figure 5. Metabolite ratios and brain distribution of ketamine and norketamine in male and cycling female rats. (a) Ratio of norketamine to ketamine area under the concentration-time curve (AUC) values in plasma and brain of male (n=3-4/timepoint) and female (n=7-8/timepoint) rats (brain regions were averaged due to overall similarity). (b,c) Cumulative brain-to-plasma AUC ratios for ketamine and norketamine, respectively, over time in male and female

rats. Data expressed as ratios of AUC0-t values calculated using pooled group data from biological replicate measurements at each timepoint—plasma (ng/mL) and brain tissue (ng/g) concentrations were converted into micromolar (μM) units beforehand for direct comparison between analytes. AUC0-180 Ratio: total analyte exposure across all timepoints measured for each analysis, depicted by gray vertical bar. N.Q.: not quantifiable, ketamine detected in brain samples < LLOQ 90-180 min; here, AUC0-60 represents total exposure up to this timepoint, indicated by a dashed vertical bar for comparison.

Tables

Table 1. Pharmacokinetic parameters for ketamine and metabolites in plasma and brain tissue of male and female rats.

Parameter	k_{el}	$t_{1/2}$	T_{max}	C_{max}	AUC_{0-t}	$AUC_{0-\infty}$	$AUMC_{0-t}$	$AUMC_{0-\infty}$	MRT_{0-t}	$MRT_{0-\infty}$	V_z/F	Cl/F
Unit	1/min	min	min	ng/ml	ng•min/L	ng•min/L	ng•min^2/L	ng•min^2/L	min	min	L/kg	L/min/kg
Plasma												
Ketamine												
Male	0.0156	44.42	5	100.52	2.86	3.10	127.52	186.09	44.60	60.05	51.69	0.807
Female	0.0116	59.99	5	135.83	5.48	6.07	276.24	434.03	50.45	71.53	35.66	0.412
Norketamine												
Male	0.0124	55.76	30	190.68	14.54	16.85	808.15	1409.02	55.58	83.63	11.94	0.148
Female	0.0095	72.72	30	311.80	28.04	34.41	1937.45	3751.16	69.10	109.03	7.62	0.073
Dehydronorketamine												
Male	–	–	30	9.88	0.61	–	38.48	–	62.67	–	–	–
Female	–	–	90	5.49	0.66	–	62.28	–	94.02	–	–	–
mPFC												
Ketamine												
Male	0.0471	14.72	5	198.47	9.09	–	288.26	–	31.72	–	–	–
Female	0.0504	13.74	10	402.87	10.92	–	274.57	–	25.15	–	–	–
Norketamine												
Male	0.0223	31.08	10	170.64	9.78	–	405.59	–	41.45	–	–	–
Female	0.0170	40.66	10	413.26	26.07	–	1233.84	–	47.33	–	–	–
HPC												
Ketamine												
Male	0.0487	14.24	10	199.21	9.12	–	289.12	–	31.70	–	–	–
Female	0.0503	13.78	10	375.79	10.72	–	277.72	–	25.91	–	–	–
Norketamine												
Male	0.0239	29.01	10	170.25	9.10	–	355.52	–	39.06	–	–	–
Female	0.0163	42.42	10	374.46	21.69	–	1043.47	–	48.12	–	–	–

Table 2. Pharmacokinetic parameters for ketamine and metabolites in plasma and brain tissue of diestrus and proestrus female rats.

Parameter	**k_{el}**	**$t_{1/2}$**	**T_{max}**	**C_{max}**	**AUC_{0-t}**	**$AUC_{0-\infty}$**	**$AUMC_{0-t}$**	**$AUMC_{0-\infty}$**	**MRT_{0-t}**	**$MRT_{0-\infty}$**	**V_z/F**	**Cl/F**
Unit	1/min	min	min	ng/ml	ng•min/L	ng•min/L	ng•min^2/L	ng•min^2/L	min	min	L/kg	L/min/kg
Plasma												
Ketamine												
Diestrus	0.0122	56.70	5	123.93	5.56	6.06	270.64	400.96	48.64	66.15	33.74	0.412
Proestrus	0.0110	63.15	5	147.73	5.38	6.07	281.36	468.94	52.33	77.27	37.53	0.412
Norketamine												
Diestrus	0.0108	64.21	30	333.05	27.39	31.99	1817.55	3070.51	66.36	96.00	7.24	0.078
Proestrus	0.0085	81.22	30	290.54	28.63	37.03	2050.42	4548.97	71.62	122.83	7.91	0.068
Dehydronorketamine												
Diestrus	–	–	90	7.30	0.73	–	72.73	–	99.40	–	–	–
Proestrus	–	–	60	4.87	0.59	–	51.59	–	87.50	–	–	–
mPFC												
Ketamine												
Diestrus	0.0505	13.74	10	404.82	10.91	–	274.31	–	25.14	–	–	–
Proestrus	0.0504	13.74	10	400.93	10.92	–	274.83	–	25.17	–	–	–
Norketamine												
Diestrus	0.0167	41.57	10	415.86	26.49	–	1255.24	–	47.39	–	–	–
Proestrus	0.0175	39.67	10	410.66	25.63	–	1210.16	–	47.21	–	–	–
HPC												
Ketamine												
Diestrus	0.0500	13.87	5	373.87	10.69	–	276.91	–	25.90	–	–	–
Proestrus	0.0504	13.76	10	380.44	10.74	–	278.37	–	25.92	–	–	–
Norketamine												
Diestrus	0.0163	42.47	10	383.14	22.99	–	1115.64	–	48.53	–	–	–
Proestrus	0.0164	42.38	10	362.88	20.39	–	971.90	–	47.67	–	–	–

Table 3. Comparison of metabolite and brain-to-plasma ratios in male and female rats following low-dose ketamine administration (total exposure ratios at final detected timepoints are bolded for emphasis and comparison).

	Metabolite-to-Parent Ratios									Brain-to-Plasma Ratios					
Parameter	**AUC_{NK} / AUC_{K}**			**AUC_{DHNK} / AUC_{K}**			**$AUC_{NK+DHNK}$ / AUC_{K}**			**AUC_{brain} / AUC_{plasma}**					
AUC Units	(umol•min/L)			(umol•min/L)			(umol•min/L)			(umol•min/L)					
										Ketamine			**Norketamine**		
Minutes	Male	Female	*F:M*	Male	Female	*F:M*	Male	Female	*F:M*	Male	Female	*F:M*	Male	Female	*F:M*
Plasma															
5	1.88	1.54	*-1.22*	0.01	0.01	*-1.20*	1.91	1.55	*-1.23*	–	–	–	–	–	–
10	2.27	1.82	*-1.24*	0.04	0.01	*-3.47*	2.31	1.84	*-1.26*	–	–	–	–	–	–
30	3.68	3.02	*-1.22*	0.14	0.03	*-5.37*	3.82	3.05	*-1.25*	–	–	–	–	–	–
60	**4.86**	**4.09**	***-1.19***	**0.19**	**0.05**	***-3.93***	**5.06**	**4.14**	***-1.22***	–	–	–	–	–	–
90	5.16	4.65	*-1.11*	0.21	0.08	*-2.67*	5.37	4.73	*-1.13*	–	–	–	–	–	–
180	**5.40**	**5.44**	***-0.99***	**0.23**	**0.13**	***-1.78***	**5.64**	**5.57**	***-1.01***	–	–	–	–	–	–
mPFC															
5	0.85	1.09	*1.28*	–	–	–	–	–	–	1.97	2.93	*1.48*	0.89	2.07	*2.32*
10	0.88	1.09	*1.24*	–	–	–	–	–	–	2.27	3.28	*1.45*	0.88	1.96	*2.23*
30	0.94	1.49	*1.59*	–	–	–	–	–	–	3.15	2.97	*-1.06*	0.80	1.47	*1.82*
60	**0.95**	**2.00**	***2.09***	–	–	–	–	–	–	**4.05**	**2.75**	***-1.47***	**0.79**	**1.34**	***1.69***
HPC															
5	0.80	0.91	*1.15*	–	–	–	–	–	–	1.97	2.73	*1.38*	0.83	1.62	*1.94*
10	0.83	0.96	*1.16*	–	–	–	–	–	–	2.35	3.06	*1.30*	0.86	1.62	*1.87*
30	0.93	1.32	*1.42*	–	–	–	–	–	–	3.17	2.84	*-1.11*	0.80	1.24	*1.55*
60	**0.96**	**1.68**	***1.74***	–	–	–	–	–	–	**3.94**	**2.69**	***-1.46***	**0.78**	**1.10**	***1.41***
Brain (Avg)															
5	0.82	1.01	*1.22*	–	–	–	–	–	–	1.97	2.83	*1.43*	0.86	1.84	*2.14*
10	0.87	1.03	*1.19*	–	–	–	–	–	–	2.31	3.17	*1.37*	0.88	1.79	*2.03*
30	0.95	1.41	*1.49*	–	–	–	–	–	–	3.16	2.91	*-1.09*	0.81	1.36	*1.67*
60	**0.97**	**1.84**	***1.91***	–	–	–	–	–	–	**3.99**	**2.72**	***-1.47***	**0.79**	**1.22**	***1.54***

Figures

Figure 1

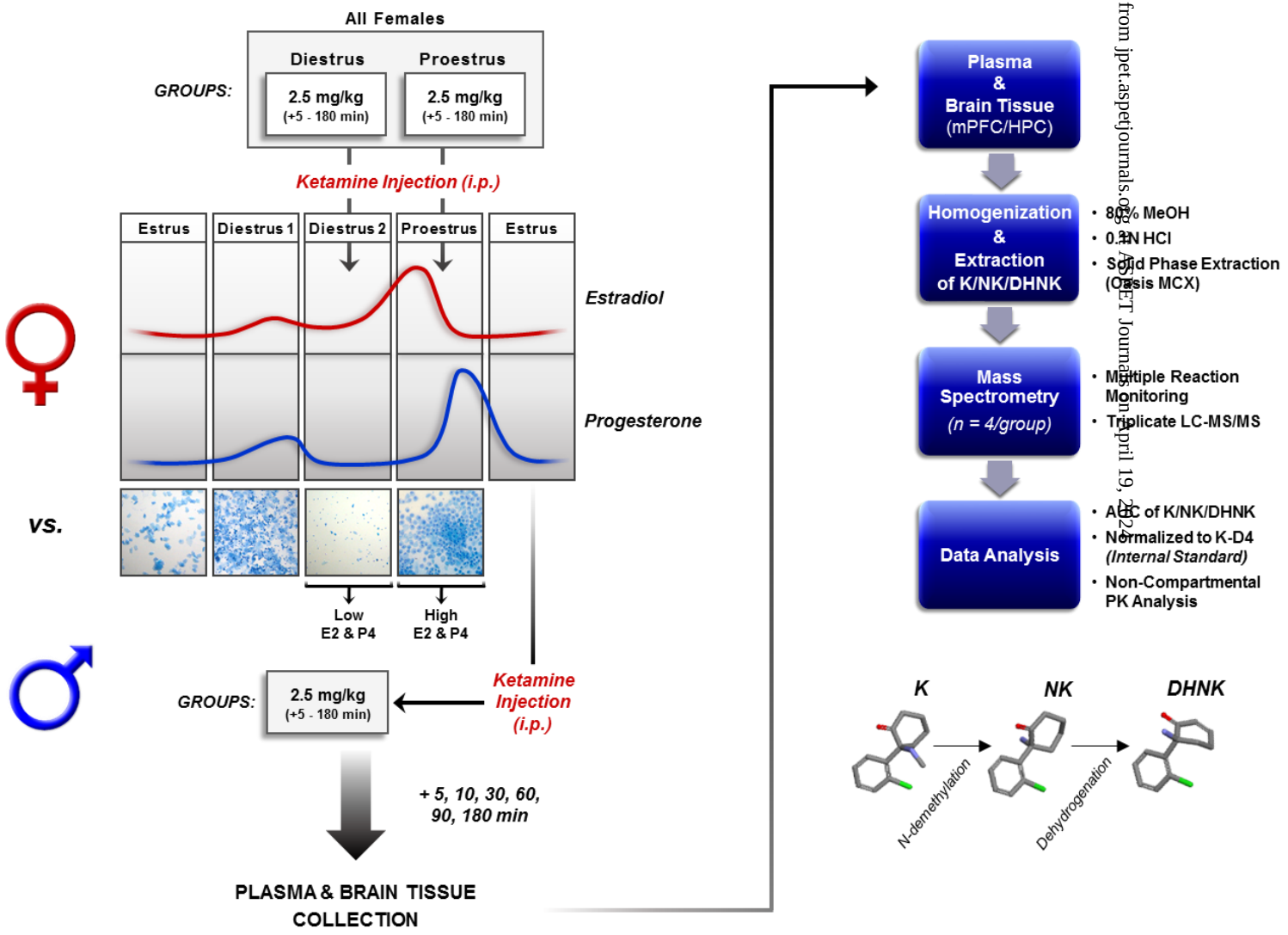

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Figure 2

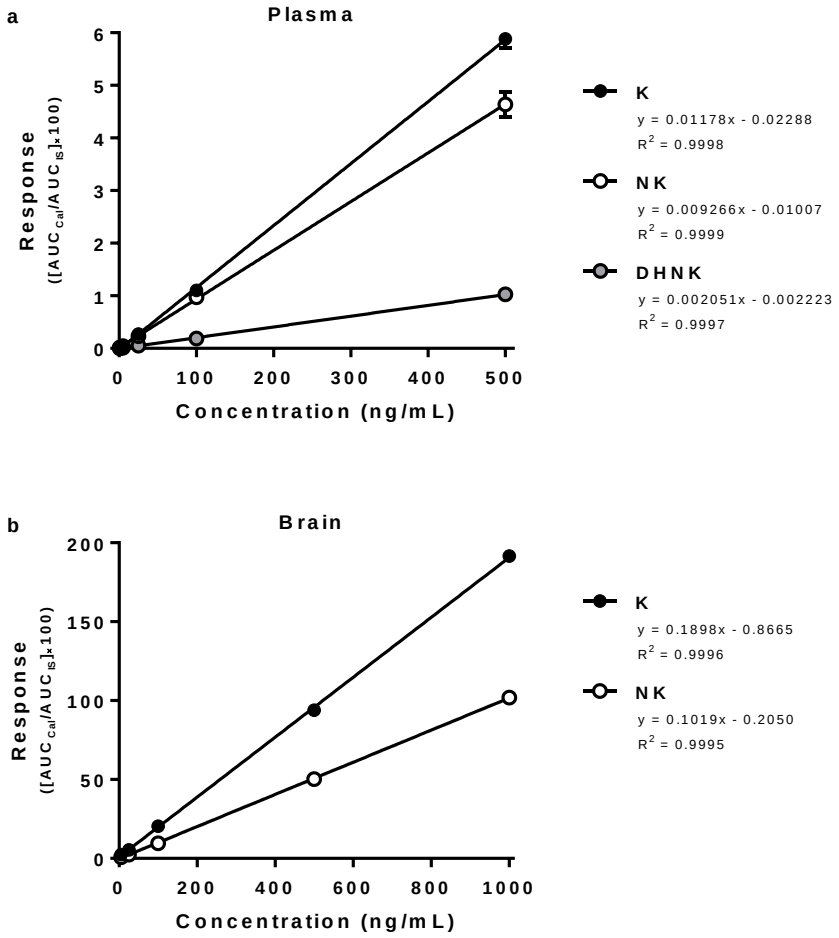

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Figure 3

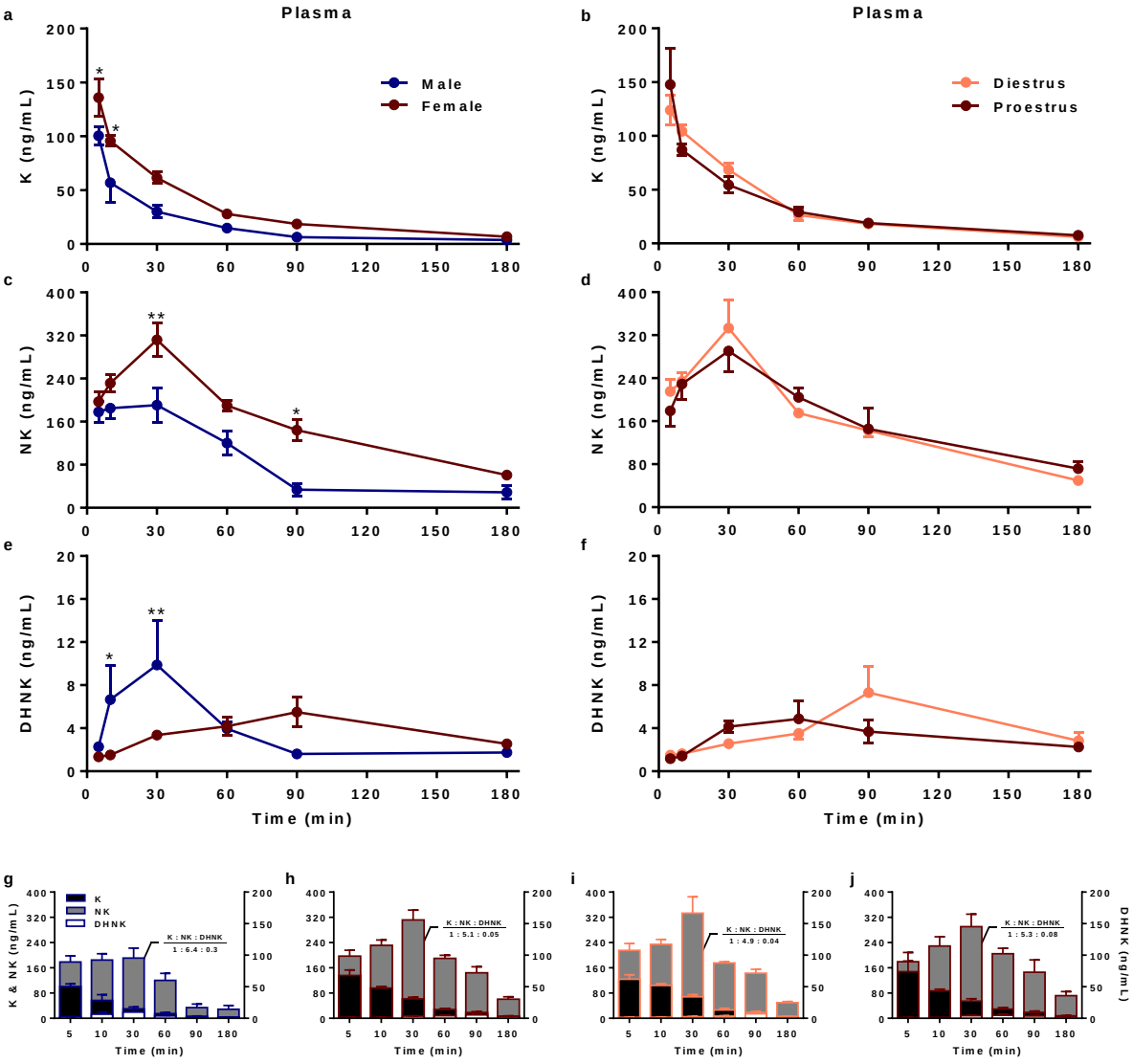

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Figure 4

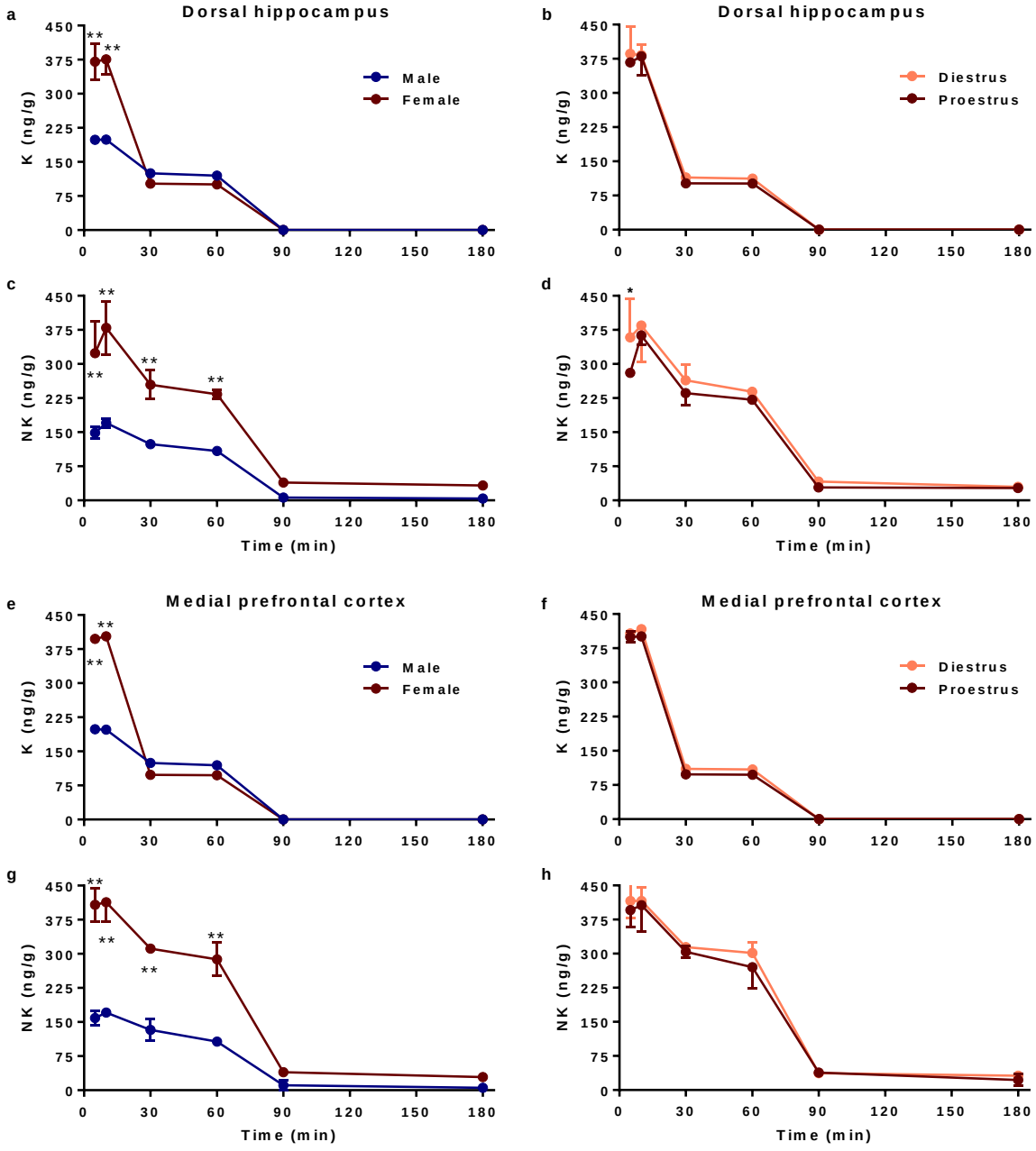

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Figure 5