Sex differences in effects of ketamine on behavior, spine density and synaptic proteins in socially isolated rats

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Abstract

Background—The mechanistic underpinnings of sex differences in occurrence of depression and efficacy of antidepressant treatments are poorly understood. Here we examined the effects of isolation stress and the fast acting antidepressant ketamine, on anhedonia and depression-like behavior, spine density and synaptic proteins in male and female rats.

Methods—We used chronic social isolation stress (IS) paradigm to test the effects of ketamine (0, 2.5 and 5mg/kg) on behavior and levels of synaptic proteins Synapsin1, PSD95 and GluR1 in male and female rats in diestrus. mPFC spine density was also examined in males and in females that received ketamine either during diestrus or proestrus phase of their estrous cycle.

Results—Males showed anhedonia and depression-like behavior after 8 weeks of IS, concomitant with decreases in spine density and levels of Synapsin1, PSD95 and GluR1 in the mPFC; changes that were reversed by a single injection of ketamine (5 mg/kg). Females, after 11 weeks of IS, showed depression-like behavior but no signs of anhedonia. Although both doses of ketamine rescued depression-like behavior in female rats, the decline observed in synaptic proteins and spine density in IS and in diestrus females, could not be reversed by ketamine. Spine density was higher in females during proestrus than in diestrus.

Conclusions—Our findings implicate a role for synaptic proteins Synapsin1, PSD95 and GluR1, and mPFC spine density in the antidepressant effects of ketamine in IS males but not in IS females, suggesting dissimilar underlying mechanisms for efficacy of ketamine in the two sexes.

Six key words for indexing

Sex-difference; ketamine; social-isolation; mPFC; anhedonia; depression

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Introduction

Interesting gender differences exist in a) the prevalence rate of depression (1, 2) b) the symptoms of depressive disorders (3, 4) and c) the efficacy of antidepressant medication (5–7). Depression is twice as prevalent amongst women as in men. Specific symptoms of depression such as anxiety, bulimia and suicidal ideation are more common in women than in men, who predominantly display symptoms of alcoholism and substance abuse (8, 9). Pre-clinical research has also shown robust sex differences in animal models of depression (10). In spite of the existence of compelling evidence for sex differences in depression, the mechanisms underpinning these remain largely unexplored. Studies have attributed behavioral differences between males and females to sexual dimorphism of the underlying neuronal circuitry and neuroendocrinal systems (11) which in turn have genetic (12) and epigenetic (13) underpinnings. Although some clinical studies have reported higher efficacy of SSRIs in women than in men (5), collectively, for both sexes, the current available antidepressants with slow onset of therapeutic effects, have serious limitations (14, 15). However, recently, ketamine the NMDA receptor antagonist classically used as an anesthetic has emerged as a fast acting antidepressant (16, 17). Acute injections of ketamine produce rapid antidepressant effects within few hours (18–20). Interestingly, previous studies from our lab on rats (21) and others on mice (22) have shown females to be more sensitive to ketamine than males. Indeed, a single injection of 2.5 mg/kg dose of ketamine induced antidepressant-like effect in female but not in male rats, that responded to doses of 5 mg/kg and above (21). To further investigate sex differences in antidepressant actions of ketamine, we examined its effects on a) anhedonia and depression-like behavior, b) spine density in the mPFC and c) molecular changes in the mPFC synaptoneurosomes, in male and female rats that were exposed to chronic social isolation stress (IS). Isolation stress is known to evoke depression and anhedonia-like behavior in rats (23). Previous studies from our lab have established the suitability of this stress paradigm for comparative studies between males and females (24), because, unlike chronic unpredictable (25) and chronic mild (26) stress paradigms that were employed to delineate molecular pathways affected by ketamine in male animals, chronic IS does not disrupt the estrous cycle in female animals. Our results highlight interesting differences between males and females in their response to IS and ketamine treatment and implicate alternative underlying mechanisms for efficacy of ketamine in male and female rats.

Materials and Methods

Animals

Adult male (250–270 g) and female (200–225 g) Sprague Dawley rats (Charles River, USA) were maintained either under pair-housed (PH) condition or in solitary cages under isolation stress (IS) on a 12 hour/12 hour light/dark cycle (lights on at 0500 h) with *ad libitum* access to food and water. All animal protocols were carried out in accordance with the NIH Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Florida State University.

Experimental designs

Effect of ketamine on anhedonia and depression-like behavior in male and female rats subjected to isolation stress—Male (n = 8/group) and female (n = 6/group) rats were housed either under pair housed (PH) condition or under isolation stress (IS). During this period of time, PH and IS groups of animals were tested on sucrose preference test (SPT) periodically (3 alternate days/week) to check for the emergence of behavioral abnormalities induced by IS. Once a significant decline in preference for sucrose was observed in the IS group, a decline that was stable and maintained over the next week, animals were injected with saline/ketamine (2.5, 5 mg/kg; Butler Schein Animal Health, Inc.) and tested for anhedonia-like behavior on the SPT after 3 hours (day1) and 27 hours (day2) and for depression-like behavior on the FST on the third day post injection (Figure 1A). Female rats were lavaged for determination of estrous cycle stage and those having consistent cycle lengths of 4–5 days were injected with ketamine and tested behaviorally during diestrus.

Effect of ketamine on mPFC spine density in male rats and during different phases of estrous cycle in female rats subjected to isolation stress—To investigate effects of ketamine on spine density in animals exposed to IS, bilateral stereotactic surgeries were performed to infuse HSV- GFP into the mPFC of PH and IS rats of both sexes (n = 4/group). Female rats were lavaged to determine estrous cycle stage. 3–4 days after surgery, rats were injected with saline/ketamine (2.5, 5 mg/kg). Female rats were injected with ketamine either on diestrus when gonadal hormone levels are lowest or on proestrus when gonadal hormone levels are at peak. Rats were perfused 3 hours post drug injections. GFP immunohistochemistry was performed to detect fluorescence. Dendrites belonging to the apical tuft of layer V pyramidal neurons of the mPFC were imaged using Zeiss Confocal microscope under 63X oil objective. Spines were counted using Neurolucida Explorer.

Effect of ketamine on synaptic proteins in male and female rats subjected to isolation stress—PH and IS male (n = 6/group) and female (n = 5/group) rats that underwent behavioral testing, were sacrificed 1 hour after FST and brain tissues were collected for Western blot assay of synaptic proteins; Synapsin1, Post synaptic density protein 95 (PSD95) and Glutamate receptor 1 (GluR1) on synaptoneurosomal fractions isolated from mPFC.

Behavioral tests

Sucrose preference test (SPT)—The SPT, a two-bottle choice paradigm was performed as described earlier (21, 27). Rats were habituated to drinking from two bottles prior to testing. For testing of baseline preference for sucrose and for the SPT after the period of stress, rats were given access to two pre-weighed bottles, one of which contained water and the other contained 0.25% sucrose for the first 2 hours of the dark cycle. The bottles were weighed at 17:00 and 19:00 hours and the preference for sucrose over water was used as a measure of anhedonia.

Forced swim test (FST)—The FST, a two day procedure was performed as described previously (28, 29). On day 1 and day2, rats were placed in 30×45 cm Plexiglas cylinders filled with water, maintained at 25 °C for 15 and for 5 minutes respectively and their behaviors were videotaped, and analyzed for immobility time by a scorer blind to the treatment conditions. Immobility was defined as minimum movement required for remaining afloat (30). Female rats received the pre-test and test when they were in the diestrus stage of their cycle, when the levels of gonadal hormones are at their lowest. Therefore the FST test was conducted 3 days after ketamine injection in male rats, and 3–5 days after ketamine injection in female rats.

Virus delivery and immunohistochemistry for GFP staining

To visualize dendrites and spines in the pre-limbic (PL) region of the mPFC, both male and female rats were given bilateral stereotactic injections of the HSV- GFP viral vector [p1005+ HSV plasmid expressing GFP under the control of CMV promoter] following standard methods (31). Animals were anesthetized using Isoflurane (Henry Schein Animal Health, OH, USA) during surgeries. Bregma coordinates used for the surgery were as follows: +3.1 mm anterior, +/− 0.6 mm lateral, and 3.9 mm ventral to bregma. The virus was delivered at a rate of 0.1 μl/min using 24-gauge syringe needles (Hamilton), for a total volume of 1.0 μl per animal, on each side. The animals were sacrificed 3–4 days after viral infection when transgene expression is maximal.

Coronal sections (50 μm) of the mPFC, encompassing the site of HSV-GFP injection, were generated using a Vibratome (Leica Microsystems, Germany). To visualize the spines, immunohistochemistry for GFP was performed on the sections. Briefly; after blocking (5% normal goat serum and 0.3% Triton X) sections were incubated overnight with Chicken-anti-GFP (1:500; Abcam), washed and incubated with Alexa488-conjugated goat-anti-chicken (1:1000; Life Technologies) for 3 hours. Labeled pyramidal neurons of the PL were imaged under 63x oil objective (Zeiss Plan-Apochromat, NA = 1.40) of a Zeiss LSM880 confocal microscope. The fluorescent tag was excited using an Argon/Krypton 488nm laser line. For spine density analysis, Z-stacks of images were obtained, consisting of 2–4 scans at 3x optical zoom, 0.5 μm step sizes.

Spine density measurements

Spine density was sampled in the proximal segment [a segment known to be affected by chronic stress exposure (32)] of the apical tuft dendrites of layer V pyramidal neurons of the PL region of the mPFC. Dendritic spines were qualitatively classified into one of the following three types: mushroom, thin and stubby. Spine density was analyzed using Neurolucida Explorer (version 9, MBF Bioscience) and expressed as the number of spines per 10 μm dendritic segments. Dendritic branches, 30–50 μm long, from the branch point, were included in the analysis. Consistent with previously reported spine density analyses (32), ~12 neurons from each rat, (n = 4 rats in each treatment group) were quantified. In cases where the established criteria for inclusion in analyses were not met by the visible neurons of both hemispheres, data was obtained from neurons only in one of the two hemispheres.

Synaptoneurosome preparation and western blot

The prelimbic region of the mPFC was tissue punched in a cryostat and frozen at −80 °C until further processing. Synaptoneurosomal fractions were prepared and collected from the tissue as previously described (21, 32). Briefly, tissue was homogenized in a solution containing 0.32 M sucrose, 20 mM HEPES (pH 7.4), 1 mM EDTA and protease inhibitors and centrifuged at 4 °C for 10 minutes at 2800 rpm. The pellet (nuclear fraction) was removed. The supernatant was centrifuged at 4 °C for 10 minutes at 12,000 rpm and the pellet (crude synaptoneurosomal fraction) was collected and sonicated in 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, and protease inhibitors. Equal concentrations of proteins (10 mg) were loaded into 12% Acrylamide gel for electrophoresis. Immunoblots were incubated overnight with Synapsin1 (1:1000), PSD95 (1:1000), GluR1 (1:500) or GAPDH (1:5000) antibodies (Cell Signalling Technology), washed and incubated for 1 hour with goat anti-rabbit IR-Dye680LT (Li-COR Biosciences; 1:20,000) fluorescent secondary antibody, and visualized using Odyssey infrared imaging system (Li-COR Biosciences). Quantification was performed using NIH ImageJ software.

Statistical analysis

Data was analysed using repeated measures and two-way analysis of variance (ANOVA) followed by Fisher's *post-hoc* wherever appropriate. *p* values <0.05 were considered statistically significant.

Results

Effect of ketamine on anhedonia and depression-like behavior in male and female rats subjected to isolation stress

Male IS rats started showing a significant decline in sucrose preference from week-7 onwards, which was stable across week-8 [effect of housing condition; $F_{(1,46)}=50.1$, $p<0.05$] (Figure 1B). In male rats, the IS evoked decline in sucrose preference [housing; $F_{(1,42)}=7.76$, $p<0.05$] was completely rescued by an acute injection of ketamine (5mg/kg) on day1 of SPT [effect of treatment; $F_{(2,42)}=3.24$, $p<0.05$] but not on day2 (Figure 1C). IS also elicited an increase in immobility time [housing; $F_{(1,42)}=9.68$, $p<0.05$], indicative of depression-like behavior in the FST in male rats (Figure 1D). Ketamine (5mg/kg) reduced immobility time significantly in the FST in male rats [treatment; $F_{(1,42)}= 4.77$, $p<0.05$]. The 2.5mg/kg dose of ketamine did not improve behavior in the SPT and FST in males, consistent with previous findings (21).

Surprisingly, IS females showed no decline in sucrose preference even after 11 weeks of isolation (Figure 1E). A single injection of ketamine did not have an effect on sucrose preference in female rats, at either dose (Figure 1F). However, IS evoked a significant increase in immobility time in the FST [$F_{(1,65)}=74.39$, $p<0.05$] and a single injection of both doses of ketamine elicited a reduction in immobility time in PH as well as in IS female rats [$F_{(2,65)}=7.95$, $p<0.05$] (Figure 1G). Efficacy of both, the lower (2.5 mg/kg) as well as the higher (5 mg/kg) doses of ketamine in reversing the behavioral deficits observed in FST in females, strengthens our hypothesis that female rats are more sensitive to the antidepressant effects of ketamine than their male counterparts.

Effect of ketamine on mPFC spine density in male rats and during different phases of estrous cycle in female rats subjected to isolation stress

To investigate the effect of IS on mPFC spine density, we counted spine numbers by confocal imaging of the apical tuft of pre-labelled layer V pyramidal neurons of the mPFC (Figure 2B). Our results showed that 8 weeks of IS, induced a significant decline in spine density in the proximal segment of the apical tuft in male rats [housing; $F_{(1,18)}=9.68$, $p<0.05$] (Figure 2C, D). This deficit was completely reversed by the 5 mg/kg but not the 2.5 mg/kg dose of ketamine [treatment; $F_{(2,18)}=3.16$, $p<0.05$] (Figure 2D), consistent with the improvement in behavior observed in male rats in SPT and FST with the same dose. Further, analysis of spine morphology revealed a decline in mushroom [housing; $F_{(1,18)}=13.10$, $p<0.05$] and thin [housing; $F_{(1,18)}=4.5$, $p<0.05$] spine density in male IS rats (Figure 2E, F). Ketamine (5 mg/kg) increased the density of thin (treatment; $F_{(2,18)}=2.45$, $p<0.05$), but not mushroom spines (treatment; $F_{(2,18)}=1.52$, $p=0.24$) in male rats (Figure 2E, F).

We also investigated the effect of ketamine on spine density changes evoked by IS in female rats. Female rats were lavaged for tracking of estrous cycle. After 11 weeks of stress, bilateral stereotactic injections of HSV-GFP were performed. After 3–4 days, animals were injected with saline/ketamine (2.5, 5 mg/kg) either during diestrus or during proestrus and sacrificed 3 hours later (Figure 2G). IS evoked significant declines in the densities of total ($F_{(1,36)}=8.83$, $p<0.05$), mushroom ($F_{(1,36)}=15.49$, $p<0.05$) and thin spines ($F_{(1,36)}=5.46$, $p<0.05$) in female rats irrespective of the cycle stage (main effect of housing condition) (Figure 2I–K). Total ($F_{(1,36)}=18.87$, $p<0.05$), mushroom ($F_{(1,36)}=18.66$, $p<0.05$) and thin spine ($F_{(1,36)}=12.97$, $p<0.05$) densities were significantly lower in both PH and IS female rats during diestrus than in proestrus (main effect of cycle stage) (Figure 2I–K). However, in female rats, ketamine did not alter spine density in the mPFC (Figure 2I–K). Density of stubby spines remained unaltered in males ($F_{(1,18)}=0.22$, $p=0.8$) and females ($F_{(1,36)}=0.28$, $p=0.59$) under all conditions.

Effect of ketamine on synaptic proteins in male and female rats subjected to isolation stress

We examined the influence of IS on specific synaptic proteins in synaptoneurosome preparations of the mPFC (Figure 3A, B). Our results showed that levels of Synapsin1 ($F_{(1,30)}=47.31$, $p<0.05$) PSD95 ($F_{(1,30)}=19.02$, $p<0.05$) and GluR1($F_{(1,32)}=9.26$, $p<0.05$), were significantly reduced in the mPFC of IS males (Figure 3C–F). A single injection of ketamine (5mg/kg) reversed the decline observed in the levels of Synapsin1 ($F_{(2,30)}=27.39$, $p<0.05$) and PSD95 ($F_{(2,30)}=3.37$, $p<0.05$) and attenuated the decline in GluR1($F_{(2,32)}=1.70$, $p=0.07$) in male rats (Figure 3C–F), consistent with our results in behavioral and spine density experiments. Similarly, declines in the levels of Synapsin1 ($F_{(1,26)}=12.67$, $p<0.05$), PSD95 ($F_{(1,26)}=11.94$, $p<0.05$) and GluR1 ($F_{(1,6)}=8.17$, $p<0.05$) were also observed in the mPFC of IS females (Figure 3G–J). However, unlike in the males, ketamine did not elevate the levels of these proteins in the females, consistent with ketamine's lack of effect on SPT and spine density in female rats.

Discussions

The consequences of social isolation stress are distinct and different, based on the time and duration of administration (33). Studies have investigated the effect of early-life (34–37), and adolescent (38, 39) social isolation of varying time-lengths on behavior and underlying mechanisms. Here we showed for the first time, the effect of chronic social isolation (8–12 weeks) administered during adulthood, on behavior, spine density and synaptic molecules in the mPFC in male as well as in female rats. Our study is unique because it incorporates the variable of sex, which had not been taken into consideration in previous reports. We further investigated the effect of ketamine, in male and female rats exposed to chronic IS in adulthood. The effect of ketamine and IS on spine density was studied in diestrus as well as in proestrus females, giving us further insights on the influence of ovarian hormones on spine density in ketamine treated IS rats. Our experimental design enabled us to unveil several differences between males and females exposed to the same stressor (albeit for different exposure time) and treatment paradigm.

Our results showed that while 7 weeks of IS in adulthood evoked a strong anhedonia-like behavior in male rats, in the female rats it did not. This result is in agreement with a previous report in which anhedonia-like behavior was not observed in female Wistar rats that underwent chronic IS in adulthood (40). Clinical (41) and preclinical studies (42) have shown that circulating levels of estrogen in females can modulate the threshold for sweetness detection and preference for sucrose. Given that the female rats used in our experiment for SPT had undisrupted estrous cycles, the effect of IS alone on sucrose preference, in the presence of fluctuating levels of ovarian hormones remained unresolved. Future experiments examining sucrose preference during specific stages of the estrous cycle, or in overectomized and hormone-supplemented rats may provide a better understanding of the influence of ovarian hormones on anhedonia-like behavior in IS females. In the FST, although both sexes showed depression-like behavior after chronic IS, male rats responded only to the higher dose of ketamine, while the females responded to both (2.5 mg/kg and 5 mg/kg). This result corroborated previous findings from our lab which demonstrated that female rats have higher sensitivity towards ketamine and respond to the lower dose, to which the males do not (21). Additionally, in the FST, significant improvements in behavior with ketamine were observed also in the PH groups. PH males injected with the higher dose and PH females injected with either dose of ketamine showed significant lowering of immobility time in the FST. While the lower dose of ketamine did not influence the behavior of either sex in the SPT, a prominent sex difference in the efficacy of this dose emerged in the FST. Given that FST is a stressful behavioral test, the unmasking of a sex difference particularly in the FST may have resulted from the additional/acute stress experienced during the test.

Several studies have linked chronic stress exposure to decline in spine density and myelination in the mPFC (32, 37, 43–45). Recently, ketamine was shown to elicit a rapid reversal of chronic unpredictable stress (CUS) evoked decline in spine density in the mPFC in male mice (32). Given the large body of literature that implicates mPFC and dendritic spines as possible substrates for the action of stress, we examined the effects of IS and ketamine on mPFC spine density in male and female (diestrus and proestrus) rats. Previous studies have shown that spine density in the female mPFC decreases during diestrus, when

the circulating levels of estrogen and progesterone are low, and increases during proestrus when the hormone levels are high, in cycling female rats (46). In our study, a similar pattern of spine density fluctuations was witnessed in the mPFC of female rats even after 11 weeks of IS. It was indeed interesting to note lower spine densities in both PH and IS females during diestrus, than during the proestrus phase of the cycle. Clinical depression is also reported more commonly in women during phases of life when levels of estrogen and progesterone are at their lowest, such as during the post-partum and menopausal phase (47). Our results add to the existing pool of knowledge that advocates a direct correlation between hormonal levels, spine density and mood. Ketamine evoked a rapid (within 3 hours) and complete reversal of the IS mediated decline in spine density in male rats. While the decline in total spine density was a cumulative effect of the decline in thin spines and mushroom spines, the increase in spine density by ketamine within 3 hours, was mediated mostly through an increase in thin spines (new, immature spines), while the mushroom spines (established, mature) remained unaffected. Previous studies have shown increases in number of mushroom spines in the mPFC of CUS exposed mice, after 24 hours of ketamine treatment (32). Although our results show a rapid increase in the number of thin, immature spines within 3 hours of ketamine treatment, it remains to be investigated whether a longer delay after ketamine treatment (24 hours) would enable maturation of the thin spines into mushroom spines in IS rats. It would also be interesting to investigate mushroom spine densities in male rats after 3 days of ketamine treatment, given the fact that we observed a rescue of PSD95 levels, a marker for mature spines 3 days post ketamine injections. On the other hand, ketamine had no effect on spine density in the mPFC of female rats. Studies in the past have shown that effects of stress on spine density are circuit, sex and stressor specific (48–50). For example, while 10 days of restraint stress elicited no change in spine density in mPFC neurons that project to the basolateral amygdala (BLA) in male rats (51), the same stressor increased spine density in BLA projecting neurons of mPFC layer III in female rats (52). However, past studies have not investigated sex effects on the action of antidepressants on spine density. Our result demonstrates for the first time that sex differences exist in the effects of stress and antidepressants on spine density. However, one of the limitations of this study is the fact that animals tested for spine density analysis, were not examined for behavioural changes induced by IS.

Observing stress effects on spine density in the mPFC encouraged us to investigate levels of synaptic proteins in the mPFC of IS rats. We examined the levels of well characterized pre and post-synaptic molecules, Synapsin1, PSD95 and GluR1 that have previously shown a decline in the male mPFC following chronic stress (32, 53) and in post-mortem studies on MDD patients [Synapsin1; (54), PSD95; (55), GluR1; (56)]. Our results demonstrated sex differences in the long term (3 days) effects of acute ketamine injection on synaptic proteins. A single injection of ketamine was effective in reversing the IS evoked decline in synaptic proteins in the mPFC of male, but not female rats. Increases in Synapsin1, PSD95 and GluR1 levels in the mPFC of male mice exposed to CUS were previously shown with ketamine and considered crucial for spinogenesis and the rapid antidepressant effects of ketamine (32). Although our results in male rats are in agreement with Li *et al*, we hypothesize the existence of alternate underlying mechanisms for the behavioral efficacy of ketamine in female rats. Another study implicated reduction in eEF2 kinase activity which

triggers increases in BDNF translation in the hippocampus in the rapid antidepressant effects of ketamine in male mice (26). Examining the effect of ketamine on the eEF2-BDNF pathway in the hippocampus of female rats was beyond the scope of this study. However, we speculate the involvement of other brain areas such as the hippocampus, and/or pathways in the antidepressant effects of ketamine in female rats. Very recent studies have shown sex differences in expression of several NMDA and AMPA receptor subunit genes in post-mortem mPFC tissue of patients suffering from MDD (57) and in rats with a history of prenatal chronic mild stress (53). An altered AMPA to NMDA receptor function balance has also been implicated in rapid antidepressant effects of ketamine in male mice (58). Future experiments examining possible sex differences in the expression of glutamate receptor genes and AMPA receptor activity in specific neuronal circuits may provide further insights for differential efficacy of ketamine in male and female rats.

In conclusion, our findings highlight a) sex differences in the effect of chronic IS on behavior, spine density and synaptic proteins, b) sex differences in the action of ketamine on behavior, spine density and synaptic proteins in animals subjected to chronic IS and c) in females, estrous cycle stage specific differences in the effect of chronic IS on spine density in the mPFC. We implicate a role for mPFC synaptic proteins and spine maturation in the antidepressant effects of ketamine in male rats subjected to IS in adulthood. Our results indicate that the efficacy of ketamine is probably mediated via dissimilar underlying mechanisms in the two sexes, and opens up several avenues for investigations in the future.

Acknowledgments

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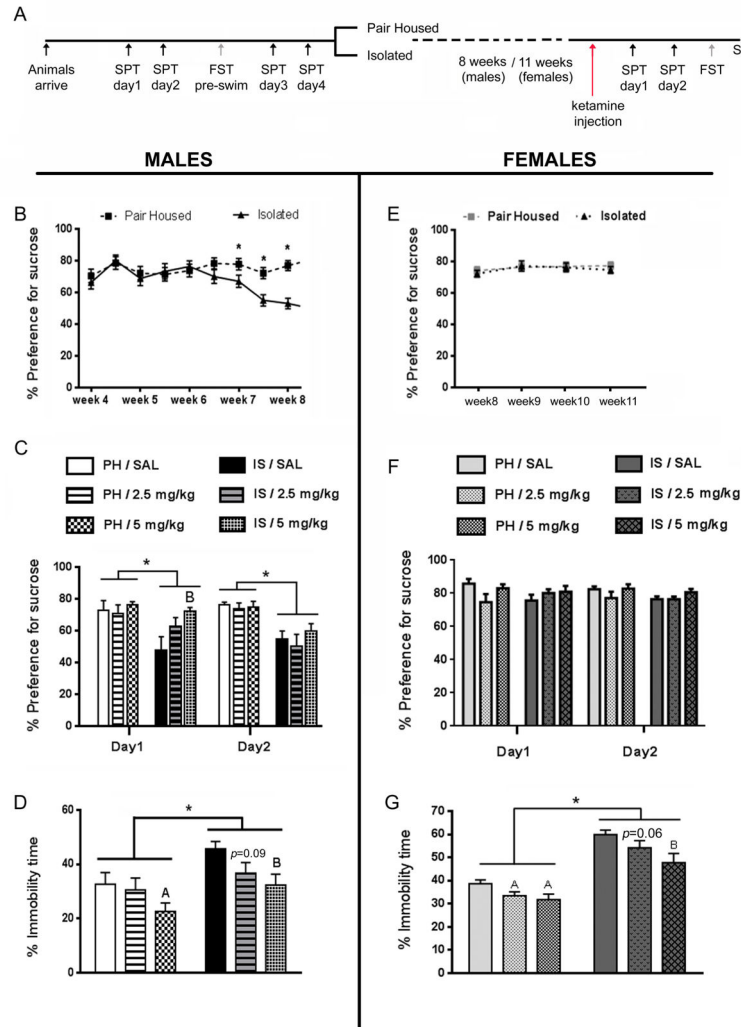
Figure 1.
Isolation stress and ketamine affect male and female rats differently, in tests for anhedonia
and depression-like behavior. **(A)** Schematic representation of the experimental design.
Animals were tested for their baseline behaviors on the sucrose preference test (SPT) and
pre-swim for the forced swim test (FST), prior to grouping into pair-housed (PH) and
isolation stress (IS). PH and IS rats lived in respective housing conditions for 8 weeks
(males) or 11 weeks (females). Ketamine was injected after week 8 in males and week 11 in
females when they were in diestrus. SPT was performed 3 hours (day1) and 27 hours (day2)
after ketamine injection, followed by FST on the next day for male rats and the next 1–2
days for females (when they were in diestrus). Animals were sacrificed (S) 1 hour after FST.
(B) IS induced a significant and stable decline in sucrose preference after 7 weeks in male
rats. **(C)** This deficit could be rescued by an acute injection of ketamine (5mg/kg) on day1 of
SPT, but not on day2. **(D)** IS also evoked an increase in immobility time, indicative of
depression-like behavior in the FST in males. A single injection of ketamine (5mg/kg)
reduced immobility time significantly in the FST in both PH and IS male rats. **(E)** IS did not
evoke a decline in sucrose preference in the female rats even after 11 weeks of stress. **(F)**

Ketamine did not increase preference for sucrose in female PH and IS rats, at either dose (2.5 and 5mg/kg). **(G)** IS evoked a significant increase in immobility time, in the FST in females rats. A single injection of both doses of ketamine (2.5 and 5mg/kg) caused a reduction in immobility time in the FST in PH as well as IS female rats. Data are mean ± SEM for percent preference for sucrose in SPT and percent immobility time in FST [n = 8/ group (males), 6/group (females)]. (*p<0.05, ^{A}p<0.05 compared to PH/SAL, ^{B}p<0.05 compared to IS/SAL, repeated measures ANOVA for SPT and two way ANOVA for FST, Fisher's *post-hoc* test)
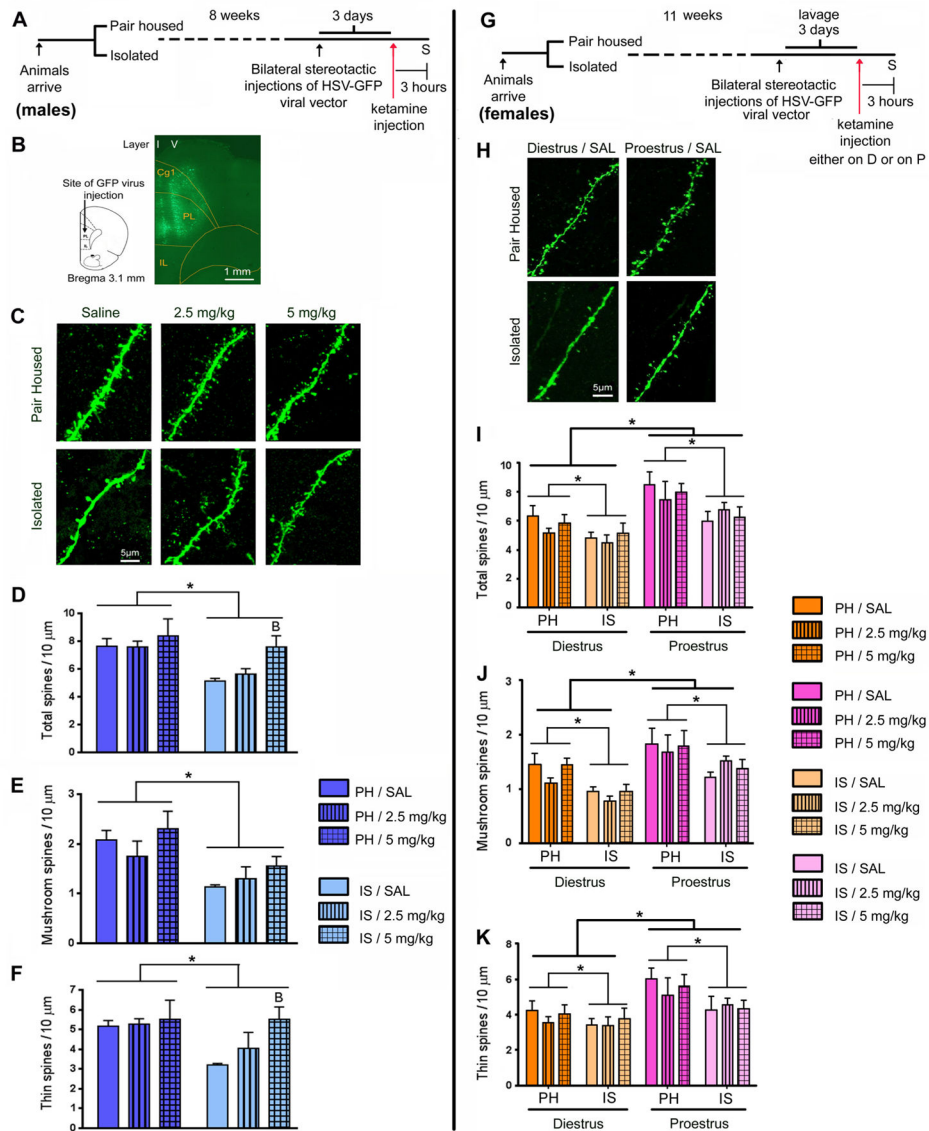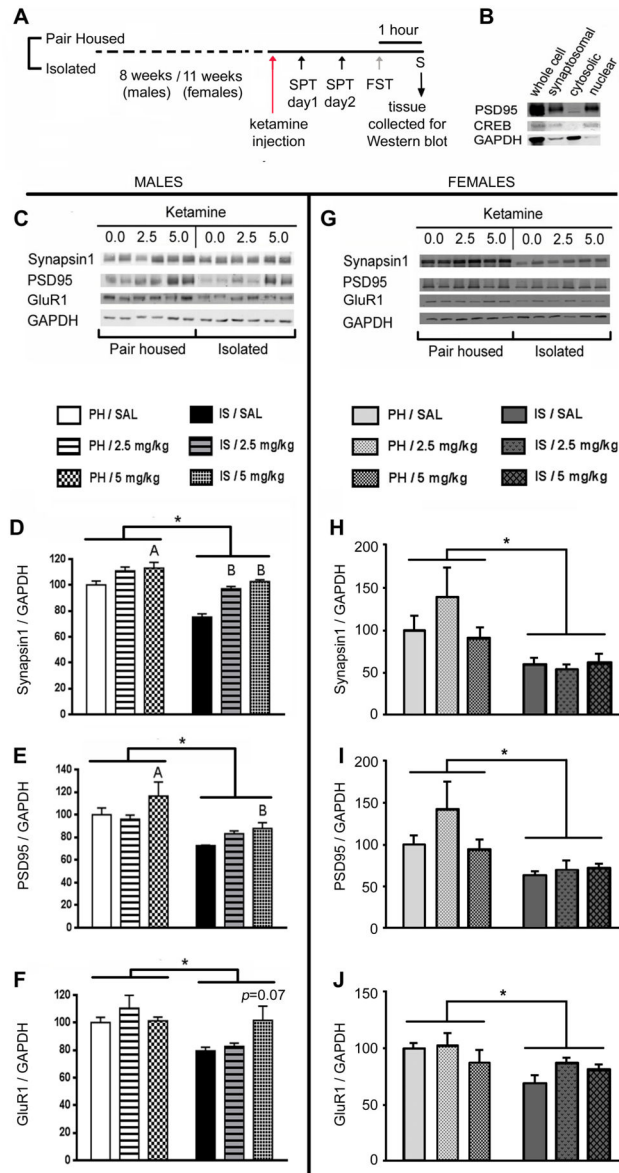
Figure 2.

Ketamine rescued IS evoked mPFC spine density deficits in males but not in female rats. **(A)** Schematic representation of the experimental design with male rats. Animals were grouped into PH and IS. After 8 weeks of stress, bilateral stereotactic injections of HSV-GFP were performed. After 3 days, when virus expression was at its maximum, animals were injected with ketamine. 3 hours after ketamine injection, animals were sacrificed (S). **(B)** Map of PL and IL regions in the mPFC of the rat, showing the site of injection of the HSV-GFP virus. GFP fluorescence was detected by immunostaining of sections. Shown is a low magnification (10x) fluorescence image of a GFP expressing mPFC section. Green fluorescence can be seen both in layer I (apical tuft) and in layer V (cell body) of the PL region of the mPFC. **(C)** Representative images are shown of high magnification Z-stack projections of proximal segments of the layer V pyramidal cell apical tuft dendrites of male rats (scale bar: 5 μm). **(D)** IS decreased spine density in the proximal segment of the apical

tuft. This deficit was completely reversed by ketamine treatment (5 mg/kg) in male rats. Analysis of spine morphology revealed a decline in **(E)** mushroom and **(F)** thin spine density in IS animals. A single injection of ketamine (2.5 and 5 mg/kg) increased the density of **(F)** thin, but not **(E)** mushroom spines in the mPFC of male rats. **(G)** Schematic representation of the experimental design with female rats. Animals were grouped into PH and IS. Animals were lavaged for tracking of estrus cycle. After 11 weeks of grouping, bilateral stereotactic injections of HSV-GFP were performed. After 3 days, animals were injected with ketamine, either during diestrus (D) or proestrus (P). 3 hours after ketamine injection, animals were sacrificed (S). **(H)** Representative images are shown of high magnification Z-stack projections of apical tuft dendrites in female rats (scale bar: 5 μm). **(I–K)** IS evoked a significant decline in the density of spines of all sub categories in female rats irrespective of the cycle stage (effect of housing condition). **(I)** Total spine density, **(J)** mushroom as well as **(K)** thin spine densities were significantly lower in both PH and IS groups of female rats during diestrus than in proestrus (effect of cycle stage). However, in female rats, ketamine did not cause an increase in spine density in the mPFC. Density of spines was analyzed using Neurolucida Explorer (version 9; MBF Bioscience). Results are the mean ± SEM (~12 cells from four rats in each group; *p<0.05, ^{B}p<0.05 compared to IS/SAL, two-way ANOVA, Fisher's *post-hoc* test).

Figure 3.
Ketamine rescued IS evoked decline in synaptic protein levels in males but not in female rats. **(A)** Schematic representation of the experimental design. Animals were grouped into PH or IS. After 8 weeks (males) or 11 weeks (females) of stress, animals were injected with ketamine (females were injected when they were in diestrus). After completion of behavioral tests(i.e 3 days in males and 3–5 days for females) after ketamine injection, animals were sacrificed and brains were collected for Western blot assays. **(B)** Synaptoneurosomal fractions collected for Western blot assays were enriched in post synaptic density proteins such as PSD-95. **(C)** Representative Western blot showing that IS decreases levels of Synapsin1, PSD95 and GluR1 in the mPFC of male rats, and the ability of a single injection of ketamine to reverse this effect. IS evoked a significant decline in levels of **(D)** Synapsin1, **(E)** PSD95 and **(F)** GluR1in male rats; deficits that could be rescued by a single injection of

ketamine (5mg/kg). **(G)** Representative Western blot showing that IS decreases levels of Synapsin1, PSD95 and GluR1 in the mPFC of female rats too, but a single injection of ketamine does not reverse this effect. IS evoked a significant decline in levels of **(H)** Synapsin1, **(I)** PSD95 and (J) GluR1 in female rats; however, these deficits could not be rescued by a single injection of ketamine. Levels of individual proteins were quantified using NIH ImageJ. Values represent mean ± SEM (n = 5–7/group) Levels of GAPDH were quantitated to control for differences in amounts of protein loading. ([*]$p<0.05$, [A]$p<0.05$ compared to PH/SAL, [B]$p<0.05$ compared to IS/SAL, two-way ANOVA, Fisher's *post-hoc* test)