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Sex-specific effects of social isolation stress and ketamine on hippocampal plasticity

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Abstract

Chronic social isolation stress (SIS) induces lasting negative effects on the brain, including memory deficits, cognitive impairments, and mood alterations such as depression and anxiety. All these symptoms, at least in part, reflect reduced hippocampal function. In both clinical and preclinical studies, subanesthetic doses of the NMDA receptor antagonist, ketamine (KET), was shown to have rapid and lasting antidepressant effects. Animal studies have shown that biological sex and levels of gonadal hormones alter the behavioral effects of KET, with ovarian hormones increasing sensitivity to the antidepressant-like effects of KET. Since the hippocampus plays a key role in mediating some of the effects of SIS, and considering that KET at low doses has been shown to rescue some of the behavioral deficits of isolation rearing this study aimed to assess the effects of isolation stress on pre- and post-synaptic hippocampal functions in male and female rats reared in SIS, as well as determine whether some of the physiological deficits can be rescued with a single injection of sub-anesthetic doses of KET. To do this, Sprague-Dawley rats were raised from weaning in either social isolation or with same-sex cage mate for 5 to 7 weeks. Male and female rats in either diestrus or proestrus received a single injection of KET (0, 2.5, or 5.0 mg/kg) three hours prior to termination and collection of acute hippocampal slices for *ex vivo* electrophysiological field potential recordings. Long-term potentiation (LTP) and paired pulse facilitation (PPF) outputs were assessed in a canonical CA3-CA1 dorsal hippocampal circuit. Our data show that SIS inhibits hippocampal LTP without affecting PPF in male rats, an effect that was rescued by KET. In female rats, isolation stress did not alter LTP, but did reduce PPF - especially when females were tested in diestrus-, an effect that was rescued by KET at the highest dose. Our data thus suggest sex differences in the contribution of pre- and postsynaptic hippocampal compartments in response to stress and KET.

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7. Contributions:

MK and YZ designed research. JL and KS performed experiments and analyzed data. ABG analyzed the data. JL, MK and YZ wrote the paper.

6. Conflict of Interest:

The authors declare no conflict of interest.

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Keywords

social isolation; ketamine; long term potentiation; paired pulse facilitation; hippocampus; plasticity; estrous cycle; sex differences

1. Introduction

In humans, early-life social isolation resulting from neglect or abandonment and solitary confinement leads to depression, anxiety and cognitive deficits (Mulder et al 2018, Liang et al 2016, Coid et al 2003, Khan and Leventhal 2020). Like humans, rodents also require continuous interaction with conspecifics to maintain homeostasis and normal brain functions (Hawkley and Cacioppo, 2010; Cacioppo et al., 2011). Therefore, early life SIS can have numerous negative physiological impacts in social species (Mumtaz et al., 2018). For example, early life SIS leads to deficits in recognition and spatial memories (Bianchi et al 2006; Wang et al 2019), depressive- and anxiety-like behaviors (Leussiss & Andersen 2008), and increased aggressive behaviors (Vale & Montgomery 1997).

Early life stress has been clearly shown to alter the hippocampus anatomy and physiology (Teicher, M. H. & Samson, J. A.; 2016; Frodl, T. et al., 2017; Lambert, H. K. et al.2019; De Bellis, et al., 2013). This highly interconnected brain area, which continues to develop during childhood, is extremely sensitive to stress (Khalaf-Nazzal, R. & Francis, F., 2013). It is thus not surprising that early life stress, such as SIS, will alter some of the essential functions of the hippocampus such as cognition and emotion (Teicher, M. H. & Samson, J. 2016; Lambert, H. K. et al.2019; De Bellis, et al., 2013).

One of the important physiological functions of the hippocampus that could be altered by SIS is Long Term Potentiation (LTP), which is a form of synaptic plasticity. LTP studies test the adaptability of a specific brain circuit to changes in inputs. An electrical current stimulates the presynaptic terminal into firing, causing the release of glutamate, which activates AMPA and NMDA receptors on the postsynaptic terminals, leading to the production of a field excitatory post-synaptic potential (fEPSP). With repeated high-frequency stimulation (HFS), the synaptic responses change over time to produce larger fEPSPs. This plasticity in the synapse is termed LTP and reflects the synapses' ability to adapt to stimuli. LTP thus is an important candidate to the cellular mechanisms of learning and memory (Cooke SF, Bliss TV 2006). SIS has been shown to decrease hippocampal LTP in male mice (Wang et al 2019), and the non-competitive NMDA receptor antagonist ketamine (KET), when administered at lower doses, was able to rescue LTP deficits in a rat model of the studies of depression (Lily R Aleksandrova et al., 2020). Of note, KET, when administered at subanesthetic doses, has rapid and long lasting anti-depressant effects (Berman et al 2000, Duman 2018, Sterpenich et al 2019). Since stress in general reduces LTP (Lily R Aleksandrova et al., 2020; Pereda-Pérez, N. Popovi , B.B. Otalora, *et al.* 2013; P. Kehoe, J.D. Bronzino 1999), while chronic administration of the antidepressant fluoxetine enhances it (K.G. Bath, D.Q. Jing, I. Dincheva, *et al.*2012), we hypothesize that acute KET administration at subthreshold doses may rescue synaptic plasticity deficits induced by SIS in male and female rats. We decided to include both sexes because our previous work

and the work of others clearly show sex differences in KET's antidepressant-like effects (Carrier and Kabbaj, 2013; Franceschelli et al 2015) and a significant role of female gonadal hormones in mediating these sex differences (Carrier and Kabbaj, 2013). Furthermore, since estrous cycle stages have been shown to have strong influences on hippocampal LTP, with females in proestrus showing greater hippocampal LTP than females in diestrus (Warren et al., 1995), another aim of this study was to examine LTP in female rats at different stages of their estrous cycle. In this work, we will be also assessing Paired-pulse facilitation (PPF) which is a short-term, pre-synaptic form of plasticity. PPF is thought to be caused by an influx of calcium ions (Ca^{2+}) into the terminals *via* the opening of the voltage dependent channels. When there is a short interval between two stimulations, the residual Ca^{2+} from the first stimulation adds to the Ca^{2+} influx from the second stimulation, increasing the Ca^{2+} concentration on the second pulse, thus increasing probability of neurotransmitter release. This method thus allows a relative measure of presynaptic terminals' short-term plasticity. Hence, performing PPF and LTP measurements in conjunction provides more detailed information about the effect that a treatment has on a synapse.

2. Materials and Methods

2.1 Animals

44 Male and 84 female Sprague Dawley rats aged twenty-two days (P22) were obtained immediately post weaning from Charles River Laboratories (Raleigh, NC). All animals were housed under controlled temperatures and 12-hour light/dark cycles (with lights on at 0900) and were allowed *ad libitum* access to standard rat chow and water. All procedures were carried out under strict accordance to the NIH Guide for the Care and Use of Laboratory Animals and the protocol was approved by the Florida State University Institutional Animal Care and Use Committee.

2.2 Social Isolation Stress

Upon rats' arrival to the animal facility on P22, they were randomly assigned to be housed either in a same sex pair or in social isolation with opaque barriers between cages to prevent visual contact between animals. Animals were maintained in these conditions for five to seven weeks, only being handled for a weekly body mass measurement and cage/bedding changes (Figure 1). Beginning on the fourth week of housing, female rats were vaginally lavaged once daily during the light cycle to assess estrous cycle stage. Cycle stage was assessed by cell cytology under a light microscope (Goldman et al 2007; Saland and Kabbaj, 2018). After the fifth week of isolation (P56), female animals were selected for hippocampal LTP recording any day they were in either Diestrus 1 or Proestrus. Male and female rats were counterbalanced and were age-matched at time of termination. Rats were 8–10 weeks old when terminated (Figure 1).

2.3 Drug administration

KET hydrochloride (Ketathesia, racemic, Henry Schein Animal Health Inc.) in an injectable solution was diluted to 2.5 and 5.0 mg/mL in sterile saline vehicle (VEH). Animals were weighed and injected intraperitoneally (i.p.) with 1.0ml/kg body mass of either VEH, 2.5 mg/kg KET, or 5.0 mg/kg KET. Immediately following the injection, animals were quietly

transported in their home cage from the housing facility to the electrophysiology recording space to rest for exactly 3 hours. Care was taken to maintain a warm and quiet environment for the animals during this time.

2.4 Tissue Preparation

Solutions and acute hippocampal slices were prepared similarly as described in Qiao et al (2014). Exactly 3 hours after i.p. injection, rats were rapidly decapitated. The brain was rapidly dissected and placed into ice-cold carbogenated cutting solution containing: 230 mM sucrose, 2.5 mM KCl, 10 mM MgSO₄, 1.25 mM Na₂HPO₄, 26 mM NaHCO₃, 0.5 mM CaCl₂, and 10 mM D-glucose. Parasagittal brain slices containing dorsal hippocampus were cut (400µm) using a vibrating blade microtome (Leica VT1200S). Hippocampal slices were then incubated in carbogenated artificial cerebrospinal fluid (ACSF: 124 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1.3 mM MgSO₄, 1.1 mM KH₂PO₄, 1 mM NaHCO₃, and 10 mM glucose) at 37°C for 1 hours, then incubation temperature was restored to room temperature.

2.5 Electrophysiological Recordings

Hippocampal slices were individually transferred to the recording chamber. Carbogenated ACSF warmed to physiological temperature (Warner Instruments TC-344B) was continuously perfused onto the stage using a simple gravity siphon, and a vacuum cleared the waste. Tissue was held in place using a slice anchor (Warner Instruments, SHD-26H/2). Slices were visualized using a Nikon Eclipse FN1 at 40x with the image projected to a digital screen using a Nikon DS-Qi1Mc digital camera and NIS-Elements AR 3.2 64-bit software.

Field potentials were evoked using a concentric bipolar stimulated electrode (FHC CBAPC75) placed in the Schaeffer collaterals of CA3, and stimuli were issued by a Master-8 stimulus generator coupled to an AMPI Iso-Flex power box. Field potentials were recorded with a silver electrode in a glass pipette (World Precision Instruments, 4IN Thinwall GL 1.5 OD/1.12ID) made using a Narshige PC-10 pipette puller and backfilled with 4M NaCl. The recording electrode was coupled to an Axon Multiclamp 700B (Molecular Devices) and placed in the CA1 stratum radiatum. Synaptic transmission strength was measured by assessing the initial slope of fEPSPs using stimuli of monophasic 100 µs pulses at 0.033Hz. The responses were tested over a range of stimulus intensities from 0.2 to 0.65 mA. Stimuli was adjusted to produce 50–60% of maximum response. LTP was then induced with two trains of 50 square pulses (100Hz) delivered 30 seconds apart. Slices that did not exhibit an initial increase in slope of the downward deflection of the fEPSP following HFS were considered unhealthy and not included for analysis.

Paired-pulse facilitation and LTP were never measured in the same slice. Synaptic transmission strength was measured in the same manner as previously described, with the stimuli adjusted to 50–60% maximum response before paired pulses were initiated. Two monophasic pulses of 100µs were applied in a single sweep, with the pulses being delivered at intervals of 20ms, 50ms, and 100ms. Sweeps were separated by 30 seconds.

2.6 Statistical Analysis

For statistical purposes, “Experimental condition” was defined as follows: Pair-housed Saline, Isolated Saline, Isolated KET 2.5mg/kg and Isolated KET 5mg/kg. Following HFS, LTP was defined as a significant change of the fEPSP slope from baseline. LTP data are expressed as mean response over the measured time \pm SEM, with statistical significance assessed by three-way mixed model ANOVA with post hoc Tukey corrected tests. Due to the large variability of the initial 10 minutes following HFS, these data were not included in the analyses. Paired-pulse facilitation and input/output data were expressed as means \pm SEM at each time interval/intensity. Both were analyzed using a three-way mixed model ANOVA test with post hoc Tukey corrected tests. Data were analyzed in R Studio (lme4 package) and GraphPad Prism.

The approach taken to estimate the degrees of freedom is the Kenward-Roger method, implemented in the lme4 R package. This method improves the calculation of the F value in small to moderate samples. In order to improve the computation of the slope differences for LTP, the repeated measures regarding Pre-HFS and post-HFS were averaged, as recommended by Haleko and Højsgaard (2014). Supplementary table 1 shows individual F values of the adopted 3-way ANOVAs along with effect sizes and individual n's.

3. Results

To determine the impact of SIS/KET treatment on long-term synaptic plasticity, we used HFS to induce LTP in CA3-CA1 synapses in acute hippocampal slices. Three-way repeated measures ANOVA revealed a significant effect of Sex ($F_{(1,88.5)} = 25.910$, $p < 0.001$), Experimental Condition ($F_{(3,114.9)} = 33.194$, $p < 0.001$) and Time (Pre HFS vs. Post-HFS) ($F_{(1,8837.9)} = 1219.844$, $p < 0.001$), as well as a three-way interaction between these three factors ($F_{(3,8837.9)} = 16.137$, $p < 0.001$).

Since there was a significant three-way interaction between Estrous Cycle, Experimental Condition and Time ($F_{(3,5302.0)} = 37.3101$, $p < 0.001$), we analyzed the effect of KET and housing conditions on LTP separately in males, females during diestrus and females during proestrus.

In males (Fig. 2A), there were significant changes in fEPSP from baseline in all experimental conditions except Isolated Saline ($p > 0.05$). The magnitude of increase from baseline in fEPSP was greater in Isolated KET 5mg/kg than Isolated Saline ($p < 0.05$).

In proestrus females, there were significant changes from baseline in all experimental conditions, as well as greater increase from baseline in Isolated KET 5mg/kg when compared to Isolated Saline ($p < 0.05$) (Fig. 2B).

In diestrus females, there were significant changes in fEPSP from baseline in all experimental conditions, but no group differences were observed in the increase from baseline (Fig. 2C).

To assess potential presynaptic plasticity alterations by KET and SIS, we measured PPF in the CA3-CA1 synapses of male and female rats in diestrus and proestrus. PPF was

assessed in separate slices from those used for LTP measurements. As expected, there was a main effect of stimulus interval ($F_{(2,118)} = 8.3019$, $p < 0.05$). There was also an interaction between Experimental Condition and Stimulus interval ($F_{(6,118)} = 3.7778$, $p < 0.05$). To further investigate this, and based on our *a priori* assumption of Sex and Cycle differences, the data was analyzed with a two-way mixed model ANOVA. In male rats and proestrus females, there were no differences between the 4 groups at any stimulus interval (Fig. 3A, 3B). In diestrus females however (Fig. 3C), a main effect of Stimulus Interval was observed ($F_{(2,38)} = 4.7280$, $p < 0.05$), as well as a two-way interaction between Experimental Condition and Stimulus Interval ($F_{(6,38)} = 2.5824$, $p < 0.05$). Pair-housed diestrus females treated with saline had a greater PPF ratio than Isolated KET 2.5mg/kg at the 20ms interval ($p < 0.05$). A trend was also observed when comparing PPF ratios in Pair-housed *vs.* Isolated saline at 20ms interval ($p = 0.07$). The observed effects indicate that the effects of SIS at the pre-synaptic site of the CA3-CA1, are only evident during the diestrus phase, when circulating levels of hormones are lower. Our hypothesis is that lower hormones levels in females increase the vulnerability to the effects of SIS at the pre-synaptic site, and that KET (5 mg/kg) abolishes the effects of isolation stress.

We have also assessed the effects of KET and SIS on Input Output Curves in males and females. As expected, there was a main effect of Stimulus Intensity ($F_{(9,1304.42)} = 24.9168$, $p < 2.2e-16$). However, there were no differences between experimental conditions within each Sex level (Male, Female Diestrus, Female Proestrus) (Fig. 4).

4. Discussion

In this work, we examined the effects of SIS on hippocampal plasticity in both sexes of Sprague-Dawley rats and examined whether low-dose KET can rescue stress-induced deficits. Using slice electrophysiology, we explored the interactions between sex, estrous cycles in females, and KET on hippocampal plasticity following chronic social isolation.

In male rats, SIS led to a complete loss of LTP that was rescued dose-dependently by an acute KET treatment. The increased LTP in isolated male rats by KET is, at least in part, in agreement with a previous report showing that high dose of this drug (10 mg/Kg, *i.v.*) enhanced LTP in male rats under theta-burst, but not HFS-induced LTP (Widman *et al.* 2018). It is important to note however that Widman *et al.*, used a high dose of KET (10mg/kg), administered intravenously. This is a high dose that is within the anesthetic range of KET and thus its effects cannot be considered related to the antidepressant-like effects of KET, which usually occur below the anesthetic range in rats (Li *et al.*, 2010; Radford *et al.*, 2018; 2020). Radford *et al.*, reported that KET at this dose (10 mg/kg, *i.v.*) induced sedation, elevated plasma corticosterone levels and increased short-term fear memory (Radford *et al.*, 2018; 2020), which are not consistent with an antidepressant-like effects in rodents. Another apparent difference between Widman *et al.* and our study was the fact that we examined the effects of KET in chronically stressed rats, while in their study they used brain tissue from unstressed naïve rats (Widman *et al.*, 2018). Interestingly, in their study Widman *et al.* show that KET induced LTP when the slices were potentiated using theta burst stimulation (TBS), but not when using HFS. It is known that TBS activates different intracellular pathways than HFS, with TBS-induced LTP being dependent on BDNF/TrkB signaling (Chen *et al.* 1999,

Edelman et al 2015). It is also possible that KET induces BDNF dependent LTP under TBS conditions (although this needs to be demonstrated at antidepressant relevant doses of KET), but BDNF independent form of plasticity under HFS conditions. This latter possibility is however unlikely because other recent studies have shown that KET, at low antidepressant dose, induced BDNF/TrkB dependent LTP under HFS conditions in the hippocampus. For example, in an animal model of inflammation (LPS induced depression rat model), KET administration (10 mg/kg, i.p.) had antidepressant effects that were dependent on increased expressions of p-CREB and BDNF in the hippocampus and prevented the impairment of LTP induction caused by LPS. In this study, LTP induced by HFS was clearly dependent on BDNF signaling (Tang et al, 2020 *J of Neuroinflammation*). Our results in male rats clearly show that SIS leads to an impairment of LTP in the hippocampus, an effect that was rescued by administration of low doses of KET. It is tempting, when comparing our study to Tang et al. study, to propose that SIS may be causing neuroinflammation that leads to impairment in LTP, which is then rescued by low dose KET. Indeed, previous studies have shown that SIS in male rats and mice have a serious impact on the blood brain barrier integrity and is associated with increased inflammatory markers in the hippocampus (Alshammari et al., 2020, Du Preeze et al., 2020; Calcia et al, 2016). Since PPF, which is a form of short-term plasticity dependent on presynaptic mechanisms (Kamiya and Zucker, 1994; Zucker and Regehr, 2002), was not affected in male rats, we suggest that isolation and the rescuing effects of KET on LTP function are dependent on postsynaptic function of NMDA receptors. The postsynaptic contribution in male rats was also demonstrated by the fact that there were no effects of isolation stress and/or KET in the I/O curve.

Females in Diestrus and Proestrus exhibited LTP that was not affected by the stress history or KET, except for Proestrus female rats which showed increased LTP response at the highest dose of KET. This suggests that KET-induced increased LTP in Proestrus might depend on the presence of high circulating levels of estrogens and progesterone. Previous studies from our group in both rats and mice showed that these hormones are critical in enhancing KET's antidepressant effects in females (Dossat et al., 2018; Saland et al., 2016, Carrier and Kabbaj, 2013).

Unlike male rats, female isolated rats, especially when tested in diestrus, showed reduced PPF when compared to group-housed control rats. This suggests that SIS alters the short-term presynaptic response in female rats without affecting LTP. The effects of SIS at the pre-synaptic site are only evident during the diestrus phase, when circulating levels of hormones are lower. Our hypothesis is that lower hormone levels in females increase the vulnerability to the effects of SIS at the pre-synaptic site, and that KET (5 mg/kg) abolishes the effects of isolation stress. Also, there were no effects of isolation stress and/or KET in I/O curve in females.

It is important to note that one of the limitations of our study is that we did not include group-housed rats treated with ketamine. However, we believe that ketamine treatment in group housed rats will have no effects on LTP as shown recently in a study by Alexandrova et al. who compared Wistar Kyoto rats (WKR: a model of human depression) to Wistar control rats response to KET (5 mg/kg, i.p.). In saline treated groups, WKR had lower LTP compared to Wistar controls. In WKR, KET increased hippocampal LTP but did not

affect LTP in Wistar controls, despite inducing antidepressant like effects in both groups (Aleksandrova *et al.*, 2020). Regardless, it will be important to run these control groups in the future to verify that LTP is not affected by low doses KET in group housed male and female SD rats.

Overall, our data indicate that there is a sexually dimorphic response to SIS. Females present a presynaptic dysfunction that prevents rapid facilitation that is partially ameliorated in proestrus, while males present a post-synaptic deficit following isolation stress that is ameliorated by KET. We thus have shown that the interaction between SIS and low dose KET can alter hippocampal plasticity differently between males and females. Further investigation is needed to determine the mechanisms underlying sex differences in presynaptic and postsynaptic contributions following stress and response to antidepressants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

- Social isolation stress and low doses of ketamine alter hippocampal plasticity differently between male and female rats.
- In male rats, social isolation inhibits hippocampal long-term potentiation, an effect that was rescued by ketamine.
- In female rats, social isolation altered the short-term presynaptic response.
- Females in Diestrus and Proestrus exhibited long-term potentiation (LTP) that was not affected by the stress history or ketamine, except for Proestrus female rats which showed increased LTP response at the highest ketamine dose.

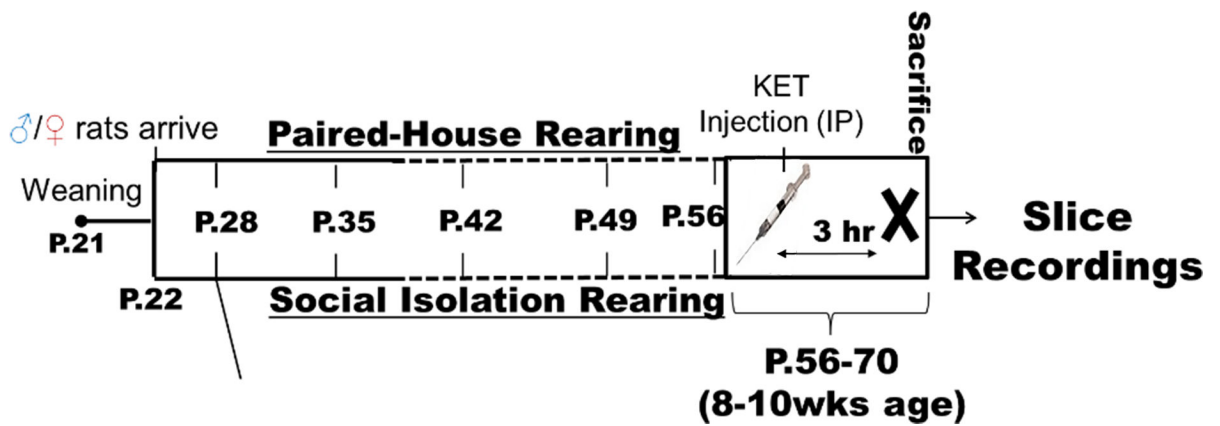


Figure 1. Experimental Timeline

Animals were isolated post-weaning (P22) for a minimum of 5 weeks. Animals aged 56–70 days were selected for experiments. Estrous stage was assessed on P49 until the day of experiment: females were selected for recordings when either diestrus I or proestrus phase was confirmed that morning. The experimental animals received a single acute injection of KET (0, 2.5, or 5.0 mg/kg, i.p.) at lights onset. Three hours later, acute brain slices containing dorsal hippocampus were prepared for electrophysiological field recordings.

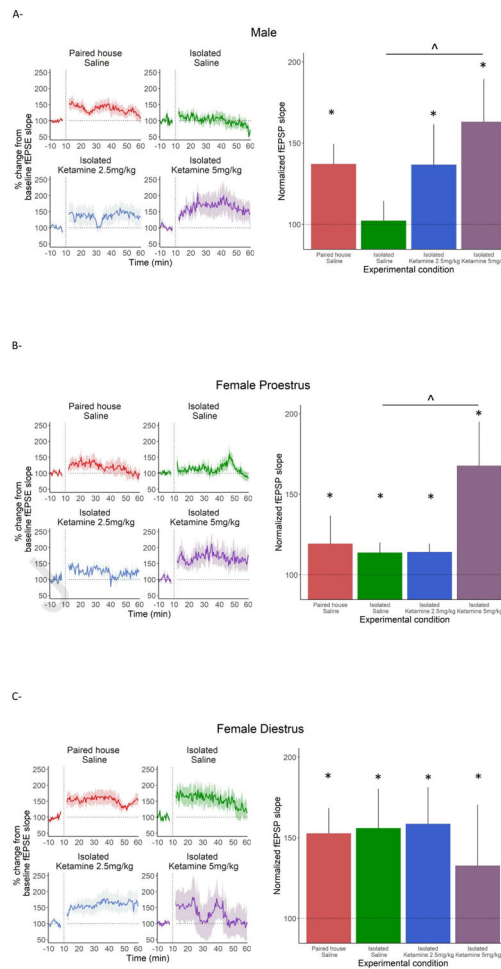


Figure 2. Long-term potentiation recordings

* indicates difference from baseline. ^ indicates difference between groups.

(A) All male groups except Isolated Saline differ from baseline following HFS. The Isolated KET 5mg/kg has a significantly greater increase from baseline than Isolated Saline ($p < 0.05$). (Paired with saline treatment $n=5$, Isolated with saline treatment $n=5$, Isolated with 2.5 mg/kg KET treatment $n=5$, Isolated with 5.0 mg/kg KET treatment $n=5$)

(B) All proestrus female groups increased from baseline. The Isolated KET 5mg/kg treatment had a significantly greater increase from baseline than Isolated Saline ($p < 0.05$) (Paired with saline treatment $n=6$, Isolated with saline treatment $n=4$, Isolated with 2.5 mg/kg KET treatment $n=6$, Isolated with 5.0 mg/kg KET treatment $n=4$)

All diestrus female groups increased from baseline but none differed from each other in responses. (Paired with saline treatment $n=5$, Isolated with saline treatment $n=5$, Isolated with 2.5 KET mg/kg treatment $n=4$, Isolated with 5.0 mg/kg KET treatment $n=4$).

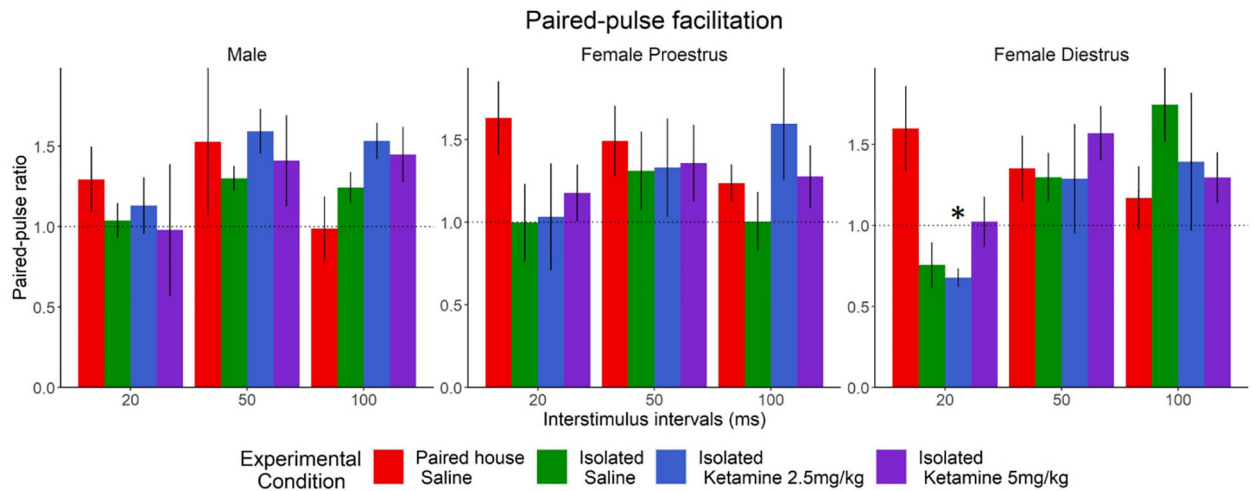


Figure 3. Paired Pulse Facilitations

A main effect of stimulus intensity was present, but there were no differences between sex or treatment in the (A) male (Paired with saline treatment $n=7$, Isolated with saline treatment $n=12$, Isolated with 2.5 mg/kg treatment $n=11$, Isolated with 5.0mg/kg treatment $n=14$) or (B) proestrus females (Paired with saline treatment $n=12$, Isolated with saline treatment $n=13$, Isolated with 2.5 mg/kg treatment $n=9$, Isolated with 5.0 mg/kg treatment $n=7$). (C) Diestrus females show a trend toward decreased facilitation at the 20ms interval, with the Isolated Saline ($p=0.07$) and Isolated with 2.5mg/kg KET ($p<0.05$) groups. This decrease is not present with the 5.0mg/kg treatment (Paired with saline treatment $n=10$, Isolated with saline treatment $n=9$, Isolated with 2.5 mg/kg treatment $n=12$, Isolated with 5.0 mg/kg treatment $n=12$).

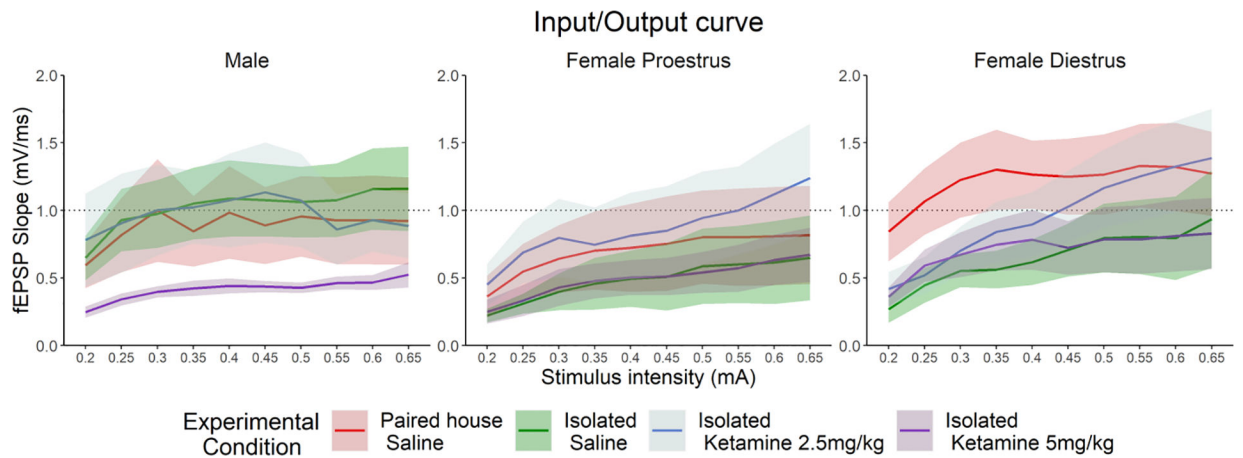


Figure 4. Input Output Curves

There was a main effect of stimulus intensity across all groups, but there were no differences by sex or treatment in the (A) male (Paired with saline treatment n= 7, Isolated with saline treatment n= 12, Isolated with 2.5 mg/kg treatment n= 11, Isolated with 5.0 mg/kg treatment n= 14), (B) proestrus females (Paired with saline treatment n= 12, Isolated with saline treatment n= 13, Isolated with 2.5 mg/kg treatment n= 9, Isolated with 5.0 mg/kg treatment n= 7), or (C) diestrus females (Paired with saline treatment n= 10, Isolated with saline treatment n= 9, Isolated with 2.5 mg/kg treatment n= 12, Isolated with 5.0 mg/kg treatment n= 12).