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RESEARCH

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Aromatase inhibition and ketamine in rats: sex-differences in antidepressant-like efficacy

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Abstract

Background Ketamine has been recently approved to treat resistant depression; however preclinical studies showed sex differences in its efficacy. Sex steroids, such as estrogens and testosterone, both in the periphery and locally in the brain, are regarded as important modulators of these sex differences. Therefore, the present study evaluated how inhibiting the biosynthesis of estrogens with letrozole (an aromatase inhibitor) could affect the observed sex differences in ketamine's antidepressant-like-response.

Methods We performed several consecutive studies in adult Sprague–Dawley rats to evaluate potential sex differences in the antidepressant-like effects of ketamine (5 mg/kg, 7 days, i.p.), letrozole (1 mg/kg, 8 days, i.p.) and their combination (letrozole pre-treatment 3 h before ketamine). Acute and repeated antidepressant-like responses were ascertained in a series of behavioral tests (forced-swim, novelty-suppressed feeding, two-bottle choice for sucrose preference).

Results The main results proved clear sex differences in the antidepressant-like response induced by ketamine, which was observed following a repeated paradigm in adult male rats, but rendered inefficacious in female rats. Moreover, decreasing estrogens production with letrozole induced on itself an antidepressant-like response in female rats, while also increased ketamine's response in male rats (i.e., quicker response observed after only a single dose). Interestingly, both the antidepressant-like effects induced by ketamine in male rats or letrozole in female rats persisted over time up to 65 days post-treatment, suggesting long-term sex-directed benefits for these drugs.

Conclusions The present results demonstrated a sex-specific role for aromatase inhibition with letrozole in the antidepressant-like response induced by ketamine in male rats. Moreover, letrozole itself presented as a potential antidepressant for females with persistent effects over time. Clearly, the production of estrogens is key in modulating, in a sex-specific manner, affective-like responses and thus deserve further studies.

Highlights

- There are clear sex differences in the antidepressant-like effects of ketamine.
- Ketamine showed efficacy in adult male rats while rendered ineffective in females.
- Aromatase inhibition with letrozole improved the effects observed in male rats.
- Aromatase inhibition with letrozole induced an antidepressant-like effect in females.
- The antidepressant-like effects of ketamine or letrozole persisted up to 2 months.

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Keywords Ketamine, Letrozole, Estrogens, Sex, Antidepressant

Plain language summary

Ketamine is a novel fast-acting antidepressant recently approved for treatment-resistant depression. Since preclinical studies showed sex differences in its efficacy, probably driven by sex hormones (estrogens and testosterone), we evaluated the antidepressant-like effects of ketamine in male and female rats when the biosynthesis of estrogens was inhibited. To do so, we utilized letrozole, an inhibitor of the aromatase enzyme responsible for the conversion of testosterone into estrogens. The results showed, in line with the prior literature, sex-differences in the antidepressant-like response of ketamine; with efficacy in male rats and a lack of response for females. Aromatase inhibition with letrozole induced a faster response for ketamine in male rats, while did not change the lack of response for females. However, aromatase inhibition on itself was capable of inducing an antidepressant-like response in female rats. Interestingly, both ketamine's and letrozole's antidepressant-like effects in male and female rats respectively showed long-term beneficial effects, up to 65 days post-treatment.

Background

Esketamine, the S-enantiomer of ketamine, was approved in 2019 by the FDA (https://www.accessdata.fda.gov/drugsatfda_docs/label/2019/211243lbl.pdf) for treatment-resistant depression. Its great clinical outcome for depressed patients that are resistant to at least two other pharmacological options, confront with the scarce knowledge regarding the potential long-term impact of its repeated treatment (e.g., safety and tolerability; recently reviewed by [1]). In this context, a great effort has been placed in the last years attempting to further characterize ketamine's antidepressant-like actions, beyond its initial binding as a non-competitive antagonist to the N-Methyl-D-Aspartate (NMDA) receptor (e.g., [1–3]).

Additionally, further defining potential sex differences in ketamine's therapeutic actions is a priority since targeted treatments might be needed for each sex; in fact, both prior clinical and preclinical reports suggest different responses for males and females, with even contradictory data regarding which sex might benefit the most from the treatment. For example, when analyzing recent reviews with clinical data, no consensus could be reached, since ketamine is sometimes presented as more effective for males (e.g., [4]), but others for females (e.g., [1]), or even with no significant sex differences in its therapeutic effects (e.g., [5]). Similarly, results from preclinical studies are also inconsistent, with alternate responses for ketamine for either male or female rodents, that seemed to depend on the dose administered [6–8], the interaction with prior early-life stress exposure [9], and/or the time-duration of the response (e.g., [10]). These sex differences in efficacy reinforce the need of including sex as a biological variable in all preclinical studies [11–13] and might be explained by disparities in the pharmacokinetics and pharmacodynamics effects of the drug [14, 15], which could in turn be influenced by a variety of factors including the level of sex steroids, such as estrogens and testosterone, both in the periphery and locally in the brain (e.g., [1]).

In this context, recent studies not only supported a possible involvement of estrogens in the sex-differences reported in the pathogenesis of depression (e.g., [16]), but also in the sex-specific changes in efficacy of certain antidepressants (e.g., [16, 17]). However, how the levels of estrogens might be affecting the antidepressant-like response of ketamine is still unknown. One pharmacological tool to evaluate the potential role of estrogens biosynthesis in ketamine's actions is letrozole, an aromatase inhibitor that prevents estrogens biosynthesis via androgen aromatization (e.g., [18]), which was previously used to evaluate if estrogens would interfere in the antidepressant-like response of fluoxetine in rats [19]. Against this background, the present study utilized male and female Sprague–Dawley rats to characterize the role of inhibiting estrogens biosynthesis with letrozole in the antidepressant-like response induced by ketamine, by screening the effects through several consecutive behavioral tests (e.g., forced swim test, novelty-suppressed feeding test, sucrose preference) typically used at the preclinical level to ascertain improvements in core symptoms related to depressive-like behavior (i.e., behavioral despair, hedonic-like responses), and that have captured ketamine's effects in rats (e.g., [9]).

Methods

Animals

A total of 145 adult Sprague–Dawley rats (72 male and 73 female) were used in 3 consecutive studies (see Fig. 1). All rats that were around 3 months old at the beginning of the procedures were bred in the animal facility at the University of the Balearic Islands and were housed in standard cages (2–4 rats per cage) with ad libitum access to a standard diet and tap water in a controlled temperature

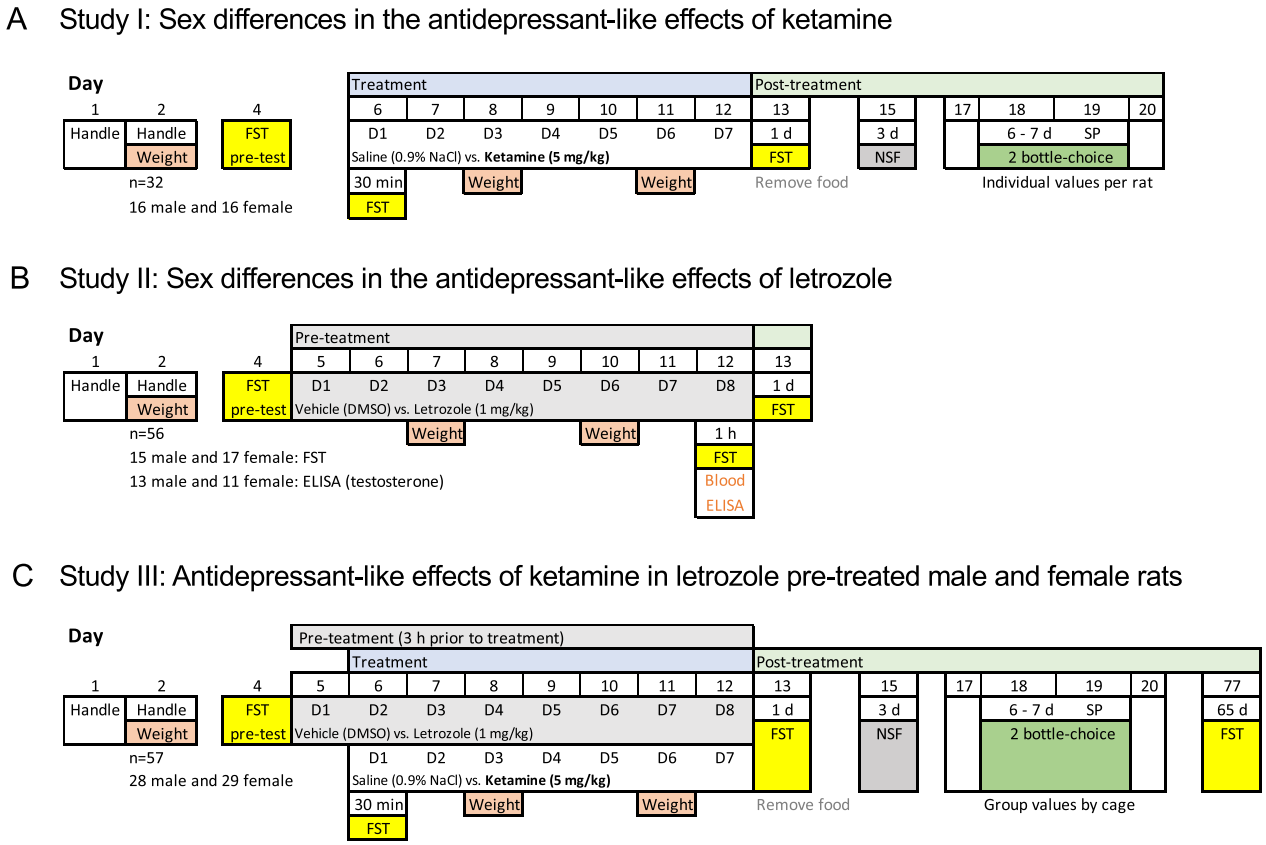

Fig. 1 Experimental timeline. *FST* forced-swim test, *NSF* novelty suppressed feeding, *SP* sucrose preference, *D* day of treatment, *d* day of post-treatment

(22 °C) and humidity (70%) vivarium (12:12 h light/dark cycle). Procedures were performed under the ARRIVE guidelines [20] and following the EU Directive 2010/63/EU of the European Parliament and of the Council, after approval by the Local Bioethical Committee (University of the Balearic Islands) and the regional Government (Conselleria Medi Ambient, Agricultura i Pesca, Direcció General Agricultura i Ramaderia, Govern de les Illes Balears). Rats were used to being handled by the experimenter prior to any procedures and body weight was monitored across time. All efforts were made to reduce the number of rats used and their suffering. The specific stages of the estrous cycle were not monitored during the experimental procedures since the cyclicity of females was not part of our research question (see [12]), but also in neuroscience research female rats are not more variable than male rats due to hormonal estrous cycle (e.g., [21, 22]).

Pharmacological drug treatments

The potential sex differences in the antidepressant-like effects of ketamine were assessed in adult male (n = 16) and female (n = 17) rats that were treated for 7 consecutive days (1 injection per day, i.p., 1 ml/kg) with saline (0.9% NaCl) or ketamine (Richter Pharma, Austria; dose of 5 mg/kg dissolved in saline, selected from [9]) (see Study I; Fig. 1A). To characterize the effects of letrozole (Study II; Fig. 1B), adult male (n = 28) and female (n = 28) rats were treated for 8 consecutive days (1 injection per day, i.p., 1 ml/kg) with vehicle (DMSO) or letrozole (Novartis Pharma, Switzerland, dose of 1 mg/kg, selected from [19]). Some of these rats were exposed to behavioral phenotyping through the forced-swim test (15 male and 17 female) while the rest were used to assess testosterone levels (13 male and 11 female) following the procedures described later on. Finally, we combined both designs to evaluate the role of inhibiting the synthesis of estrogens with letrozole in the differential sex-response induced by ketamine (Study III; Fig. 1C). To do so, male (n = 28) and female (n = 29) rats were pretreated (i.p.) with letrozole (1 mg/kg/day) or vehicle (1 ml/kg/day of DMSO) for 8 consecutive days, followed 3 h later by a daily injection with ketamine (5 mg/kg/day) or saline (1 ml/kg/day of 0.9% NaCl) for 7 consecutive days (days 2–8 of letrozole treatment; see Fig. 1C).

Hormonal assay

Blood samples were collected during Study II from 13 male and 11 female rats treated with either vehicle or letrozole for 8 consecutive days (blood collection 1 h post injection; see Fig. 1B). To recover plasma, blood samples were centrifuged (4 °C, 15 min at 1500×*g*) and then stored at − 80 °C. The levels of accumulated testosterone through inhibiting the synthesis of estrogens with letrozole were used as an indicative of the hormonal status, and were quantified by standard ELISA kits (Testosterone: LDN, AR E-8000R, Nordhorn, Germany;), according to the manufacturer's instructions. The sensitivity of the assay was 0.066 ng/ml.

Forced-swim test

To evaluate the potential antidepressant-like effects of ketamine, letrozole and/or their combination, we screened the animals under the stress conditions of the forced-swim test, a test that has been the goal standard screening tool in the industry (e.g., [23]) and is still commonly used for screening novel potential antidepressants, including those exerted by ketamine (e.g., [9]). Our protocol modified the standard protocol [23] in a way that would allow us to evaluate the progression of the response over time. We have done this procedure reliably in the past years as detailed in various publications [9, 24–34], in which to account for the potential emerging effects due to repetition on learning and/or tolerance to test performance, all rats were exposed to the same conditions. In particular, each rat was placed in individual tanks (41 cm high × 32 cm diameter, 25 cm depth; specific dimensions that ensure that the animal does not touch the bottom of the cylinder with its tail) filled with water (25 ± 1 °C) during 15 min (pre-test sessions) followed by 5-min test sessions that were videotaped (see Fig. 1). As mentioned, test sessions were repeated across time in an attempt to evaluate the progression of the response: acute effects (e.g., 30 min post-injection), repeated effects (e.g., 1 h or 1-day post-treatment) and/or long-term effects (e.g., 65 days post-treatment). Videos were blindly analyzed to determine individual immobility vs. active behaviors (climbing or swimming) for each rat (Behavioral Tracker software, CA, USA).

Novelty-suppressed feeding test

This test was first described to assess differences in sensitivity to novelty in an anxiogenic-like environment [35], and could be used to score chronic antidepressant-like responses, such as the ones induced following a similar dosing-paradigm of repeated ketamine treatment (e.g., [9]). To do so, rats from Studies I and III were food-deprived prior to testing for 48 h, since motivation for food is required (see Fig. 1A, C). During testing, which was done 3 days post-treatment, rats were individually placed at one of the corners facing the wall of the arena and were left undisturbed for 5 min in a square open-field arena (60 cm × 60 cm, and 40 cm in high), under housing illumination conditions with three food pellets in the center following our prior studies (e.g., [9, 30]). Sessions were videotaped and the parameters of video analysis that were evaluated for each rat were feeding time (the time each animal spent eating the pellets in s), and distance traveled (in cm).

Sucrose preference test

This test is used as an indicator of hedonic-like responses (i.e., anhedonia, a core symptom of depression; see [36]), since animals, when given the chance, prefer a sweet solution (e.g., 1% sucrose) over water. Prior to testing, rats from Studies I and III were trained during 24 h to drink from two bottles filled with water that were placed on each side of the housing cage (5 days post-treatment). Then, for 2 consecutive days, rats could either drink from a bottle containing 1% sucrose or another one with water (6–7 days post-treatment; see Fig. 1A, C). To avoid the possible preference to a particular side of the cage, bottles were placed in alternate positions. Finally, rats were again exposed for 24 h to two water bottles to make sure no bias for either bottle was present. All bottles were weighted every day to calculate sucrose intake (g/kg) and sucrose preference (%) for each individual rat (Study I: rats were isolated prior to testing) or for groups of 2–4 rats/cage (Study III) on days 6 and 7 post-treatment.

Statistical analysis

Data was analyzed with GraphPad Prism, Version 10 (GraphPad Software, San Diego, CA). In line with the guidelines for displaying data and statistical methods in experimental pharmacology (e.g., [37, 38]), results are presented as mean values ± standard errors of the mean (SEM), and individual symbols are shown for each rat. Each set of data was evaluated either with two- (independent variables: sex and treatment or pre-treatment) or three-way (independent variables: sex, pre-treatment and treatment) ANOVAs. When sex differences emerged, each sex was analyzed separately by two-way ANOVAs (independent variables: pre-treatment and treatment) or Student's *t* test, depending on the number of groups to compare, as recommended for preclinical data set analysis and when including sex as a biological variable (see [12]). When appropriate, multiple comparisons were performed by Sidak's test. The level of significance was fixed at $p \leq 0.05$.

Results

Study I: sex differences in the antidepressant-like effects of ketamine

Before phenotyping the behavioral responses induced by ketamine, and when only considering potential sex differences in the assays evaluated (effects of Sex; Additional file 1: Table S1), some significant basal changes emerged for male vs. female rats in all tests that conditioned and/or justified the posterior analysis for each sex separately. For example, there was an overall sex difference in climbing behavior as measured 30 min post a single injection in the forced-swim test (Fig. 2A; Additional file 1: Table S1: effects of Sex), with female rats climbing less time (− 30 ± 13 s, #$p = 0.031$) as compared to male adult rats. Again, sex differences were observed when evaluating distance traveled (cm) in the novelty-suppressed feeding test, with females covering significant more distance (+ 394 ± 151 cm, ##$p = 0.003$) than male rats (Fig. 2C). Finally, a significant sex difference was also observed for sucrose intake (see Additional file 1: Table S1), with female rats consuming more sucrose (+ 3.8 ± 0.6 g/kg, ###$p < 0.001$) than male adult rats (Fig. 2D). When sex differences were observed in the overall analysis (see Additional file 1: Table S1), each sex was analyzed separately.

The acute effect of ketamine was ascertained in the forced-swim test 30 min post a single injection. The results showed that ketamine did not induce changes in immobility, climbing or swimming behaviors in adult male and female rats (Fig. 2A; Additional file 1: Table S1: lack of significant effects of Treatment or Sex x Treatment interactions). Interestingly, following a repeated 7-day paradigm of ketamine, significant Sex x Treatment interactions emerged, as measured 1-day post-treatment, both for immobility and climbing behaviors (Fig. 2B; Additional file 1: Table S1). Sidak's post-hoc comparisons revealed that ketamine induced an antidepressant-like effect exclusively in adult male rats, while rendered inefficacious in females (Fig. 2B). Particularly, in male rats, ketamine decreased immobility (− 86 ± 23 s, **$p = 0.003$) and increased climbing (+ 62 ± 24 s, *$p = 0.016$) behavior in the forced swim test (Fig. 2B) when compared to saline-treated rats. These effects were not observed later on with other tests also used to characterize the antidepressant-like response. The novelty-suppressed feeding test was performed 3 days post-treatment to measure feeding time (s) and distance traveled (cm) as indicatives of an antidepressant-like response. However, the results showed no significant effects of Treatment, nor significant Sex x Treatment interactions (Fig. 2C; Additional file 1: Table S1). Similarly, no significant effects of Treatment were observed when measuring 1% sucrose intake (g/kg) or preference (%) in the sucrose preference test in male and female rats 6–7 days post-treatment (Fig. 2D; Additional file 1: Table S1).

Study II: sex differences in the antidepressant-like effects of letrozole

Before phenotyping the behavioral responses induced by letrozole, and as a positive control of the treatment success, we evaluated testosterone levels in plasma (ng/ml), as an indicative of the correct inhibition of estrogens biosynthesis by this aromatase inhibitor. As expected, the results showed significant baseline sex differences in testosterone levels (Fig. 3A and Additional file 1: Table S1), with female rats displaying lower levels (− 4.41 ± 1.34 ng/ml of testosterone, ##$p = 0.004$) than males. Interestingly, and given the baseline sex difference in testosterone levels, when the effect of letrozole treatment was evaluated separately for each sex, it only rendered significant for females (+ 1.89 ± 0.42 ng/ml; $t = 4.51$, df = 9, **$p = 0.002$ vs. vehicle-treated female rats), but not for male rats (Fig. 3A).

The potential antidepressant-like effect of letrozole was evaluated in the forced-swim test 1 h and 1-day post-repeated treatment (8 days). Significant effects of Sex were observed for almost all data analyzed through two-way ANOVAs (see Additional file 1: Table S1). Overall, female rats displayed higher immobility (1 h: + 60 ± 12 s, ###$p < 0.001$; 1 d: + 59 ± 18 s, ##$p = 0.003$) and lower climbing (1 h: − 56 ± 12 s, ###$p < 0.001$; 1 d: − 61 ± 17 s, ##$p = 0.001$) than males (Fig. 3B, C). Thus, the response of letrozole in the forced-swim test was analyzed for each sex separately through Student's t tests. The results showed that letrozole induced an antidepressant-like effect exclusively in female rats by decreasing immobility (− 28 ± 12 s; $t = 2.27$, df = 15, *$p = 0.038$ vs. vehicle-treated female rats), and increasing climbing behavior (+ 19 ± 11 s; $t = 1.80$, df = 15, *$p = 0.046$ vs. vehicle-treated female rats; one-tailed p value) as measured 1 h post-treatment (Fig. 3B). This antidepressant-like effect induced by letrozole in female rats returned to normal 1-day post-treatment (Fig. 3C). No significant effects were observed 1 h or 1-day post-treatment for male rats (Fig. 3B, C).

Study III: antidepressant-like effects of ketamine in letrozole pre-treated male and female rats

When analyzing all raw data through three-way ANOVAs, significant sex differences emerged in almost all features evaluated (see Additional file 1: Table S2: effects of Sex). Overall, and similarly to what was described for Study I, female rats showed lower climbing rates in the forced-swim test (##$p = 0.001$; see Additional file 1: Table S2 and Fig. 3A, B), more distance traveled (cm) in the novelty-suppressed feeding test (##$p = 0.003$,

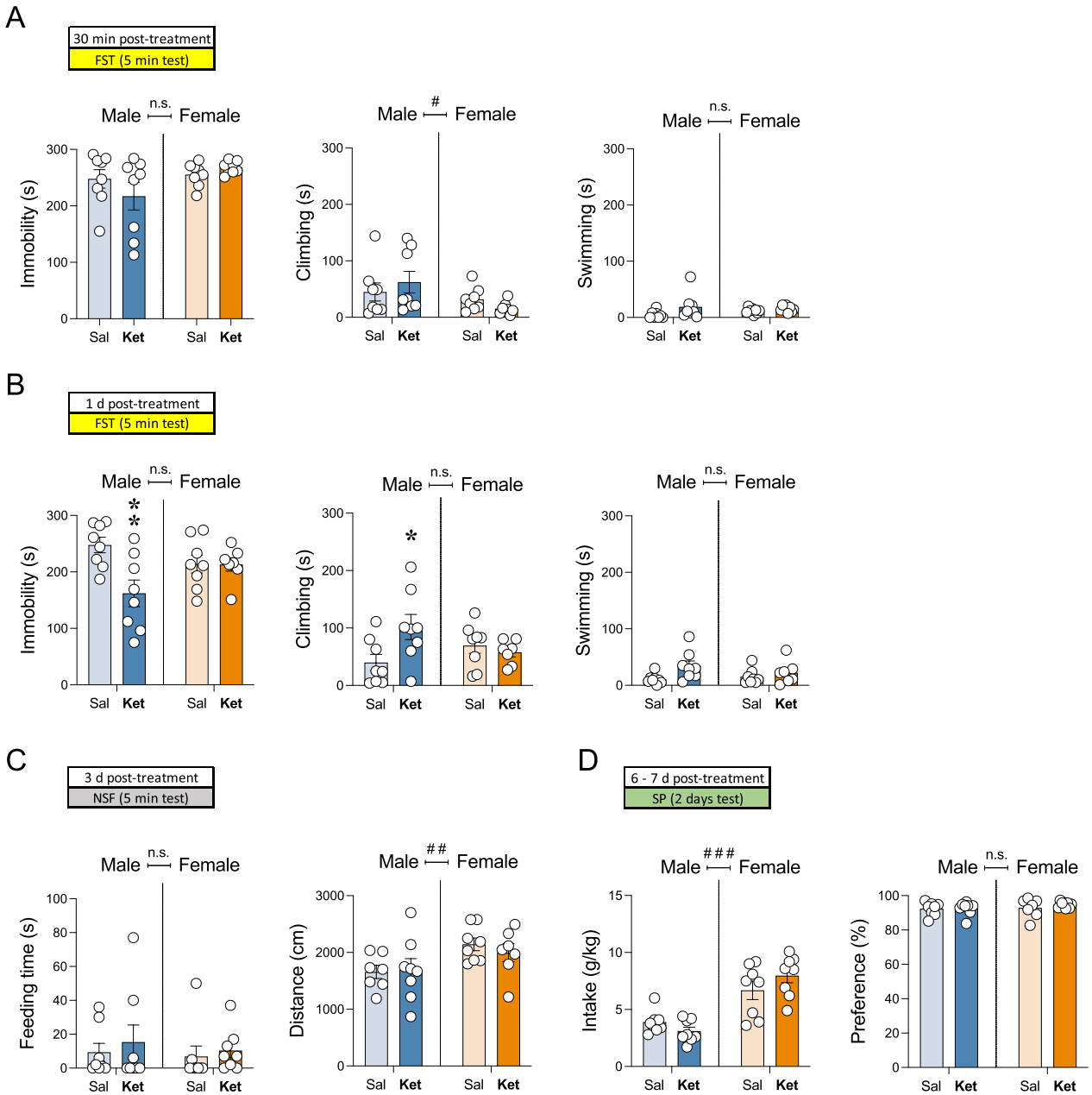

Fig. 2 Sex differences in the antidepressant-like effects of ketamine. **A** Acute (30 min post-treatment) and **B** repeated (1-day post-treatment) effects exerted by ketamine exposure in male and female rats in the forced-swim test (FST). Data represent mean ± SEM of the time spent (s) immobile, climbing, or swimming. **C** Repeated effects (3-days post-treatment) exerted by ketamine exposure in male and female rats in the novelty-suppressed feeding test (NSF). Data represent mean ± SEM of the feeding time (s) or distance traveled (cm). **D** Repeated effects (6–7 days post-treatment) exerted by ketamine exposure in male and female rats in the sucrose preference test (SP). Data represent mean ± SEM of sucrose intake (g/kg) or preference (%). **A–D** Individual values are shown for each rat (symbols). Two-way ANOVAs (independent variables: sex and treatment) were performed and results are shown in Additional file 1: Table S1. $^{###}p < 0.001$ and $^{\#}p < 0.05$ when comparing female vs. male rats (effect of Sex). Student's *t*-tests for each sex separately: $^{**}p < 0.01$ and $^{*}p < 0.05$ vs. same sex saline-treated rats. *Sal* saline, *Ket* ketamine, *Veh* vehicle, *LTZ* letrozole

Fig. 3C), and higher sucrose intake (g/kg) in the sucrose preference test ($###p < 0.001$, Fig. 3D) than male adult rats. Interestingly, these basal sex differences persisted in time, since they were still present 65 days post-treatment, when female rats still showed lower climbing rates than males in the forced-swim test ($\#p = 0.013$, Fig. 5;

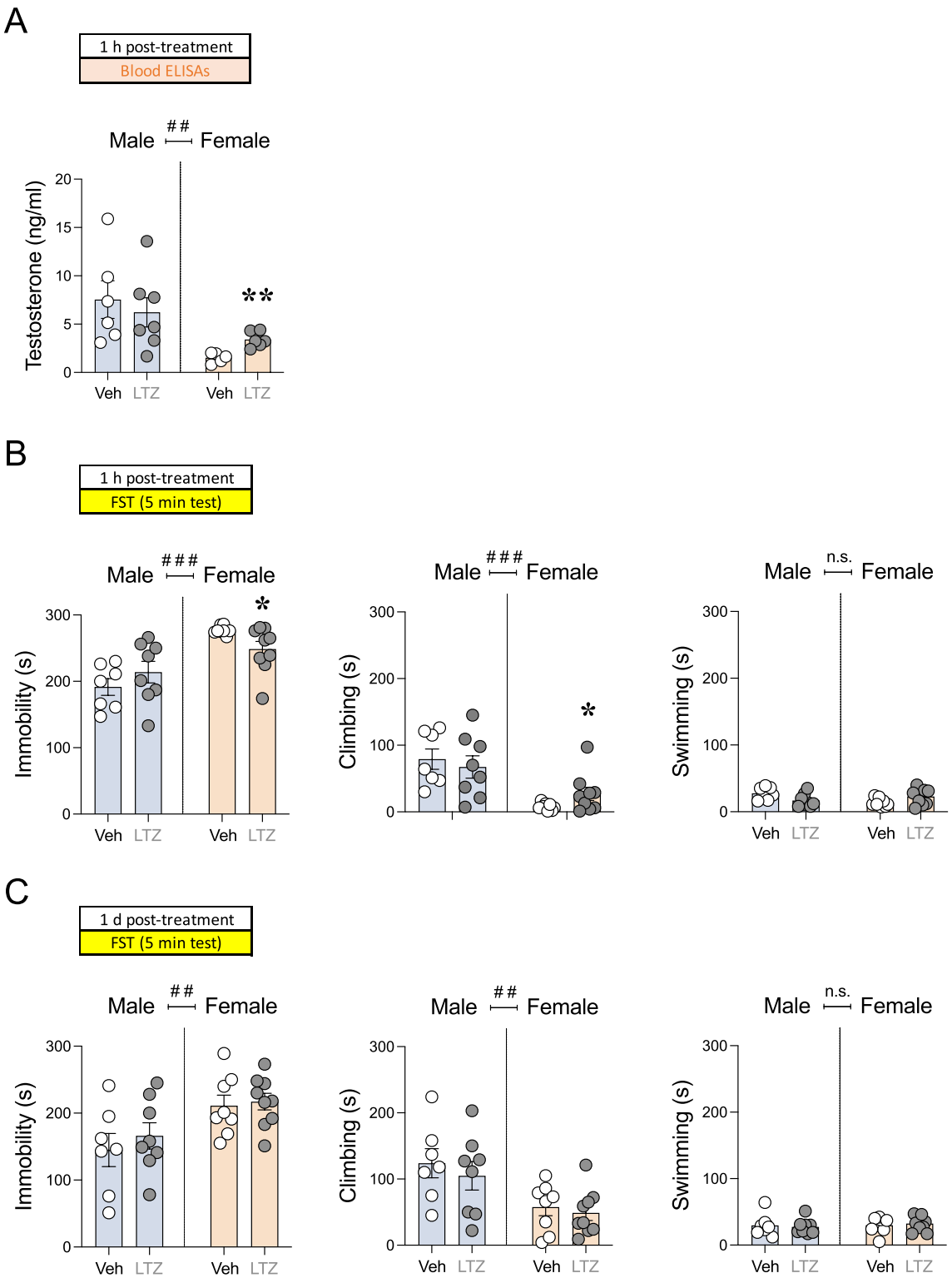

Fig. 3 Sex differences in the antidepressant-like effects of letrozole. **A** Repeated effect of letrozole (1 h post-8 days of treatment) on testosterone levels. Data represent mean ± SEM of testosterone levels (ng/ml). **B** Acute (1 h post-8 days of treatment) and **C** repeated (1-day post-8 days of treatment) effects exerted by letrozole exposure in male and female rats in the forced-swim test (FST). Data represent mean ± SEM of the time spent (s) immobile, climbing, or swimming. **A-C** Individual values are shown for each rat (symbols). Two-way ANOVAs (independent variables: sex and treatment) were performed and results are shown in Additional file 1: Table S1. $^{###}p < 0.001$ and $^{##}p < 0.01$ when comparing female vs. male rats (effect of Sex). $^{**}p < 0.01$ and $^{*}p < 0.05$ vs. same-sex vehicle-treated rats. *Veh* vehicle, *LTZ* letrozole

Additional file 1: Table S2). Because of these noticeable sex differences consistent over time, the combined effects of letrozole pre-treatment and ketamine treatment were analyzed for each sex separately through two-ways ANOVAs (Additional file 1: Table S2; Figs. 4, 5).

The response induced by acute ketamine was measured in the forced-swim test 30 min post a single injection in rats pre-treated or not with letrozole. The results showed that, comparably to what was reported for Study I, acute ketamine did not induce changes in immobility, climbing or swimming behaviors in adult male or female rats (vehicle pre-treated; Fig. 4A). However, in adult male rats pre-treated with letrozole, acute ketamine was capable of inducing an antidepressant-like response, an effect that was not observed in females. Particularly, in male rats pre-treated with letrozole ketamine decreased immobility (-88 ± 22 s, $^{**}p = 0.003$ vs. Sal-LTZ; -71 ± 22 s, $\$p = 0.020$ vs. Ket-LTZ; Fig. 4A) and increased climbing ($+69 \pm 20$ s, $^{*}p = 0.013$ vs. Sal-LTZ; Fig. 4A). Interestingly, a significant effect of Pre-treatment was observed for female rats (Additional file 1: Table S2) both for immobility and climbing as measured 1-day post-treatment. This overall antidepressant-like effect induced by letrozole in female rats (i.e., decreased immobility and increased swimming; Fig. 4B) was also observed in Study II following an 8-day treatment paradigm.

Following a repeated paradigm of ketamine, again, and similarly to the results reported in Study I, significant effects of Treatment were present both for immobility and climbing exclusively in male rats as measured 1-day post-treatment (see Additional file 1: Table S2). Sidak's post-hoc comparisons revealed that ketamine decreased immobility time both in vehicle (-77 ± 27 s, $^{*}p = 0.047$ for Veh-Sal vs. Veh-Ket:) and letrozole (-98 ± 26 s, $^{**}p = 0.007$ for LTZ-Sal vs. LTZ-Ket) pre-treated male rats (Fig. 4B). In the novelty-suppressed feeding test (as measured 3 days post-treatment), no significant effects of Treatment were detected (see Additional file 1: Table S2) when measuring feeding time (s) or distance traveled (cm), independently of Pre-treatment, in male and female rats (Fig. 4C and Additional file 1: Table S2). Similarly, no significant effects of Treatment or Pre-treatment (Additional file 1: Table S2) were observed for 1% sucrose intake (g/kg) or preference (%) in the sucrose preference test in groups of male and female rats and as measured 6–7 days post-treatment (Fig. 4D).

Finally, the potential long-term antidepressant-like effect of these drugs was evaluated in the forced-swim test 65 days post-treatment for each sex separately. Interestingly, the results in male rats showed an overall effect of Treatment, demonstrating a persistent antidepressant-like response of ketamine (vs. saline-male treated rats, and independently of Pre-treatment) by decreasing immobility (-58 ± 25 s; $p = 0.003$) and increasing climbing ($+54 \pm 24$ s; $p = 0.004$) in the forced-swim test (see Additional file 1: Table S2 and Fig. 5). Moreover, in female rats there was on overall effect of Pre-treatment (see Additional file 1: Table S2; Fig. 5), suggesting a persistent antidepressant-like effect induced by letrozole (vs. vehicle-female treated rats) both for immobility (-46 ± 27 s; $p = 0.028$) and climbing ($+42 \pm 25$ s; $p = 0.038$) in the forced-swim test (see Additional file 1: Table S2, and Fig. 5).

Discussion

This study proved that estrogens production is key in modulating some sex-specific antidepressant-like responses in Sprague–Dawley rats. While ketamine was efficacious after a repeated paradigm exclusively in male rats, its combination with letrozole, an inhibitor of estrogens biosynthesis, induced a faster efficacy as it was observed right after a single dose. However, although ketamine rendered inefficacious in female rats, decreasing estrogens production with letrozole induced on itself an antidepressant-like response, which paralleled significant increases in testosterone levels, and suggesting a beneficial role for letrozole in females. Interestingly, both the antidepressant-like effects induced by ketamine in male rats and letrozole in female rats persisted over time (i.e., up to 65 days post-treatment) suggesting long-term sex-directed antidepressant-like effects for these drugs.

(See figure on next page.)

Fig. 4 Antidepressant-like effects of ketamine in letrozole pre-treated male and female rats. **A** Acute (30 min post-treatment) and **B** repeated (1-day post-treatment) effects exerted by ketamine exposure in male and female rats pre-treated with vehicle or letrozole in the forced-swim test (FST). Data represent mean ± SEM of the time spent (s) immobile, climbing, or swimming. **C** Repeated effects (3-days post-treatment) exerted by ketamine exposure in male and female rats pre-treated with vehicle or letrozole in the novelty-suppressed feeding test (NSF). Data represent mean ± SEM of the feeding time (s), or distance traveled (cm). **D** Repeated effects (6–7 days post-treatment) exerted by ketamine exposure in male and female rats pre-treated with vehicle or letrozole in the sucrose preference test (SP). Data represent mean ± SEM of the sucrose intake (g/kg) or preference (%). **A–C** Individual values for each rat or **D** cage values (groups of 2–4 rats) are shown (symbols). Three-way ANOVAs (independent variables: Sex, pre-treatment and treatment) or two-way ANOVAs (independent variables: pre-treatment and treatment) were performed and results are shown in Additional file 1: Table S2. $^{###}p < 0.001$ and $^{##}p < 0.01$ when comparing female vs. male rats (effect of Sex). $^{**}p < 0.01$ and $^{*}p < 0.05$ for LTZ-Ket vs. LTZ-Sal and $^{\$}p < 0.05$ for LTZ-Ket vs. Veh-Ket. *Sal* saline, *Ket* ketamine, *Veh* vehicle, *LTZ* letrozole

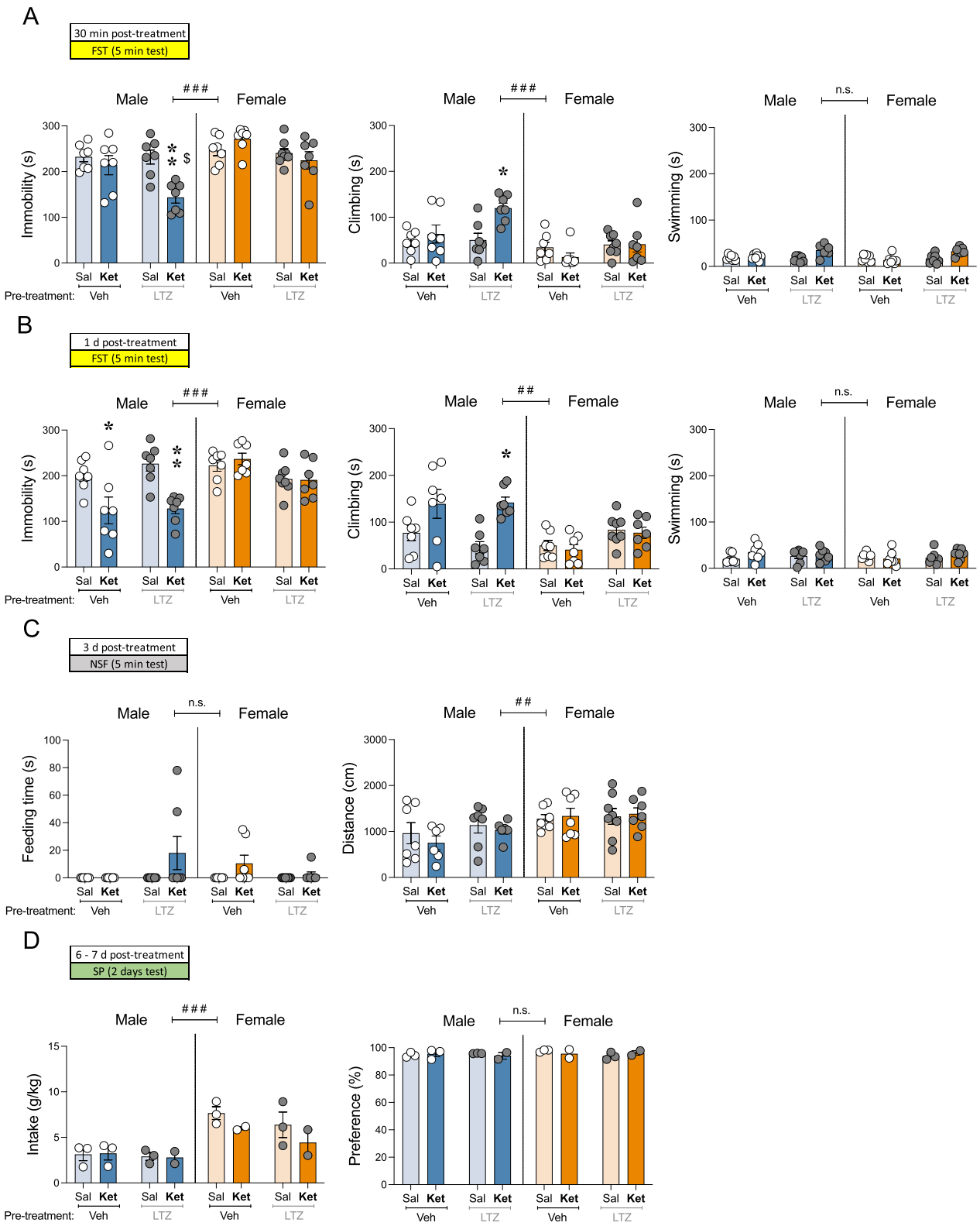

Fig. 4 (See legend on previous page.)

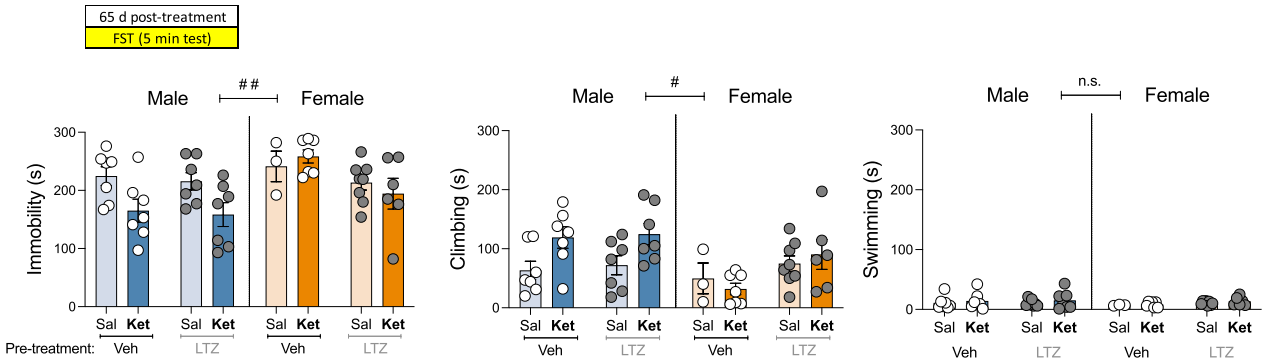

Fig. 5 Long-lasting antidepressant-like effects of ketamine or letrozole in rats. Repeated (65-day post-treatment) effects exerted by ketamine exposure in male and female rats pre-treated with vehicle or letrozole in the forced-swim test (FST). Data represent mean ± SEM of the time spent (s) immobile, climbing, or swimming. Three-way ANOVAs (independent variables: sex, pre-treatment and treatment) or two-way ANOVAs (independent variables: pre-treatment and treatment) were performed and results are shown in Additional file 1: Table S2. $^{\#\#\#}p < 0.01$ and $^{\#}p < 0.05$ when comparing female vs. male rats (effect of Sex). *Sal* saline, *Ket* ketamine, *Veh* vehicle, *LTZ* letrozole

The present results showed clear and interesting sex differences in the pharmacological actions exerted by ketamine. Particularly, while ketamine showed certain efficacy in male rats (following a repeated treatment of 5 mg/kg during 7 days), female rats were unresponsive to the expected beneficial antidepressant-like effects of ketamine. In line with our results prior data showed a beneficial response after a repeated paradigm with low doses of ketamine (5, 10 and 15 mg/kg) in Wistar adult male rats [39]. Moreover, our own prior study in adolescent Sprague–Dawley rats, showed that the same dose of ketamine (5 mg/kg) induced an acute antidepressant-like effect (after a single dose) which was sex- and stress-dependent, since it was observed in naïve male rats and in maternally-deprived female rats [9]. Other acute doses tested of ketamine also worked in male adult rats (5, 10, 15 mg/kg) [6, 40]. However, and contrarily to our results, other studies showed that female rats were more sensitive to the antidepressant-like effects of ketamine than males, as they responded to lower doses (e.g., 2.5 mg/kg) in the forced-swim test (see review by [1]). These inconsistent results go along with prior clinical and preclinical studies reporting some sex-related differential responses for ketamine's actions, with not a clear outcome in terms of which sex benefits the most from this particular treatment (reviewed by [1]), and suggested that the disparities reported could be due to differences in the dose/treatment regimens, in the behavioral tests utilized to score the results at different time-points after treatment (i.e., length of efficacy), and/or in the animals or particular strains used, which overall introduce a higher degree of variability in the findings. Therefore, the present study presents the limitation to have only explored one dose of ketamine (5 mg/kg), which was selected based on our prior studies that proved efficacy in adolescent male naïve rats and adolescent female rats that were maternally deprived at early age [9], proving similar effects when the drug was administered during adulthood, and suggesting the need to further study other doses to better comprehend these sex disparities.

Sex hormones appear to be critical mediators of the antidepressant-like response of ketamine and needs further research, especially because it is overlooked in much of the available literature. For example, gonadal hormones have been implicated in the possible modulation of the antidepressant-like effect induced by ketamine at low doses, since the response was abolished in ovariectomized female rats and also recovered after receiving a hormone replacement treatment [6, 41]. In this context, the present study aimed at evaluating whether inhibiting the synthesis of estrogens through the use of letrozole, an aromatase inhibitor, would improve the antidepressant-like response of ketamine in male rats and/or would allow for ketamine to induce an antidepressant-like effect in female rats. To do so, we first characterized the response induced by letrozole alone, both at the blood hormonal and behavioral levels, since prior preclinical studies already showed that inhibiting the synthesis of estrogens either with letrozole [19, 42], or formestane [43], could induce an antidepressant like-response in female rats, and increase hippocampal neurogenesis (i.e., as a neuroplasticity sign of an antidepressant-like action; [44]). Our results showed that a sustained treatment with letrozole (8 days) significantly increased the blood levels of testosterone in female rats as expected, since blocking ovarian aromatization results in a high accumulation of testosterone in females. In male rats, however, the levels did not change, since a high amount of testosterone is expected to be metabolized to non-aromatizable androgens, such as dihydrotestosterone [42]. In fact, and similarly to the

present results, this prior study, in which we based the paradigm of letrozole pre-treatment followed, found that while the activity of the enzyme was decreased by treatment, no overall changes were observed in testosterone levels in male rats (see [42] and references within for further details). In fact, when letrozole was used to inhibit the biosynthesis of estrogens in adolescent rats, testosterone levels showed increased significant levels also for male rats [45]. This could be caused either by the lower starting basal levels of testosterone in adolescent vs. adult rats or by the time-point at which blood was collected (1 h post-treatment in the present study vs. 1-day post-treatment in the adolescent study, see [45]). Interestingly, this same treatment with letrozole, in line with prior results [19, 42], induced an antidepressant-like effect exclusively in female rats, which parallel the increased in testosterone levels observed 1 h post-treatment. Remarkably, the repeated administration of testosterone was previously shown to induce an antidepressant-like response in the forced-swim test [46] and to increase hippocampal neurogenesis (see review by [47]), suggesting a role for this hormone in the beneficial effects induced by letrozole in female rats.

In regards to the antidepressant-like effects of ketamine in letrozole pre-treated male and female rats, the results showed, as hypothesized, that letrozole improved the response of ketamine in male rats (i.e., effect observed right after a single dose in the forced swim test). Moreover, and replicating the data presented before, a repeated paradigm of ketamine was needed to induce an antidepressant-like response in vehicle-pretreated rats, as observed 1-day post-treatment. At this time point, combining letrozole and ketamine did not show a higher response than just ketamine, suggesting probably a maximum effect in the assay evaluated. However, letrozole did not improve the lack of response induced by ketamine in female rats, which still rendered inefficacious, but once again induced an overall antidepressant-like effect. The mechanism by which letrozole may potentiate the antidepressant-like effects of ketamine in adult male rats is unknown. One could speculate that letrozole could either be acting synergistically on the same molecular pathways targeted by ketamine (e.g., [2]), such as the modulation of neurotrophic factors like BDNF (and related-signaling) in the prefrontal cortex (e.g., [3]), common to several antidepressants [46, 47], or in additional molecular pathways, producing the enhanced physiological response observed. Moreover, and as discussed in the context of the beneficial effects of letrozole observed in females, it is possible that the increase in testosterone that accompanies aromatase inhibition might be responsible for the improved antidepressant-like response (i.e., [48, 49]) induced by ketamine. Moreover, the inhibition of estrogens biosynthesis by letrozole could be affecting the pharmacokinetic and pharmacodynamic profiles of ketamine, since hormonal fluctuations are postulated as the main cause of variability in these processes for any drug [50]. Further studies will be centered in evaluating the molecular mechanisms whereby letrozole increased the efficacy of ketamine in adult male rats.

Finally, one of the most remarkable results presented in this study is the fact that, both the antidepressant-like effects induced by ketamine in adult male rats or letrozole in adult female rats persisted over a long time, since they were still present up to 65 days post-treatment, suggesting long-term sex-directed benefits for these drugs. This is relevant in the context that no prior studies have evaluated persistent effects for so long. For example, a single dose of ketamine induced an acute antidepressant-like effect that long-lasted up to 7 days [51], but nothing further has been evaluated after that time. Moreover, no studies have described the antidepressant-like effects of letrozole across time. Therefore, these findings might be the first ones demonstrating persistent and long-lasting effects for ketamine and letrozole up to 65 days post-treatment, proving a great therapeutical potential in a sex-driven manner (i.e., ketamine for male rats and letrozole for females).

Perspectives and significance

Overall, these findings have proven clear sex-differences in the antidepressant-like response of ketamine in rats, with male rats being more responsive to its beneficial effects proving long-term efficacy. Moreover, inhibiting the biosynthesis of estrogens with letrozole improved the efficacy of ketamine in male rats by advancing the response right after a single dose, in comparison to the repeated paradigm needed in normal conditions. Also, ketamine did not show an antidepressant-like potential in female rats, at least with the conditions tested, however, letrozole induced a remarkable antidepressant-like response in female rats, which persisted in time. Based on this study, ketamine should be utilized adhering to sex-specific considerations. Moreover, aromatase inhibition with letrozole presents itself as a great pharmacological option, since it improved ketamine's efficacy in male rats and induced on itself a promising beneficial response in female rats. The production of estrogens and/or the accumulation of testosterone are key players in modulating some sex-specific antidepressant-like responses in Sprague–Dawley rats. Future studies

should center in evaluating the molecular mechanisms behind these effect at key points of behavioral changes.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13293-023-00560-5.

Additional file 1: Table S1. Antidepressant-like effects induced by ketamine or letrozole in adult male and female rats. **Table S2.** Antidepressant-like effects of ketamine in letrozole pre-treated male and female rats.

Author contributions

MJG-F and SL-C were responsible for the study concept and design. SL-C conducted the experiments and analyzed all data, with the participation of JJ-P. MJG-F revised all raw data and plotted the final figures. SL-C wrote the first draft of the manuscript with inputs from JJ-P. MJG-F edited the manuscript to its final version. All authors critically contributed to the discussion of the results and approved the final version of the manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study can be made available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Experimental procedures were conducted according to the ethical guidelines for the care and use of laboratory animals. Experiments were approved by the local animal care committee (CEEA).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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