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Exploring pharmacological options for adolescent depression: a preclinical evaluation with a sex perspective

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There is an urgent need for developing novel pharmacological treatment options for adolescent depression, and to ensure an optimal translational outcome to the clinic, sex should be included as a biological variable in preclinical studies. In this context, the present study compared the antidepressant-like potential of ketamine and cannabidiol, with the clinical standard fluoxetine, in adolescent rats exposed to maternal deprivation (as a model of early-life stress), while including a sex perspective. Moreover, changes in drug efficacy over time were evaluated by re-exposing rats to the same dose regimens during adulthood. Antidepressant-like responses were scored through a battery of distinctive tests (forced-swim, novelty-suppressed feeding, and sucrose preference) across time. The main results proved an antidepressant-like potential for ketamine and cannabidiol in adolescent rats, although their efficacy was dependent on sex and prior stress exposure, as well as on treatment length and the behavioral feature analyzed. In general, while all tested antidepressants in male rats improved certain affective-like features, female rats were mainly unresponsive to the treatments performed (except for certain benefits induced by ketamine), demonstrating the need for further characterizing proper treatments for this particular sex. Moreover, when rats were re-exposed in adulthood to the same drug regimens as in adolescence, a drop in efficacy was observed. These findings may have translational ramifications in that ketamine or cannabidiol could be moved forward as antidepressants for the adolescent depressed population, but not before further characterizing their potential long-term safety and/or beneficial vs. harmful effects for both sexes.

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INTRODUCTION

Major depression affects around 5–6% of adolescents (13–18 years old; [1]), and its treatment is a major cause of concern since, other than a psychological approach (including internet-based platforms), only fluoxetine is recommended as a pharmacological option. The reason behind this lack of options for this age population relies on the fact that the intrinsic mechanisms mediating adolescent depression seem to vary from those in adulthood, since the brain is still in development, and pharmacological treatments often fail to respond, show lower efficacy (e.g., [2–5]), and/or could even induce a harmful response (i.e., increased suicide ideation; [6]). Moreover, the underlying sex differences in relation to puberty and depression [7, 8] (i.e., common rates during early adolescence [9–11], but increased propensity at later ages for females [12–14]), together with the differences in efficacy reported for certain antidepressants with sex (see [15, 16]), as well as the lack of preclinical studies performed in females [17], especially for the adolescent population, ensures a poor translational outcome to the clinic.

The goal of this study was therefore to characterize novel pharmacological antidepressants for adolescence using a rodent model of early-life stress (e.g., [18–20]) and while including sex as a biological variable [21, 17, 8]. In this context, ketamine, an NMDA receptor antagonist, was recently approved by the FDA for treatment-resistant depression and suicidality in adults [22], and

while its use in younger populations is currently limited to anesthetic and analgesic purposes [23], an increasing number of clinical reports [24–26] suggested a potential use for this drug in adolescent depression, although special caution is still needed in terms of safety and long-term potential concerns in addiction-liability [27, 28]. In this context, recent studies are centered in evaluating the differential effects of ketamine as influenced by sex (some studies showed improved responses in rodents for females [29–32] or males [33], while others even showed no sex differences in humans [34, 35]), by age [36, 25] and prior stress exposure (e.g., [32, 36–39]), as well as the potential dose-related molecular actions behind these disparities [40–43]. Another good candidate to be further explored as a potential antidepressant for adolescent depression is cannabidiol [44–46], a non-psychotomimetic active compound extracted from the plant *Cannabis Sativa*. Prior preclinical studies in rodents have also proven sex-, age- and stress-related antidepressant-like effects induced by cannabidiol [47, 4, 48, 49], without clear potential harmful behavioral effects [50].

Within this framework, ketamine and cannabidiol emerged as possible novel pharmacological options for adolescent depression and were evaluated in the present study in comparison with the standard fluoxetine [51–56] in adolescent rats exposed to early life stress (i.e., maternal separation as a potential triggering factor for several mood disorders [57, 58]), while including sex as a

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bias towards any side of the cage. The test was performed in whole cages to avoid the stress of individually housing rats (i.e., social isolation) for extended periods of time (e.g., [67]), since the experimental design lasted several months (see Fig. 1). On day 8 post-treatment rats were presented again with two water bottles to ensure an equal preference for both of them.

Data analysis and statistics

Data were analyzed with GraphPad Prism, Version 9.3.0 (GraphPad Software, Inc., USA). Results are presented as mean values \pm standard errors of the mean (SEM), with symbols representing individual values for each rat, in line with the recommendations for displaying data and statistical results in experimental pharmacology (e.g., [68, 69]). All data generated was evaluated by three-way ANOVAs (independent variables: sex, early-life, and treatment) and/or, when assessing each sex separately, by two-way ANOVAs (independent variables: early-life and treatment). When appropriate, multiple comparisons were performed by Tukey's test. The level of significance was fixed at $p \leq 0.05$.

RESULTS

Early-life stress as a mild model of negative affect

In an attempt to behaviorally characterize the animal model utilized, as well as the impact of repeating behavioral tests across time, before reporting the effects of drug treatment, the impact of early-life stress was evaluated over time in the cohorts of vehicle-treated rats (a total of $n = 49$ controls and $n = 49$ MD rats; see Supplementary Fig. S1). The progression of affective-like responses through time (adolescence vs. adulthood), both in the forced-swim and novelty-suppressed feeding tests, suggested the absence of significant responses as induced by maternal deprivation for both sexes (see Supplementary Fig. S1). Some differences emerged in test performance with time, such as lower immobility and higher climbing rates in the forced-swim test (i.e., indicative of a more active/escaping behavior, which for female rats was presented as higher swimming rates), and higher latencies to center paired with lower feeding times and distance traveled in the novelty-suppressed feeding test (i.e., indicative of a worse behavioral outcome probably caused by the loss of novelty with test repetition; Supplementary Fig. S1). Interestingly, some basal sex differences also emerged in the novelty-suppressed feeding test, such as an overall lower latency to the center and a higher feeding time and distance traveled for female vs. male rats (see Supplementary Fig. S1).

Remarkably, when also considering treatment in the analysis (three-way ANOVAs with independent variables: sex, early-life, treatment), early-life stress-induced significant changes in negative affect, as observed in the forced-swim and novelty-suppressed feeding tests (see Supplementary Table S1). More importantly, the drugs tested responded in a different way depending on whether rats were previously exposed to early-life stress or not (treatment \times early-life condition interactions, see Supplementary Table S1).

Antidepressant-like effects during adolescence

Given the observed sex and test repetition (or age maturity) differences in effect for all cohorts of vehicle-treated rats (see Supplementary Fig. S1), as well as the sex differences reported when analyzing all data through three-way ANOVAs (see Supplementary Table S1), the effects of drug treatments were reported in control and maternally deprived rats for each sex separately, and analyzed through two-way ANOVAs (independent variables: early-life condition and treatment; see Supplementary Table S2) at each day of testing (Fig. 1).

Particularly, in male adolescent control rats, both ketamine and cannabidiol-induced an acute antidepressant-like effect as measured in the forced-swim test 30 min post a single injection (i.e., decreased immobility); while ketamine increased active behaviors in general (climbing and swimming), cannabidiol exclusively

increased climbing behavior (Fig. 2A). These effects were not observed in male rats exposed to maternal deprivation early in life. Contrarily, fluoxetine induced an acute antidepressant-like effect in adolescent male rats but only in those exposed to maternal deprivation (i.e., decreased immobility and increased swimming; Fig. 2A). Moreover, as for the repeated drug treatments, only cannabidiol rendered efficacious in male adolescent rats, both in controls and maternally deprived rats, by proving an antidepressant-like effect as measured in the forced-swim test (i.e., decreased immobility and increased climbing; Fig. 2B). Interestingly, most of the drugs tested during adolescence in the forced-swim test were not effective in female rats (neither acutely nor repeatedly; Fig. 2), except for acute ketamine that exerted an antidepressant-like effect in maternally deprived rats (i.e., decreased immobility and increased swimming; Fig. 2A).

The novelty-suppressed feeding test was performed during adolescence 3 days post-treatment. The results showed that ketamine increased the time rats spent feeding (i.e., an indicative of an antidepressant- and/or anxiolytic-like effect), but only in male adolescent control rats. Contrarily, no effects were observed for cannabidiol in male adolescent rats. Interestingly, fluoxetine also increased the time control and maternally-deprived adolescent male rats spent feeding. Again, and similarly to the results presented in the forced-swim test, ketamine was the only drug that exerted a putative beneficial effect in females, but only detected in maternally deprived rats (i.e., increased feeding time; Fig. 3A). As the data showed, the changes in feeding time (s) did not seem to be driven by changes in distance traveled (cm) or latency to center (s), since they were not altered by any treatment studied (see Fig. 3A).

Finally, no significant changes were observed when measuring 1% sucrose intake (g/kg) or preference (%) as induced by any of the drugs tested in adolescence, and as measured in the sucrose preference test in male and female rats 6–7 days post-treatment (Fig. 3B), suggesting the importance of utilizing complementary tests for scoring different aspect of affective-like behavior. Moreover, no changes were observed in daily water consumption among groups (data not shown).

Antidepressant-like effects during adulthood

The potential long-term effects of each adolescent treatment were evaluated in the forced swim test 45 days post-treatment. The results showed that neither ketamine, cannabidiol, nor fluoxetine induced persistent changes in immobility, climbing or swimming behaviors (see Supplementary Fig. S2). Then, 5 days later, rats were re-exposed to the same drug regimens as received in adolescence and were tested in the forced swim test (see Fig. 1). None of the drugs evaluated (ketamine, cannabidiol, and fluoxetine) induced an acute antidepressant-like response as measured in the forced-swim test in male and female rats, and independently of the early-life condition experienced (see Fig. 4A). Remarkably, 24 h post-repeated treatments, cannabidiol was the only drug that produced an antidepressant-like effect, observed for male control rats in the forced swim test (i.e., decreased immobility and increased climbing; Fig. 4B), similarly to the results reported in adolescence. On the contrary, repeated ketamine administration increased immobility and reduced climbing in adult male maternally-deprived rats (see Fig. 4B), indicative of a negative impact on affective-like behavior. Finally, female rats were even more unresponsive than in adolescence, since no signs of efficacy were induced by any of these treatments in adulthood (Fig. 4).

In the novelty-suppressed feeding test (as measured 3 days post-treatment in adulthood), ketamine decreased the latency to center (s) both in control and maternally deprived male rats. Moreover, ketamine increased the time rats spent feeding (s) in male controls, as well as in female rats (both control and maternally deprived), indicative of antidepressant- and/or anxiolytic-like responses (Fig. 5).

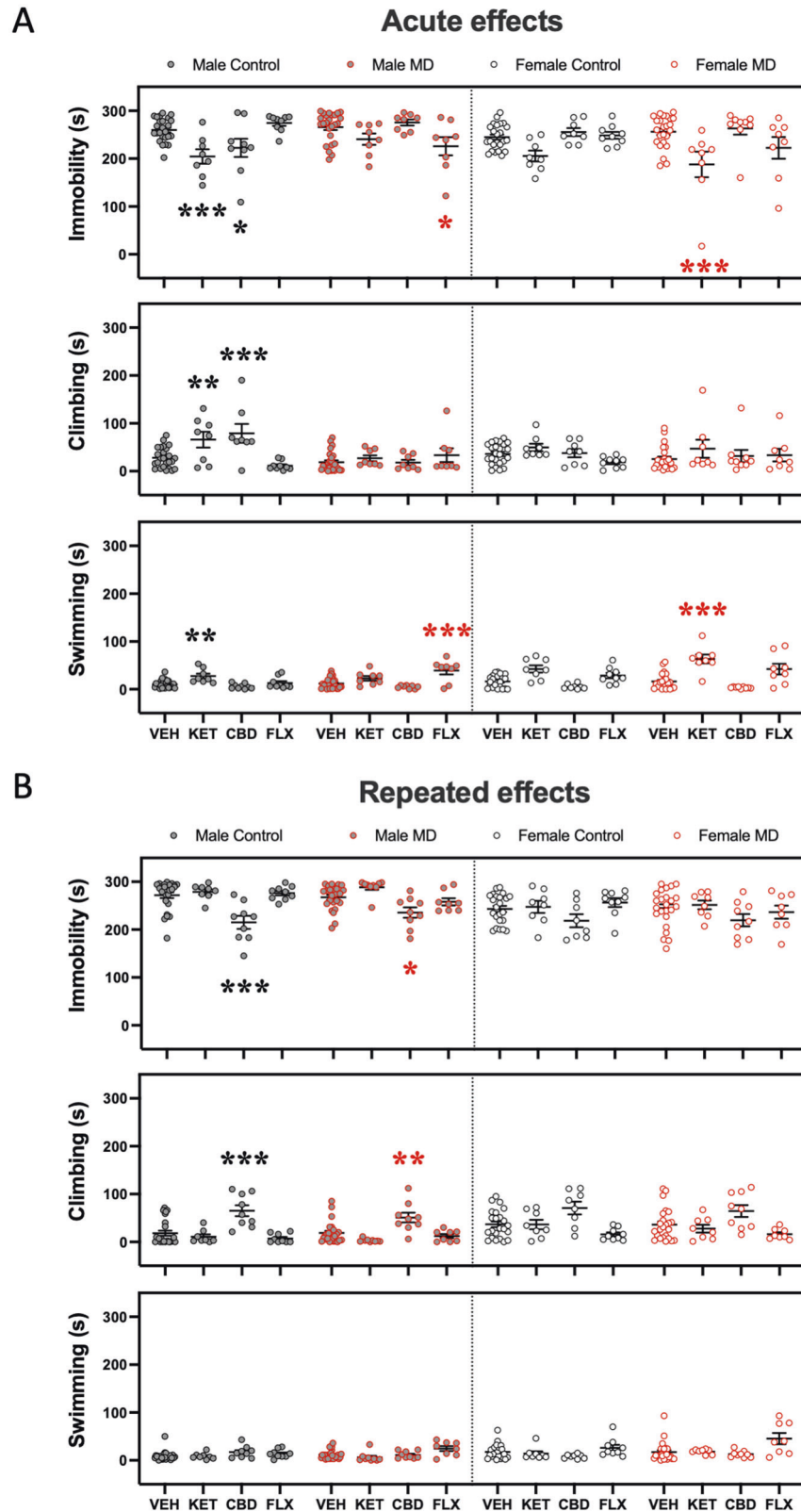
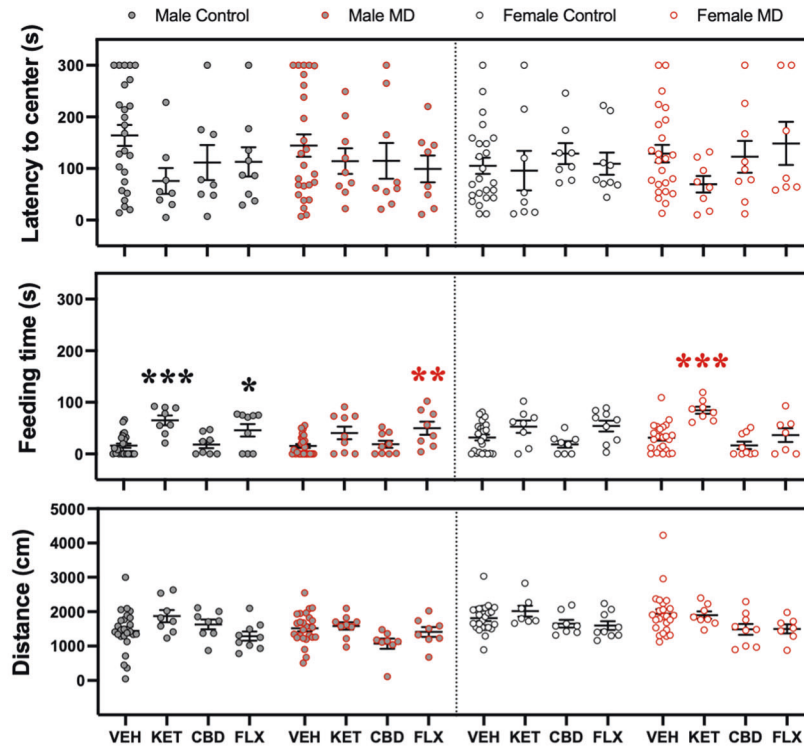


Fig. 2 Antidepressant-like effects as measured in the forced swim test (FST) during adolescence. **A** Acute (30 min post-treatment) and **B** repeated (1-day post-treatment) effects exerted by adolescent ketamine, cannabidiol, or fluoxetine exposure in male and female rats (Control or MD) in the FST. Data represent mean \pm SEM of the time spent (s) immobile, climbing, or swimming. Individual values are shown for each rat (symbols). Three-way ANOVAs (independent variables: sex, early-life, and treatment) and two-way ANOVAs (independent variables: early-life and treatment) are shown in Supplementary Tables 1 and 2 respectively. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$ vs. the appropriate control group (Control or MD) and sex.

A



B

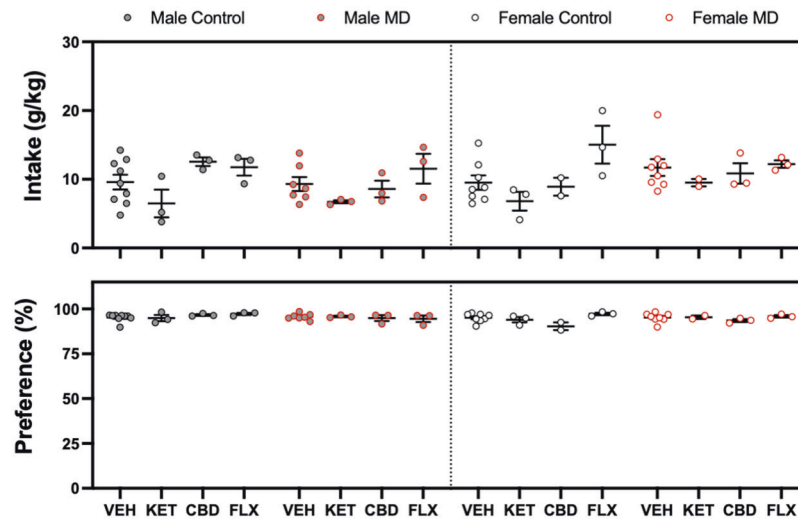


Fig. 3 Antidepressant-like effects as measured in the novelty-suppressed feeding test (NSF) or sucrose preference test (SP) during adolescence. **A** Repeated effects (3 days post-treatment) exerted by adolescent ketamine, cannabidiol, or fluoxetine exposure in male and female rats (Control or MD) in the NSF. Data represent mean \pm SEM of the latency to center (s), feeding time (s), or distance traveled (cm). **B** Repeated effects (6–7 days post-treatment) exerted by adolescent ketamine, cannabidiol, or fluoxetine exposure in male and female rats (Control or MD) in the SP. Data represent mean \pm SEM of the sucrose intake (g/kg) or preference (%). Individual values are shown for each rat (symbols). Three-way ANOVAs (independent variables: sex, early-life, and treatment) and two-way ANOVAs (independent variables: early-life and treatment) are shown in Supplementary Tables 1 and 2 respectively. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ vs. the appropriate control group (Control or MD) and sex.

Contrarily, cannabidiol and fluoxetine did not induce any beneficial effects as measured in this test both for male and female rats. In fact, they even seemed to be somehow harmful, since cannabidiol decreased the distance traveled (cm) in maternally deprived male rats, and fluoxetine increased the latency to center (s) and decreased

the distance traveled (cm) in female maternally deprived rats (Fig. 5). Food intake and water consumption were not monitored throughout the procedure so no conclusions could be extracted on the possible orexigenic and/or dipsogenic drug effects that could be confounding the present results.

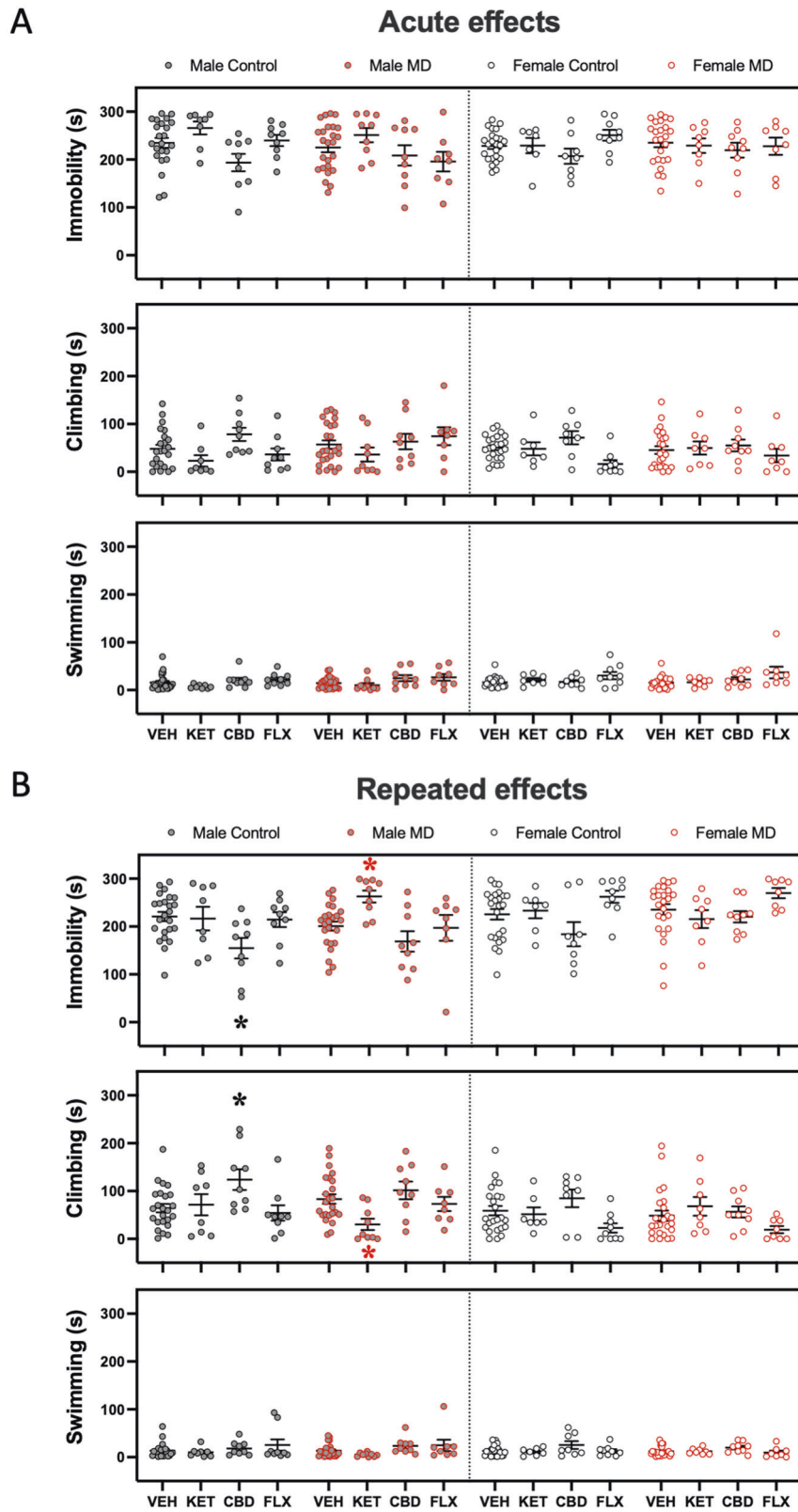


Fig. 4 Antidepressant-like effects as measured in the forced swim test (FST) during adulthood. A Acute (30 min post-treatment) and **B** repeated (1-day post-treatment) effects exerted by adult ketamine, cannabidiol, or fluoxetine exposure in male and female rats (Control or MD) in the FST. Data represent mean \pm SEM of the time spent (s) immobile, climbing, or swimming. Individual values are shown for each rat (symbols). Three-way ANOVAs (independent variables: sex, early-life, and treatment) and two-way ANOVAs (independent variables: early-life and treatment) are shown in Supplementary Tables 1 and 2 respectively. * $p < 0.05$ vs. the appropriate control group (Control or MD) and sex.

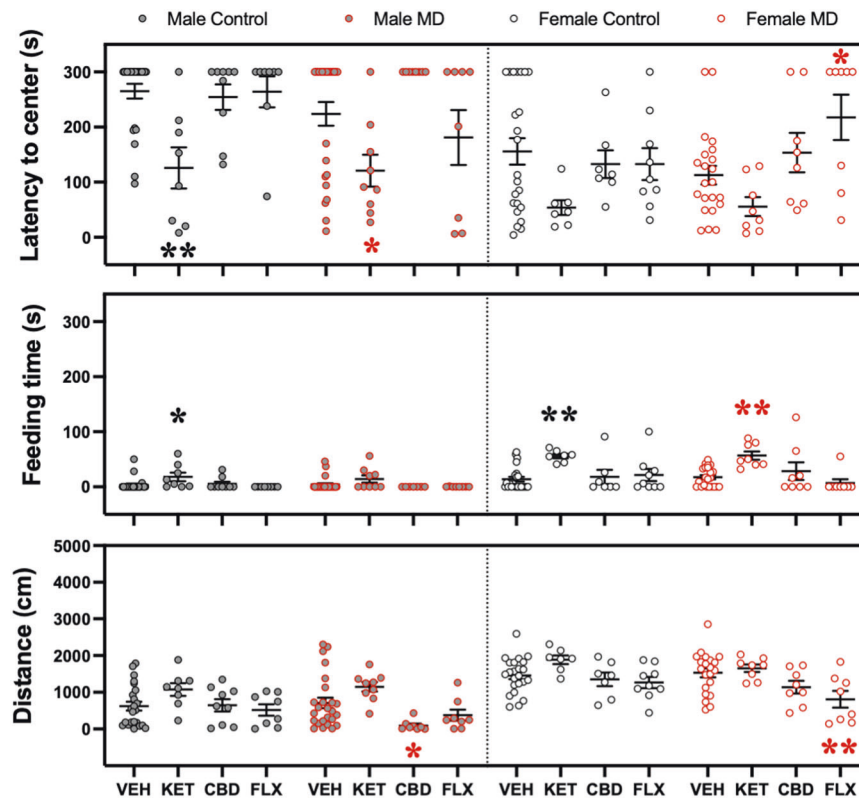


Fig. 5 Antidepressant-like effects as measured in the novelty-suppressed feeding test (NSF) during adulthood. Repeated effects (3 days post-treatment) exerted by adult ketamine, cannabidiol, or fluoxetine exposure in male and female rats (Control or MD) in the NSF. Data represent mean \pm SEM of the latency to center (s), feeding time (s), or distance traveled (cm). Individual values are shown for each rat (symbols). Three-way ANOVAs (independent variables: sex, early-life, and treatment) and two-way ANOVAs (independent variables: early-life and treatment) are shown in Supplementary Tables 1 and 2 respectively. ** $p < 0.01$ and * $p < 0.05$ vs. the appropriate control group (Control or MD) and sex.

DISCUSSION

This behavioral study proved that the antidepressant-like effects induced by ketamine, cannabidiol, or fluoxetine during adolescence were dependent on sex and prior stress exposure, as well as on treatment length and the behavioral dimension analyzed. In general, all three antidepressants improved different aspects of affective-like behavior for male rats, although prior early-life stress altered the outcome in some cases. However, female rats were mainly unresponsive to the treatments performed (except for the observed benefits induced by ketamine), demonstrating the need for further characterizing other doses, and/or finding proper treatments for this particular sex, to ensure optimal translational outcomes. The antidepressant-like effects induced during adolescence did not produce long-term responses, and when rats were re-exposed in adulthood to the same drug regimens as in adolescence, no acute effects were observed. In fact, a repeated drug regimen was needed to induce certain antidepressant-like responses by ketamine or cannabidiol, suggesting a drop in efficacy with age (adulthood vs. adolescence) following drug re-exposure.

The paradigm of early-life stress utilized in this study was mild in terms of inducing basal differences in affective-like behavior (maternally deprived vs. naive control rats), in line with some prior published literature stating that preclinically, the maternal separation paradigm, used to examine the influence of this form of early-life stress in the first weeks of life, did not always and necessarily produce a depressive-like phenotype (reviewed by [70]). In this context, reproducibility in rodent behavioral research is a topic of great debate and concern and varies for each model depending on the particular experimental conditions. Particularly, we have used this specific paradigm of maternal separation (24 h

from PND 9 to 10) in our group for some time now, publishing four independent studies including hundreds of rats [3, 48, 71, 72]. The main conclusion of all of them is that, in our hands, depriving Sprague-Dawley pups 24 h from their mom from PND 9 to PND 10, seems to be a mild procedure in terms of altering affective-like responses, at least with the behavioral tests that we normally utilize (e.g., forced-swim test, novelty-suppressed feeding, and sucrose preference). However, this lack of behavioral impact is accompanied by changes in neurochemical markers in the hippocampus (i.e., increased pro-apoptotic FADD [71]), a brain region particularly responsive to stress, suggesting a negative impact induced by this procedure. Moreover, the combination of maternal separation with a different stressor (i.e., drug administration during adolescence) induced negative affect [71, 72], in line with the literature reporting the “two-hit” hypothesis in the development of neuropsychiatric illnesses (a combination of two or more major disruptions are needed at specific time points during development). Moreover, it is important to remember that the forced-swim test is a good test to measure antidepressant-like responses, but not necessarily to phenotype a depressive-like response (reviewed by [73]). Interestingly, the fact that the model induced sex differences in response to this prior stressor, but also changes in treatment responses (as observed by the significant interactions reported), suggested an impact of the procedure and supported its suitable use for the objective of the study. In fact, we selected maternal deprivation as a simple model (with easy logistics) in which to perform the first drug screening in adolescence following an early-life stressor. To model a more robust depressive-like phenotype we would have needed other preclinical approaches that include more aggressive and/or prolonged procedures that might have even interfered with the

timing of the adolescent treatment (e.g., [19]). Therefore, early-life stress modeled with maternal separation, although failed to show baseline changes in affective-like behavior with the tests performed (as previously described in [71, 72]), proved differences in the effectiveness of the selected drug treatments, both in adolescence and adulthood, presenting itself as a good platform in which to explore novel antidepressant options for adolescence with a sex perspective.

In this regard, the present results demonstrated a great potential for ketamine as an antidepressant for adolescence, since an acute dose improved immobility in the forced swim test, and a repeated treatment increased feeding time in the novelty-suppressed feeding, both indicators of positive preclinical outcomes. However, when ketamine was re-exposed in adulthood to the same rats that received the drug in adolescence, a repeated paradigm was needed to observe the antidepressant-like response, since no acute effects were detected. Interestingly, although these effects across time were observed for both sexes, they were defined by early-life stress exposure, since ketamine showed a parallel efficacy in control male rats and maternally deprived female rats. No beneficial effects were present for maternally deprived male rats (even a deleterious effect was induced by repeated ketamine in adulthood) as well as for control female rats, suggesting sex- and stress-related interactions in the observed drug responses. In this regard, prior studies in the literature have already reported some sex- [29–33] and stress-related (e.g., [32, 36–39]) differential responses for ketamine, however, most of these earlier studies were performed in adult rodents (as opposed to adolescent), mainly in mice, and utilized other procedures to model stress-related conditions (not maternal separation). For example, a recent study reported that chronic psychological stress could also interact with ketamine to modulate its effects since it was capable of inducing an antidepressant-like effect 24 h post-injection (i.e., decreased immobility and increased swimming in the forced-swim test) in mice exposed to unpredictable chronic stress, while it induced the opposite effects in unstressed mice [38]. Moreover, when evaluating the impact that sex induced on the sustained effects of ketamine on resilience to chronic stress, a recent report in adult mice showed that while ketamine prevented chronic stress-induced changes in affective-like behaviors in males, it did not protect females [33]. Furthermore, the drop in ketamine's efficacy after re-exposure in adulthood could be mediated by the emerging differences driven by age [36, 25] and/or be dose-related [40–43]. Our data suggested a great antidepressant-like potential for ketamine in adolescence, while also demonstrated that maternal deprivation interacted with ketamine treatment to modulate its effects in female rats. Therefore, future studies should center on ascertaining the potential mechanisms behind ketamine's actions in relation to the differential effects observed by sex- and stress- (as reviewed in [40, 41]), and incorporating other doses/regimens of exposure that might still provide efficacy in adulthood, since most probably, adolescent patients will need to be further medicated at later ages. Still, caution should be taken with these data since the use of ketamine during adolescence might induce dissociative effects and long-term drug abuse problems (although low doses seem to be safe; e.g., [36, 37]), being these side effects a possible limitation to its translational use in the clinic.

In regards to the potential antidepressant-like effects of cannabidiol in adolescence, the results showed both acute and repeated efficacies as observed in the forced-swim test by decreasing immobility and increasing climbing rates, but only for male control rats. Male rats exposed to maternal deprivation needed a repeated regimen exposure for cannabidiol to exert its antidepressant-like actions in adolescence. Similarly, when cannabidiol was re-exposed to rats in adulthood, a repeated paradigm was necessary to observe the expected antidepressant-like effects in controls, while the response was no longer detected in

maternally deprived male rats. Prior preclinical studies have also reported age- and stress-related antidepressant-like effects induced by cannabidiol in male rodents [4, 47, 48]. In our case, to select the dose and length of the drug treatment, we relied on published literature on the topic, which varied from dose regimens from 7 and up to 21 days of treatment (e.g., [49]). However, we decided to start with the shortest paradigm of 7 days of cannabidiol administration at the dose of 10 mg/kg, since this was the dose and length that showed an antidepressant-like effect in our previous study in male adolescent rats (see [4]). We acknowledge that although it would have been interesting to assess how other doses and/or even longer treatments could affect the results, we first decided to check the potential sex- and/or stress-related effects of a dose known to induce a response in adolescent naïve male rats. Our results validated our prior study [4], and extended it by including cannabidiol's acute antidepressant-like potential in male rats, and its lack of efficacy in maternally deprived rats. Moreover, the present study also evaluated cannabidiol's potential in female rats, but unfortunately, no antidepressant-like responses were observed. Similarly, acute cannabidiol-induced an antidepressant-like effect in male but not in female mice [49]. Therefore, the results proved a great potential for cannabidiol (at the specific conditions tested) as a potential antidepressant for male adolescent and adult rodents in line with prior literature ([4, 48] reviewed in [44–46]), while suggesting that other doses/regimens of exposure will need to be tested to find effective treatments for females.

Finally, fluoxetine, which was selected as the clinical standard, only induced an antidepressant-like effect in adolescent male rats exposed to maternal deprivation (serving as a positive control only in this scenario), as observed acutely in the forced-swim test (i.e., decreased immobility and increased swimming) and repeatedly in the novelty-suppressed feeding test (i.e., increased feeding time). No efficacy was detected following re-exposure in adulthood, but most importantly it rendered ineffective for female rats at any age and condition tested. However, some prior studies have proven fluoxetine to work during adolescence not only in males [51, 54], but also in female rodents [53, 54], and/or to render sex- and age-related responses (e.g., [56]). In any case, the present results are really relevant since although fluoxetine is the clinical standard for adolescent depression, in our study only worked for male rats, suggesting the need for a deeper characterization for female rodents, since other doses and/or paradigms will need to be adjusted for optimal therapeutical responses and clinical translation for this particular sex.

Overall, these findings may have translational ramifications in that ketamine or cannabidiol could be moved forward as antidepressants for the adolescent depressed population, but not before further characterizing their potential long-term safety and/or beneficial vs. harmful effects. Interestingly, drugs responded differently based on prior stress exposure during early-life. Moreover, these results reinforce the need to include female rats in all preclinical studies, since the expected efficacy of the tested antidepressants was diminished or even inexistent for females (except for ketamine). The differential regulation of these antidepressants by sex deserves a broadened characterization in terms of what drives the behavioral differences and to figure out the potential molecular correlates involved in those actions. These studies are currently ongoing since they will not only include the brains of the present rats sacrificed after the behavioral phenotyping (which will only provide a "photo-finish" of the brain), but also new experiments with rats treated with the same drug paradigms and sacrificed at the specific time points in which behavior differed by sex and/or prior stress condition, so the time-course of the effects could be ascertained. So far, if one were to select one drug to move it forward to the clinic, and although ketamine is already approved by the FDA for resistant depression in adulthood and seems to work for both sexes in adolescence, it

has special surveillance for addictive and/or harmful potential, making it not an ideal drug candidate to be administered in our young population. On the other hand, cannabidiol has proven a great potential in adolescence and is already in the clinic for certain types of resistant epilepsy in children, providing some data in terms of its long-term safety. Therefore, once a better understanding is gained on its potential use at this early-life age, and further characterization is done in terms of efficacy for females, we should encourage starting clinical trials in human adolescent subjects suffering from major depression that are not responding to psychotherapy and/or the only recommended drug treatment available for this age-range (i.e., fluoxetine).

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AUTHOR CONTRIBUTIONS

MJG-F was responsible for the study concept and design with the contribution of SL-C. SL-C conducted the experiments and analyzed the behavioral data, with the participation of EH-H. MJG-F revised and interpreted all the data, drafted the final figures, and wrote the manuscript. All authors critically contributed to the discussion of the results and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare no competing interests.

ADDITIONAL INFORMATION

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