

Journal Pre-proof



Intermittent repeated stress but not ketamine changes mice response to antidepressants

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Intermittent repeated stress but not ketamine changes mice response to antidepressants

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Highlights

- Repeated ketamine anti-immobility effect lasted for 3 days after treatment.
- Repeated intermittent stress impaired fluoxetine and imipramine anti-immobility effects.
- Ketamine does not prevent stress-induced change in antidepressants' effect.

Abstract

Discovery of the rapid antidepressant effect of ketamine has been considered one of the most important advances in major depressive disorder treatment. Several studies report a significant benefit to patients that lasts up to 19 days after treatment. However, concerns arise from the long-term use of ketamine, thus a safe and effective strategy for maintaining its antidepressant effect is still necessary. To this end, our work assessed the effects of imipramine and fluoxetine after repeated ketamine treatment in male mice. Ketamine (30 mg/kg/day for 14 days) induced an anti-

immobility effect in the forced swimming (FS) paradigm, detected 1 and 3 days after treatment. Seven days after the last ketamine injection, mice received imipramine (20 mg/kg) or fluoxetine (30 mg/kg). Imipramine and fluoxetine did not change mice's immobility time, regardless of the pre-treatment (saline or ketamine). Since both drugs' anti-immobility effect was demonstrated in the classical FS test, we can assume that repeated exposure to intermittent stress inhibited the antidepressant drugs' anti-immobility effects. Moreover, pre-exposure to ketamine did not counteract stress-induced changes in mice response to antidepressants. Since exposure to forced swim and i.p. injections are stressful to rodents, each stressor's contribution to the blunted response to antidepressants was investigated. Our data demonstrated that both stressors (FS and i.p. injections) influenced the reported effect. In summary, our results showed that exposure to intermittent repeated stress inhibited the anti-immobility effect of imipramine and fluoxetine in mice and corroborated findings demonstrating that exposure to stress can blunt patients' response to antidepressants.

Abbreviations:

ACTH - adrenocorticotrophic hormone

CRF - corticotropin-releasing factor

FLU – fluoxetine

FS – forced swimming

IMI – imipramine

MDD – major depressive disorder

OF – open field

PND – postnatal day

VEH – vehicle

Keywords: stress; forced swimming; ketamine; imipramine, fluoxetine; environmental factors

1. Introduction

First described around twenty years ago, the rapid antidepressant effect of ketamine had been considered one of the most important advances in major depressive disorder (MDD) treatment [1,2]. A single subanesthetic dose of ketamine i.v. can improve patients' mood for seven days [2], decreasing especially suicidal ideation [3,4] and, after repeated treatment (six infusions over 12 days), ketamine effects lasted up to 19 days after administration [5]. These findings justified the prescription of ketamine for treatment-resistant MDD patients. The need for a safer administration route led to the development of an intranasal formulation containing the S(+) active enantiomer of ketamine, esketamine [6]. This medication has been proved to be effective in managing MDD as an alternative to i.v. injections, allowing for self-administration [7–9]. However, serious concerns arise from the perspective of long-term use of ketamine, such as tolerance, intolerable motor effects, risk of abuse, and the emergence of psychotomimetic effects [10–13]. Thus, finding a strategy for maintaining ketamine's antidepressant effect for a more extended period avoiding the harms of its continuous use is still a challenge.

To this end, several approaches have been hypothesized, including the use of a different glutamatergic drug or even monoaminergic antidepressants in an attempt to prolong ketamine's antidepressant effects or reverse relapse [14]. Data regarding the possible interaction between ketamine and classical antidepressant drugs are still scarce. Since studies have shown that previous exposure to a drug can change the response to a subsequent one [15–18], patients initially non-responsive to classical antidepressants may benefit from these drugs after ketamine treatment, making their use a suitable strategy to safely prolong the antidepressant effects of ketamine. In this

context, studies to clarify the pharmacological interactions between ketamine and other antidepressants are needed.

The antidepressant-like effect of subanesthetic doses of ketamine was demonstrated in several animal models, but data about the effect's persistence are still controversial [19–22]. Previous work from our group showed a non-persistent anti-immobility effect induced by repeated administration of ketamine in a forced swimming (FS) paradigm in mice [23]. In this work, we assessed the effects of imipramine and fluoxetine in mice previously treated with ketamine, aiming to identify possible drug interactions.

2. Material and methods

2.1. Animals

Adult male Swiss mice (PND60) from the Institute of Biomedical Sciences (ICB-UFRJ) breeding colony were used. Room temperature was kept at $23 \pm 2^\circ\text{C}$ under a 12 hours light-dark cycle (lights on at 6 am). All animals had free access to filtered water and standard diet. All procedures were previously approved by local Animal Ethical Committees (CEUA/UFRJ, approval number 83/19) and performed according to the Directive of the European Parliament and of the Council of the European Union (2010/63/EU) and the guidelines from the Brazilian National Council for Animal Experimentation Control (CONCEA).

2.2. Drugs and treatments

(S)-Ketamine hydrochloride (Cristália®, Brazil), imipramine hydrochloride (Galena, Brazil), and fluoxetine (Teuto, Brazil) were dissolved in saline (NaCl 0.9%). Fluoxetine (30 mg/kg) and imipramine (20 mg/kg) were administered orally (through gavage), and ketamine (30

mg/kg) administration was performed intraperitoneally. Doses were chosen based on previous data from our group [23–25]. Control groups received vehicle (NaCl 0.9%). All drugs were administered at 1 mL/100 g body weight.

2.3. Behavioral tests

2.3.1. Open field

In the open field (OF) task, animals were placed in the center of a circular arena ($r = 40$ cm, $h = 30$ cm) under indirect dim light and freely explored it for 10 minutes. Each mouse had its trajectory videotaped for post hoc analysis. The total distance traveled (cm) was calculated using the open-source software *MouseGlob*.

2.3.2. Forced swimming

Mice were forced to swim in transparent plexiglass cylinders ($r = 10$ cm, $h = 30$ cm) containing water up to 15 cm at 24 ± 1 °C under low indirect lighting. Forced swimming (FS) sessions lasted 3 min and were videotaped for post hoc analysis. Mice immobility time (s) was measured by trained experimenters blind to treatments and experimental conditions. A mouse was considered immobile when it remained floating with no movements except the minimum for keeping its head above water.

2.4. Experiment 1: Imipramine and fluoxetine effects in mice pretreated with ketamine

The experimental design showed in Fig. 1A was followed to investigate the effects of fluoxetine and imipramine in the immobility time of animals pretreated with ketamine. In all experimental sessions, animals performed the OF task immediately, followed by the FS. After

baseline OF and FS sessions on day 1, animals were randomly divided into two experimental groups and exposed to daily saline or ketamine i.p. injections for 14 days (from day 2 to day 15). One and three days after ketamine treatment (days 16 and 18, respectively), mice's immobility was assessed to verify the antidepressant-like effect of ketamine. Seven days later (day 22), ketamine and saline-treated mice were subdivided into three post-treatment groups: vehicle (VEH p.o.), imipramine (IMI), or fluoxetine (FLU). Drugs were administered 1 hour before the FS session.

FS sessions were performed in mice naive to any experimental intervention using the classical Porsolt forced swimming protocol to confirm our ability to detect the anti-immobility effect of imipramine and fluoxetine [26]. Briefly, animals were treated with vehicle, fluoxetine (p.o.), imipramine (p.o.), or ketamine (i.p.) and forced to swim for 6 min immediately after a 10 min OF session. All p.o. treatments were performed one hour before FS, while i.p. administrations were done 30 minutes before the swimming session.

2.5. Experiment 2: Stressors influence on mice response to antidepressants

The putative influences of repeated i.p. injections and intermittent forced swimming as potential stressors underlying the impairment in mice's responsiveness to antidepressants were evaluated following the protocol shown in Fig. 1B. Initially, animals were randomly divided into two experimental groups, each accounting for one of the stressors. All animals performed the OF task on days 1, 16, 18, and 22. Animals from the i.p. injection group received saline (0.9% NaCl) daily for 14 days (from day 2 to day 15) and were not forced to swim on days 1, 16, or 18. Animals from the FS group were not manipulated from day 2 to 15 but were exposed to FS sessions on days 1, 16, and 18. On day 22, animals were once again subdivided into three post-treatment groups to

investigate the classical antidepressant drugs' effects. Then, mice behavior was assessed using both the OF and FS. Drugs were administered 1 h before the FS session.

2.6. Statistical analysis

Data are expressed as mean $\pm$ standard mean deviation. Student's t test, one-way and two-way ANOVA for single and repeated measures samples were used. When appropriate, Tukey posthoc comparison test was also applied. The adequate hypothesis test for each dataset was selected considering the experimental design, the number of experimental groups, and data distribution. Statistical analysis was performed using *SigmaStat 3.11* (Jandel Scientific Corporation), and differences were considered significant at $p < 0.05$.

3. Results

3.1. Ketamine induced an anti-immobility effect in mice

At first, we aimed to reproduce the antidepressant-like effect of repeated ketamine treatment previously reported by us [23]. Fig. 2A shows that, at baseline (day 1), there were no significant differences between groups in both OF ($t_{43} = 0.064$, $p = 0.949$) and FS performances ($t_{43} = 0.475$, $p = 0.637$). After 14 days of ketamine or saline injections, both experimental groups presented behaviors compatible with habituation to the tasks. A significant decrease in total distance travelled (Fig. 2B, OF, Two-way RM ANOVA, $F_{\text{day}(1,43)} = 35.298$, $p < 0.001$) and an increase in FS immobility time (Fig. 2B, FS, Two-way RM ANOVA, $F_{\text{day}(1,43)} = 5.304$, $p = 0.026$) between experimental days 16 and 18 were detected regardless of drug treatment. A significant effect of drug exposure was detected in the FS task only. Mice exposed to ketamine presented a significant reduction in the immobility time when forced to swim compared to controls (Fig. 2B,

FS, Two-way RM ANOVA, $F_{\text{treatment}(1,43)} = 5.385$, $p = 0.025$), and this effect was detected both 1 and 3 days after treatment (day 16 and 18, respectively). No significant effect of treatments in total distance travelled in the open field was detected (Fig. 2B, OF, Two-way RM ANOVA, $F_{\text{treatment}(1,43)} = 0.443$, $p = 0.509$). This profile is consistent with an antidepressant-like effect induced by ketamine after repeated treatment. In this experiment, there was no difference in weight gain between groups (saline = -1.4 ± 0.6 g, ketamine = -1.6 ± 0.5 g, $t_{43} = 0.252$, $p = 0.802$).

3.2. Mice previously forced to swim did not respond to classical antidepressants

Since the antidepressant effect of ketamine in a clinical setting lasts for at least seven days after treatment and considering our previous data showing the similarity in immobility time presented by saline and ketamine treated groups one week after treatment [23], this time frame was selected for testing the effects of antidepressants in the immobility time of mice previously exposed to ketamine. Imipramine, fluoxetine, or vehicle were administered p.o. 1 hour before the behavioral assessment. As expected, both antidepressants did not change mice locomotion in the OF (Fig. 2C, OF, Two-way ANOVA, $F_{\text{post-treatment}(2,39)} = 0.022$, $p = 0.978$) and there was also no effect of ketamine pre-exposure (Fig. 2C, OF, Two-way ANOVA, $F_{\text{pre-treatment}(1,39)} = 0.189$, $p = 0.666$). Surprisingly, imipramine and fluoxetine did not change mice behavior in the FS task. Groups' average immobility times were similar regardless of the experimental intervention and no statistically significant differences were detected (Fig. 2C, FS, Two-way ANOVA, $F_{\text{pre-treatment}(1,39)} = 0.191$, $p = 0.665$; $F_{\text{post-treatment}(2,39)} = 0.063$, $p = 0.939$, $F_{\text{interaction}(2,39)} = 0.354$, $p = 0.704$).

3.3. Imipramine and fluoxetine induced an anti-immobility effect in mice not previously stressed

To confirm the effectiveness of the antidepressant doses chosen, we evaluated their anti-immobility effect using the classical Porsolt FS test [26] in mice naive to any experimental manipulation. Treatments were administered one hour before the swimming session, employing the same drugs and doses. Ketamine acute effects (30 mg/kg i.p. 30 min before tasks) were also investigated. In this experiment all tested drugs decreased mice immobility time (Fig. 3, FS, ANOVA, $F_{(3,36)} = 5.728$, $p = 0.003$). Ketamine also significantly increased mice locomotion in the OF (Fig. 3, OF, ANOVA, $F_{(3,36)} = 0.326$, $p < 0.001$), pointing to a general psychostimulant effect of ketamine acute administration. Since imipramine and fluoxetine did not significantly change mice locomotion ($p > 0.05$ at Tukey's post hoc), we can assume that these drugs induced an antidepressant-like effect in mice. Thus, considering these results and data from Fig. 2C, we can propose that exposure to intermittent stress inhibited fluoxetine and imipramine's anti-immobility effect.

3.4. Influence of different stressors in mice lack of response to antidepressant drugs

Given that mice were exposed to two different stressful manipulations (repeated i.p. injections and intermittent FS), an experiment was designed to investigate each stressor's contribution separately. Mice were divided into two experimental groups: one group was subjected to 14 days daily i.p. injections only and another one to the intermittent FS protocol only. Baseline OF performance was evaluated, showing no significant difference between groups (Fig. 4A, left panel, $t_{73} = 1.099$, $p = 0.276$). Exposure to the stressors did not differentially change mice weight gain (i.p. injection = 0.5 ± 0.3 g, FS = 1.2 ± 0.3 g, $t_{73} = 1.459$, $p = 0.149$). A reduction in OF exploration across days was observed in both groups (Fig. 4A, right panel, Two-way RM ANOVA, $F_{\text{day}(1,73)} = 10.195$, $p = 0.002$) with no significant stressor influence (Fig. 4A, right panel, Two-way

RM ANOVA, $F_{\text{stressor}(1,73)} = 1.066$, $p = 0.305$), showing mice habituation to the task. Once more, an increase in the immobility time across days was detected in the FS group (Fig. 4B, RM ANOVA, $F_{(2,72)} = 9.195$, $p < 0.001$).

When mice were acutely treated with the classical antidepressants, their locomotion was not affected (Fig. 4C, OF, Two-way ANOVA, $F_{\text{treatment}(2,69)} = 2.226$, $p = 0.116$). Previous exposure to the stressors did not change mice locomotion either (Fig. 4C, OF, Two-way ANOVA, $F_{\text{stressor}(1,69)} = 0.031$, $p = 0.860$). As expected, mice previously forced to swim showed longer immobility times when compared to mice naive to this task (Fig. 4C, FS, Two-way ANOVA, $F_{\text{stressor}(1,69)} = 24.10$, $p < 0.001$). Once more the classical antidepressants failed to induce a statistically significant anti-immobility effect in mice previously stressed (Two-way ANOVA, $F_{\text{treatment}(2,69)} = 1.936$, $p = 0.152$) and no factors interaction was found (Two-way ANOVA, $F_{\text{interaction}(2,69)} = 2.119$, $p = 0.128$). Nevertheless, a trend for reducing the immobility time induced by imipramine and fluoxetine was observed in the group previously subjected to repeated i.p. protocol, but not in the group forced to swim. Thus, both stressors contribute to the altered mice response to classical antidepressants here described, and the intermittent FS exposure seems to have a more pronounced influence.

4. Discussion

Our study's initial aim was to investigate the potential interference of ketamine pre-exposure in the subsequent response to imipramine or fluoxetine in mice using the repeated FS paradigm previously employed by our group [23]. Our data showed that repeated ketamine administration did not affect mice response to these drugs. These data contrast with those reported by Owolabi and coworkers [15], who showed that ketamine (21 days, 15 mg/kg/day i.p.) completely inhibited fluoxetine anti-immobility effect. This discordance may be due to

experimental differences. Besides ketamine dose and treatment duration, in Owolabi's study, mice were forced to swim once, whereas mice underwent intermittent FS in our study mice. Since the antidepressant-like effects of both imipramine and fluoxetine were demonstrated in our experimental conditions when mice were not pre-exposed to stress, these observations suggest that exposure to intermittent stress impaired the anti-immobility effect of the antidepressants. Moreover, since no differences were found between ketamine and saline-treated groups, we can assume that ketamine could not prevent the deleterious effect of intermittent stress over the antidepressant-like effects of the selected drugs.

Exposure to repeated FS causes physiological, endocrine, and immune changes, including an increase in serum corticosterone, adrenocorticotrophic hormone (ACTH), and corticotrophin (CRF) [27–29], confirming the stressful nature of forcing rodents to swim. In addition to FS, in the present study, mice also received repeated intraperitoneal injections, which can also be considered a source of stress. For example, repeated injections of harmless vehicle increase corticosterone plasma levels in mice [30]. Our group's previous work showed that repeated saline i.p. injections impaired mice locomotion and memory in a novel object recognition task [23]. Therefore, we investigated the relative influence of the two stressors on mice blunted response to classical antidepressants. Indeed, our data showed that both stressors seem to contribute to the reported effect, being the effect induced by the intermittent FS apparently the most pronounced. Another source of stress that must be considered is oral gavage. While it was not stressful enough to change mice's behavior by itself, as seen in the Porsolt FS test, its minor role in the repeated stress-induced change in mice response to antidepressants cannot be ruled out.

Besides the initial "behavioral despair" interpretation [26], immobility behavior developed during FS has been suggested as a strategy for stress coping or saving energy [31,32] since, after

repeated swimming, animals become immobile faster and for a longer period. Some authors have also proposed exposure to FS stress as a tool to study negative behavioral states (apathy) related to other neuropsychiatric disorders such as schizophrenia [33]. In contrast, others suggested it to be a useful model for depression-related enhanced learning of aversive memories [34,35]. Our data corroborate studies suggesting that the immobility developed during a single swim session differs from the immobility seen after repeated exposure [27,36,37]. Recent works [38,39] proposed a new repeated FS stress protocol as an animal model of depression responsive to repeated treatment with classical antidepressants or acute fast-acting antidepressants like ketamine. Our results point to the immobility behavior developed after intermittent FS as a state resistant to classical antidepressants acute effects. Since several animal models of depression use repeated exposure to stressful situations [40,41], our data support the use of the high immobility developed after repeated FS as a useful tool for identifying novel atypical antidepressant drugs.

Some examples of FS stress-induced changes in psychoactive drug effects were already described. For example, repeated FS stress increased ethanol consumption in rodents and enhanced conditioned place preference for ethanol, nicotine, and cocaine [28,42,43]. Deutsch and coworkers [44] showed that cold FS stress reduced the antiseizure efficacy of MK-801 and memantine in mice. Similarly, Briones-Aranda and coworkers [45] described that 5-HT_{1A} agonists fail to induce anxiolytic effects in mice exposed to FS stress. It is recognized that stress hormones are inducers of hepatic enzymes (mainly phase I metabolizing enzymes), and this effect seems to depend on animals' species, and stressor evaluated [46]. Thus, the contribution of changes in pharmacokinetic parameters to the absence of antidepressants anti-immobility effects after stress here reported cannot be ruled out.

Nevertheless, only a few studies demonstrating stress-induced changes over antidepressants' effects have been described. Wongwitdecha and coworkers [47] showed that the effect of desipramine on the FS test was enhanced by isolation rearing stress. However, in this study, stressed animals already presented lower baseline immobility than control animals, thus the detected effects could be due to an increase in anxiety. Corroborating our findings, Branchi and coworkers [48] showed that fluoxetine (10 mg/kg/day for three weeks) could not reverse anhedonia in mice pre-exposed to repeated stress when animals were kept under stress during drug presentation. However, when mice were kept in an enriched environment after repeated stress exposure, the anti-anhedonic effect of fluoxetine was detected. Moreover, daily ACTH treatment inhibited the immobility-reducing effects of imipramine (10 to 30 mg/kg i.p. acute and repeatedly) and desipramine (30 mg/kg i.p. acute), leading to the proposal of this procedure as an animal model of treatment-resistant MDD [49]. Taking together, these data and our results support the hypothesis that exposure to stressful situations may decrease drug efficacy in MDD and highlight the importance of considering other conditions than drug adherence for achieving therapeutic success.

This hypothesis is reinforced by clinical data showing that self-reported stressful life events are associated with differential drug response in MDD patients and that patients with lower socioeconomic status are less responsive to antidepressants [50]. Studies showed that behavioral cognitive therapy could significantly increase MDD treatment efficacy, and part of this response may be attributed to a reduction of patients' exposure to stress [51]. In this way, our data reproduction with a higher number of drugs and using different kinds of stressors will clarify this putative causal relationship between stress and lack of antidepressant response.

In summary, our work shows that exposure to intermittent repeated stress inhibited the anti-immobility effect of imipramine and fluoxetine in mice. Pre-exposure to ketamine was unable to

block the stress-induced change in mice response to antidepressants. Considering that the anti-immobility effects of both imipramine and fluoxetine were detected in the classical Porsolt FS test, our results point to the existence of different physiological changes underlying the immobility behavior single or multiple swim sessions. Finally, our data corroborate findings demonstrating that exposure to stressful situations can blunt the response to antidepressant medication.

CRedit authorship contribution statement

Milene Borsoi: Conceptualization, Data curation, Investigation, Formal Analysis, Writing - original draft, Writing – review & editing.

Luis Eduardo D. Nunes: Data curation, Investigation, Formal Analysis, Visualization, Writing - original draft, Writing – review & editing.

Amanda R. Barbosa: Data curation, Investigation, Writing – original draft.

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Stela M. K. Rates: Conceptualization, Resources, Supervision, Funding acquisition, Writing – review & editing.

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Conflict of Interest

The authors declare no conflict of interest.

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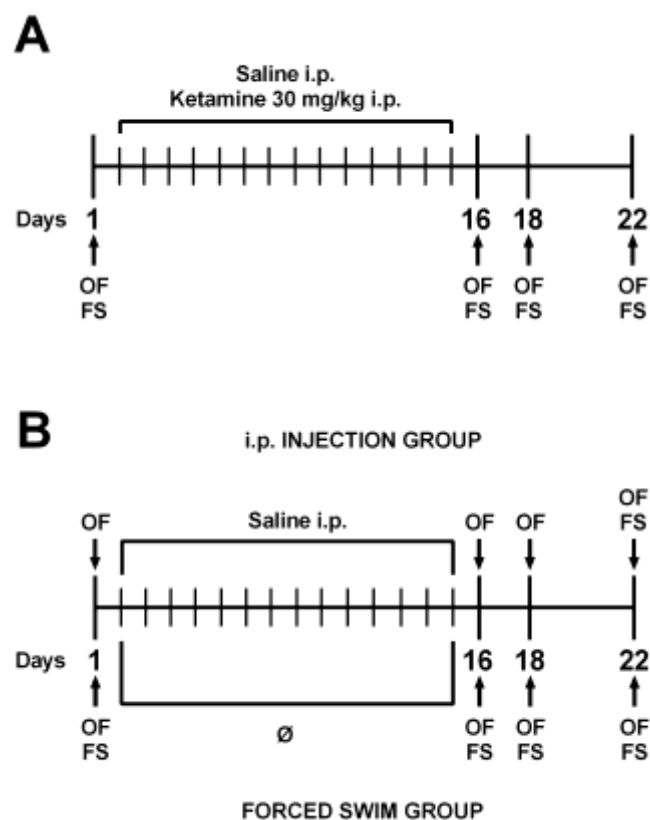


Fig 1 *Experimental design.* (A) Experiment 1: Imipramine and fluoxetine effects in mice pretreated with ketamine. (B) Experiment 2: Stressors influence in mice response to antidepressants.

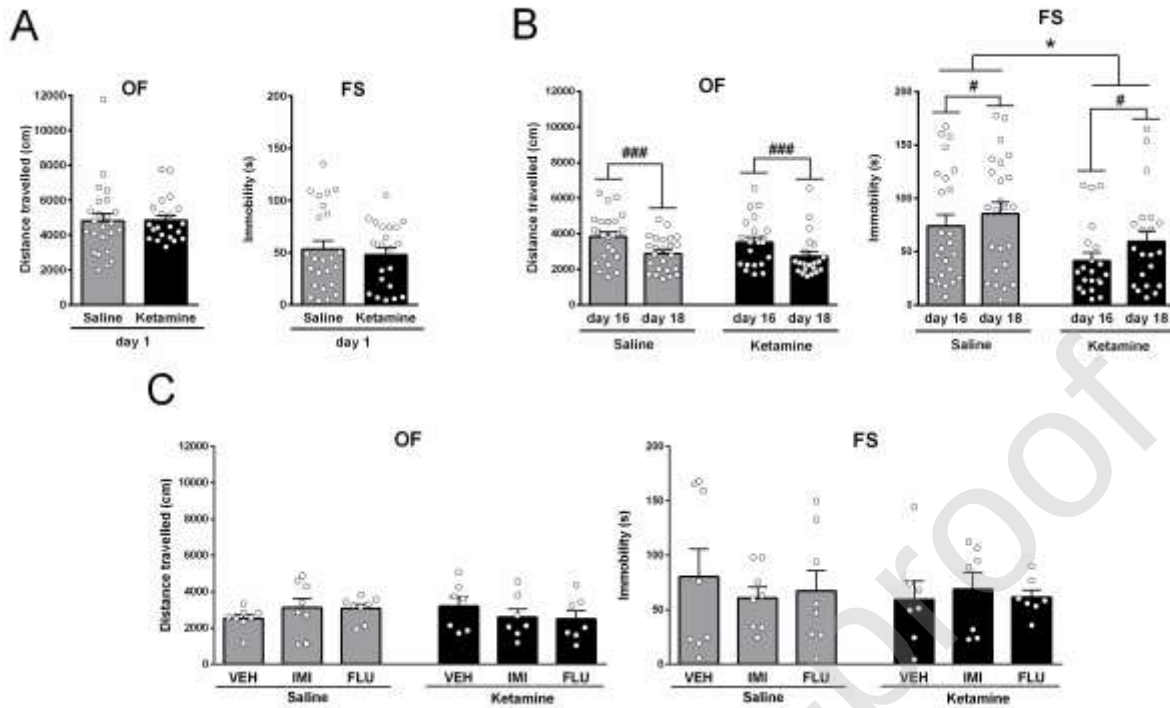


Fig 2 Imipramine (IMI, 20 mg/kg p.o.) and fluoxetine (FLU, 30 mg/kg p.o.) did not change the immobility behavior of mice pretreated with saline or ketamine (30 mg/kg/day i.p. for 14 days) and repeatedly forced to swim. Results are expressed as mean $\pm$ SEM. (A) Baseline distance traveled (cm) and immobility (s) of mice measured on day 1 ($n = 21-24$). (B) Ketamine did not change locomotion and did not prevent the immobility time increase in animals repeatedly forced to swim ($n = 21-24$). * $p < 0.05$ at Tukey posthoc test comparing saline vs ketamine groups; # $p < 0.05$, ### $p < 0.001$ at Tukey posthoc test comparing day 16 vs day 18. (C) Imipramine and fluoxetine did not change locomotion or immobility time in both saline and ketamine pretreated mice ($n = 07-08$).

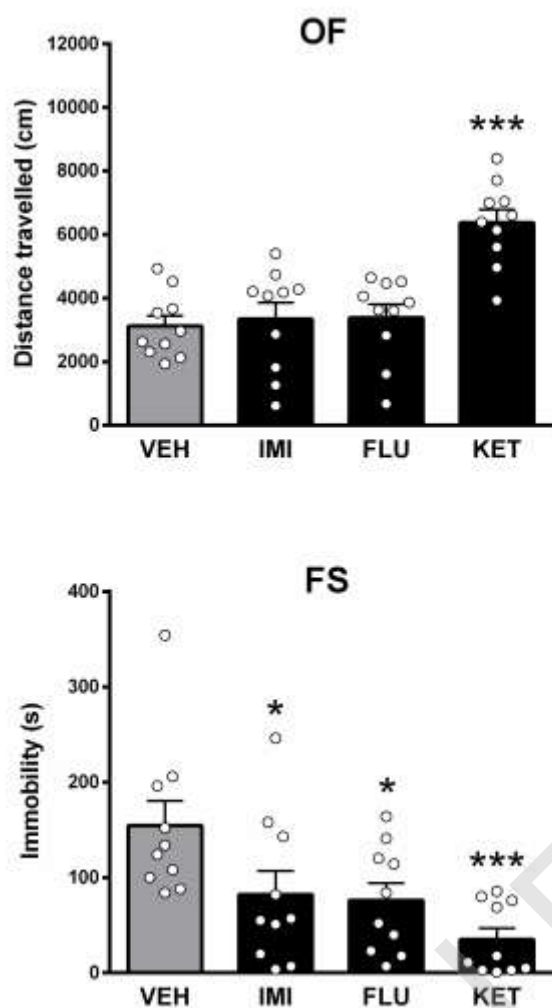


Fig 3 Effects of imipramine (IMI, 20 mg/kg p.o.), fluoxetine (FLU, 30 mg/kg p.o.) and ketamine (KET, 30 mg/kg i.p.) on mice locomotion (OF) and Porsolt's forced swimming test (FS). Results are expressed as mean $\pm$ SEM (n = 10). * p < 0.05, *** p < 0.001 at Tukey *post-hoc* test vs vehicle group.

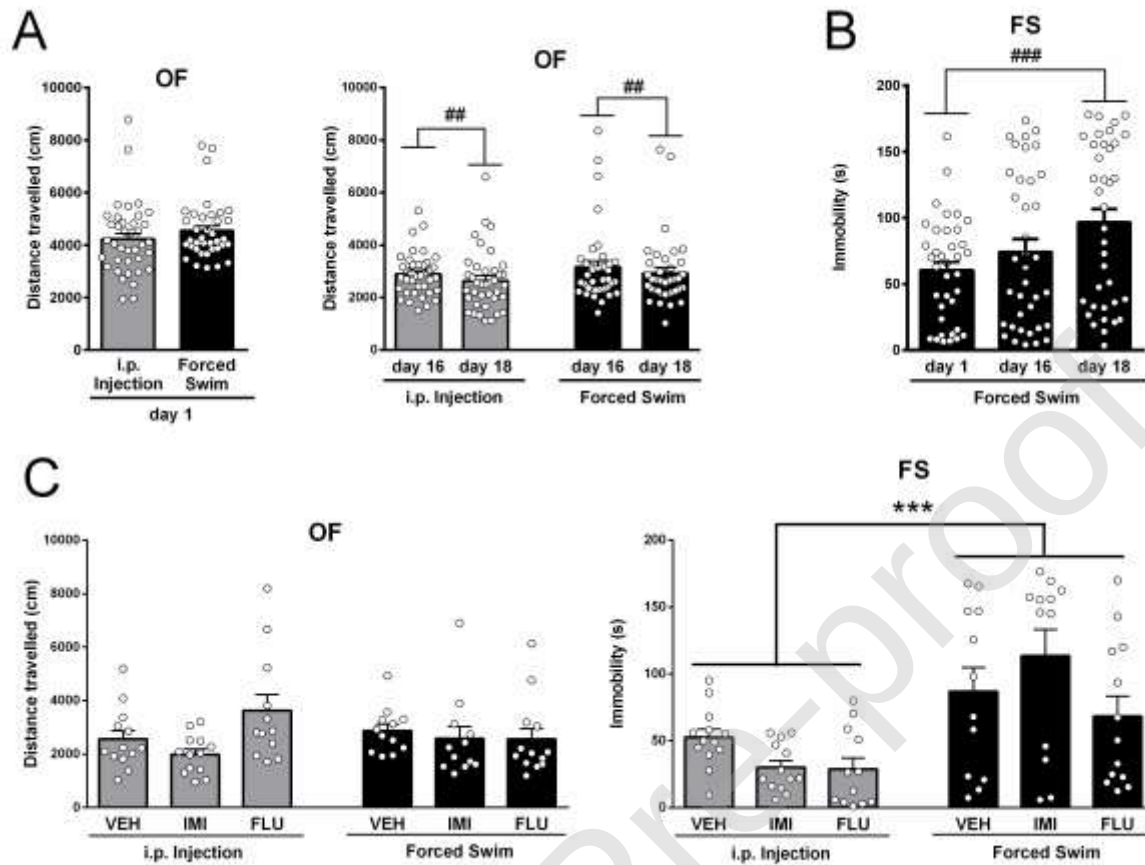


Fig 4 Imipramine (IMI, 20 mg/kg p.o.) and fluoxetine (FLU, 30 mg/kg p.o.) did not change the behavior of mice pre-exposed to repeated i.p. injection or intermittent repeated FS stress. Results are expressed as mean $\pm$ SEM. (A) Total distance traveled (cm) at baseline and after 14 days of i.p. injection or remaining undisturbed (forced swim group) ($n = 37-38$). ### $p < 0.01$ at Tukey posthoc test comparing day 16 vs day 18. (B) Immobility time at baseline and after 14 days remaining undisturbed (forced swim group) ($n = 37$). ### $p < 0.001$ at Tukey posthoc test comparing baseline vs day 18. (C) Imipramine and fluoxetine did not change locomotion or immobility time in both i.p. injection and intermittent repeated forced swim pre-exposed mice ($n = 12-13$). *** $p < 0.001$ at Tukey posthoc test comparing i.p. injection vs. forced swim groups.