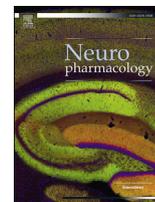




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Ketamine treatment involves medial prefrontal cortex serotonin to induce a rapid antidepressant-like activity in BALB/cJ mice

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ABSTRACT

Unlike classic serotonergic antidepressant drugs, ketamine, an NMDA receptor antagonist, exhibits a rapid and persistent antidepressant (AD) activity, at sub-anaesthetic doses in treatment-resistant depressed patients and in preclinical studies in rodents. The mechanisms mediating this activity are unclear. Here, we assessed the role of the brain serotonergic system in the AD-like activity of an acute sub-anaesthetic ketamine dose. We compared ketamine and fluoxetine responses in several behavioral tests currently used to predict anxiolytic/antidepressant-like potential in rodents. We also measured their effects on extracellular serotonin levels $[5\text{-HT}]_{\text{ext}}$ in the medial prefrontal cortex (mPFCx) and brainstem dorsal raphe nucleus (DRN), a serotonergic nucleus involved in emotional behavior, and on 5-HT cell firing in the DRN in highly anxious BALB/cJ mice. Ketamine (10 mg/kg i.p.) had no anxiolytic-like effect, but displayed a long lasting AD-like activity, i.e., 24 h post-administration, compared to fluoxetine (18 mg/kg i.p.). Ketamine (144%) and fluoxetine (171%) increased mPFCx $[5\text{-HT}]_{\text{ext}}$ compared to vehicle. Ketamine-induced AD-like effect was abolished by a tryptophan hydroxylase inhibitor, *para*-chlorophenylalanine (PCPA) pointing out the role of the 5-HT system in its behavioral activity. Interestingly, increase in cortical $[5\text{-HT}]_{\text{ext}}$ following intra-mPFCx ketamine bilateral injection (0.25 $\mu\text{g}/\text{side}$) was correlated with its AD-like activity as measured on swimming duration in the FST in the same mice. Furthermore, pre-treatment with a selective AMPA receptor antagonist (intra-DRN NBQX) blunted the effects of intra-mPFCx ketamine on both the swimming duration in the FST and mPFCx $[5\text{-HT}]_{\text{ext}}$ suggesting that the AD-like activity of ketamine required activation of DRN AMPA receptors and recruited the prefrontal cortex/brainstem DRN neural circuit in BALB/c mice. These results confirm a key role of cortical 5-HT release in ketamine's AD-like activity following the blockade of glutamatergic NMDA receptors. Tight interactions between mPFCx glutamatergic and serotonergic systems may explain the differences in this activity between ketamine and fluoxetine *in vivo*.

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1. Introduction

Ketamine, a non-competitive, glutamatergic *N*-methyl-D-aspartate receptor (NMDA-R) antagonist that binds to the phencyclidine site within this ionotropic Ca^{2+} channel, has been found to relieve symptoms within hours when administered at sub-anaesthetic

doses in treatment-resistant depressed patients (Berman et al., 2000). Since this discovery, many studies have confirmed ketamine's efficacy in humans as well as in animals. However, the mechanism of action underpinning this rapid antidepressant response in animal models still remains largely unknown.

Preclinical studies with ketamine mainly focused on the glutamatergic system. Thus, ketamine was described as a powerful antagonist at NMDA receptors (elimination half-life < 1 h; *in vitro* $\text{EC}_{50} = 760 \text{ nM}$; *in vivo* $\text{ED}_{50} = 4.4 \text{ mg/kg}$) (Lord et al., 2013; Murray et al., 2000). Antagonism of NMDA-R could be the key pharmacological feature underlying the rapid antidepressant effect of a low dose of ketamine (Krystal et al., 2013). However, the neurochemical mechanisms underlying this response are likely to be more

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Abbreviations[5-HT]_{ext} extracellular serotonin level

5-HT serotonin

8-OHDPAT 8-Hydroxy-*N,N*-dipropyl-2-aminotetralin

aCSF artificial cerebrospinal fluid

BDNF brain-derived neurotrophic factor

DA dopamine

DRN dorsal raphe nucleus

EPM elevated plus maze

FST forced swim test

i.p. intraperitoneal

mPFCx medial prefrontal cortex

MDD major depressive disorder

mTOR mammalian target of rapamycin

NBQX 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[*f*]quinoxaline-2,3-dione

NSF novelty suppressed feeding

OF open field

PCP phencyclidine

PCPA *para*-chlorophenylalanine

TPH tryptophan hydroxylase

SERT serotonin transporter

SSRIs selective serotonin reuptake inhibitors

NMDA-R glutamatergic NMDA receptor

NMDR-2A/2B glutamatergic NMDA receptor subunit 2A/2B

VTA ventral tegmental area

WAY100635 *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridyl)-cyclohexanecarboxamide

complex than a selective blockade of NMDA-R (Naughton et al., 2014). Its pharmacology has shown affinities (*and functional activity*) for PCP-site located on NMDA-R (0.5 μ M; *antagonist*), NMDR-2A, NMDR-2B binding sites, but also for non-glutamatergic neurotransmitter receptors [sigma-1 receptor (*agonist*), muscarinic, μ opioid receptor, dopamine D₂ receptor (0.5 μ M), 5-HT₂ receptor (*in vitro* 15 μ M)] (Kapur and Seeman, 2002). Thus, the fast antidepressant effect of ketamine may involve non-selective multi-system changes, including the serotonergic system, *via* direct and indirect effects (Kapur and Seeman, 2002). Indeed, recently, an *in vivo* microdialysis study performed in the prefrontal cortex of awake monkeys showed an increase in extracellular serotonin (5-HT) levels after acute ketamine injection (Yamamoto et al., 2013). Although functional interactions between glutamate and monoamines are well documented, surprisingly, an acute ketamine administration did not affect the firing activity of serotonin and dopamine neurons in rats (El Iskandrani et al., 2015).

Although ketamine antidepressant-like effects have been assessed, no study performed a behavioral characterization from the antidepressant-like effects to the anxiolytic-like effects.

The medial prefrontal cortex (mPFCx) plays a key role in ketamine's pharmacological effects, because NMDA-R, the main target with highest affinity to ketamine (Murray et al., 2000), is widely expressed in this brain region (Kamiyama et al., 2011; Sanz-Clemente et al., 2013). Artigas's group demonstrated that 5-HT release in the mPFCx depends on the excitatory glutamatergic transmission (Lopez-Gil et al., 2012). Moreover, it has been shown that mPFCx projections to the dorsal raphe nucleus (DRN) control stressful behavior (Amat et al., 2016). Indeed, a recent study demonstrated that the mPFCx is an important brain region in which deep brain stimulation produced the most profound antidepressant effects on a variety of depressive-like behavioral tests in rats (Lim et al., 2015). Thus, here, we hypothesized that serotonergic efflux in the mPFCx/DRN circuit can play a role, at least partially, in ketamine-induced rapid/long lasting antidepressant-like activity in rodents.

First, our study aimed to perform a behavioral characterization of putative long lasting anxiolytic/antidepressant-like effects ketamine (3 or 10 mg/kg, *i.p.*, 24hr before testing), in comparison to fluoxetine (18 mg/kg, *i.p.*, 24hr before testing) in male BALB/cj mice using different behavioral paradigms predictive of anxiolytic- or antidepressant-like activity. Second, we investigated the role of the serotonergic component in ketamine -induced changes in behavioral activity using *para*-chlorophenylalanine (PCPA)-induced serotonin depletion in the DRN and also following serotonin release

as measured in the mPFCx using *in vivo* microdialysis under the same experimental conditions as behavioral tests. Finally, we challenged the effects of local intra-mPFCx ketamine administration in the microdialysis and the FST measured in the same animals, and then extending to a combination with intra-DRN administration of AMPA receptor antagonist NBQX to clarify the implication of mPFCx/DRN neural circuit. The present experimental strategy offers the possibility of linking ketamine's antidepressant/anxiolytic activity to the serotonergic system in regard to the behavioral and neurochemical levels, giving furthermore persuasive evidence for the implication of a serotonergic pathway in ketamine's antidepressant mechanism.

2. Materials and methods**2.1. Animals**

Male BALB/cj mice (7–8-weeks old) weighing 23–25 g at the beginning of the experiments were purchased from Janvier Labs (Le Genest-Saint-Isle). The BALB/cj strain of mice was chosen for its baseline anxiety phenotype (Dulawa et al., 2004). They were housed in groups of five in a temperature (21 \pm 1 $^{\circ}$ C) controlled room with a 12 h light: 12 h dark cycle (lights on at 06:00 h). Food and water were available *ad libitum* except during behavioral observations. Particular efforts were made to minimize the number of mice used in the experiments. Protocols were approved by the Institutional Animal Care and Use Committee in France (Council directive # 87–848, October 19, 1987, "Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permissions # 92–196" to A.M.G.) as well as with the European directive 2010/63/EU.

2.2. Drugs and treatments

Ketamine (3 or 10 mg/kg) purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France) and fluoxetine hydrochloride (18 mg/kg) purchased from Anawa Trading (Zurich, Switzerland) were dissolved in vehicle (NaCl 0.9%) and administered 24 h prior to the behavioral tests. Drug doses and pre-treatment times were based on previous studies performed either in our laboratory for fluoxetine (David et al., 2009) or in the literature for ketamine (Li et al., 2010; Liu et al., 2012; Iijima et al., 2012; Koike et al., 2013; Zanos et al., 2015). Diazepam (1.5 mg/kg, *i.p.*, 30 min before testing) was used as a positive control, in animal paradigm predictive of anxiolytic-like effects (David et al., 2007). *Para*-chlorophenylalanine methyl ester

(PCPA, 150 mg/kg, i.p.) purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France), was dissolved in Tween 1% solution and administered twice daily (at 9:00 and 17:00) for 3 consecutive days (Fukumoto and Chaki, 2015). Ketamine or fluoxetine were then administered 24 h after the final PCPA administration and behavioral tests occurred the following day. Immediately following these tests, the animals were sacrificed and the frontal cortex was dissected for 5-HT measurements to verify the depletion of tissue content induced by PCPA as previously described (Musumeci et al., 2015). Tissue content of 5-HT was determined using an ELISA kit from ImmunoSmol (Pessac, France).

To study the mechanism underlying the serotonergic effects of ketamine, we tried to dissect the responsible neural circuits linking the mPFCx to the DRN. Thus, we performed an experiment using intra-DRN injection of NBQX, an AMPA receptor antagonist at 300 μ M (NBQX disodium salt purchased from Tocris Bioscience, Lille, France) and measured two responses, FST and microdialysis in the same animal. This dose was chosen according to Lopez-Gil et al., 2007 and Fukumoto et al., 2016. NBQX was injected 30 min before a bilateral intra-mPFCx ketamine injection (0.5 μ g). Then, dialysate samples were collected in the mPFCx 24 h after ketamine injection and the swimming duration in the FST was measured in these mice when dialysates were collected as in the protocol used in Fig. 4.

2.3. Behavioral assessment

For each behavioral tests of anxiolytic/antidepressant-like activity, a different cohort of BALB/cj mice was tested. Behavioral testing occurred during the light phase between 07:00 and 19:00.

2.3.1. Open field (OF) test

OF was performed as described previously (Dulawa et al., 2004). Briefly, motor activity was quantified in four Plexiglas open field boxes 43×43 cm² (MED Associates, Georgia, VT). Two sets of 16 pulse-modulated infrared photobeams on opposite walls 2.5 cm apart, recorded x-y ambulatory movements. Activity chambers were computer interfaced for data sampling at 100 ms resolution. The computer defined grid lines dividing centre and surround regions, with the centre square consisting of four lines 11 cm from the wall. The animals were tested for 10 min to measure the total time spent and the numbers of entries into the centre and the distance travelled in the centre divided by total distance travelled.

2.3.2. Elevated plus maze (EPM) test

The elevated plus maze (EPM) is a widely used behavioral assay for rodents and it has been validated to assess the anti-anxiety effects of pharmacological agents (for review Wolf and Frye, 2007). This test was performed as described by Mendez-David et al., 2014. The maze is a plus-shaped apparatus, with two open arms and two arms closed by walls linked by a central platform 50 cm above the floor. Mice were individually put in the centre of the maze facing an open arm and were allowed to explore the maze during 5 min. The time spent in and the number of entries into the open arms were used as an anxiety index. All parameters were measured using a videotracker (EPM3C, Bioseb, Vitrolles, France).

2.3.3. Novelty suppressed feeding (NSF) paradigm

The NSF is a conflict test that elicits competing motivations: the drive to eat and the fear of venturing into the centre of a brightly lit arena. The latency to begin eating is used as an index of anxiety/depression-like behavior, because classical anxiolytic drugs as well as chronic antidepressants decrease this measure. The NSF test was carried out during a 10-min period as previously described David et al., 2009. Briefly, the testing apparatus consisted of a

plastic box (50 \times 50 \times 20 cm), the floor of which was covered with approximately 2 cm of wooden bedding. Twenty-four hours prior to behavioral testing, all food was removed from the home cage. At the time of testing, a single pellet of food (regular chow) was placed on a white paper platform positioned in the centre of the box. Each animal was placed in a corner of the box, and a stopwatch was immediately started. The latency to eat (defined as the mouse sitting on its haunches and biting the pellet with the use of fore-paws) was measured. Immediately afterwards, the animal was transferred to its home cage, and the amount of food consumed by the mouse in the subsequent 5 min was measured, serving as a control for change in appetite as a possible confounding factor.

2.3.4. Splash test (ST)

This test was performed as previously described to assess antidepressant-like activity (David et al., 2009; Mendez-David et al., 2014). This test consisted in squirting a 10% sucrose solution on the mouse's snout. The sucrose solution dirtied the coat and induced a grooming behavior as previously shown (Ducottet and Belzung, 2004; Rainer et al., 2012). The grooming duration and latency of different behaviours (face, paws, hindquarter and shoulders) were directly recorded over a 5 min period.

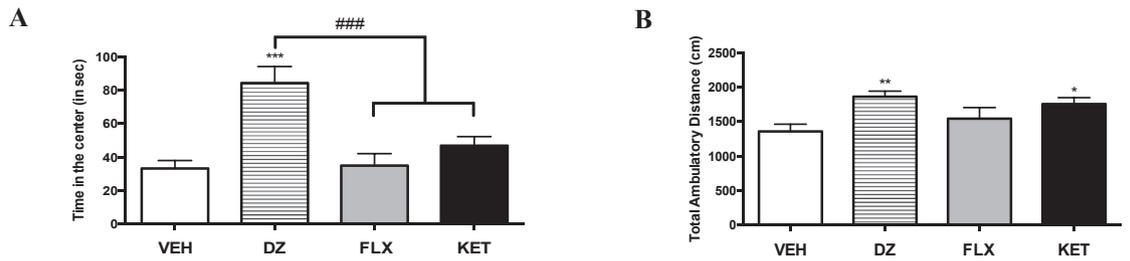
2.3.5. Forced swim test (FST)

The mouse forced swim test procedure (FST) is one of the most useful tools for antidepressants screening. Swimming, climbing and immobility behaviours were distinguished from each other according to the procedure previously described (Dulawa et al., 2004; Holick et al., 2008). Swimming behavior relies on the serotonergic system, and climbing behavior on the noradrenergic system in mouse (Holick et al., 2008). This was evidenced by the observation that desipramine, a norepinephrine reuptake inhibitor, reduces immobility duration increasing climbing behavior. In contrast, fluoxetine induced antidepressant-like effects by increasing the swimming behavior. Mice were placed individually into glass cylinders (height: 25 cm, diameter: 10 cm) containing 18 cm water, maintained at 23–25 °C for 6 min. The predominant behavior (swimming, immobility, or climbing: Holick et al., 2008) was scored for the last 4 min of the 6 min testing period using automated scoring was done using the automated X'PERT FST software (Bioseb, Vitrolles, France).

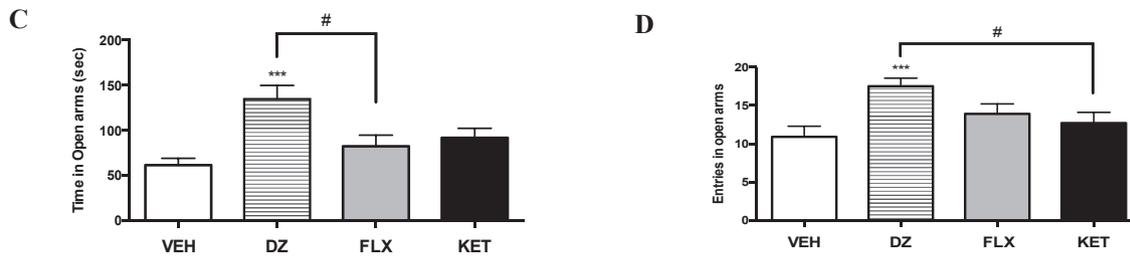
2.4. Intracerebral in vivo microdialysis

Each mouse was anesthetized with chloral hydrate (400 mg/kg, i.p.) and implanted with two microdialysis probes (CMA7 model, Carnegie Medicine, Stockholm, Sweden) located in the medial prefrontal cortex (mPFCx) and one microdialysis probe in the dorsal raphe nucleus (DRN). Stereotaxic coordinates in mm from bregma: mPFCx: A = +2.2, L = \pm 0.2, V = -3.4; DRN (with an angle of 15°): A = -4.5, L = +1.2, V = -4.7 (A, anterior; L, lateral; and V, ventral) (Calcagno and Invernizzi, 2010; Ferrer-Coy et al., 2013; Nguyen et al., 2013). On the same day, after awakening, mice received an acute ketamine dose, or fluoxetine, or their vehicle i.p. On the next day, \approx 24 h after ketamine administration, the probes were continuously perfused with an artificial cerebrospinal fluid (aCSF, composition in mmol/L: NaCl 147, KCl 3.5, CaCl₂ 2.26, NaH₂PO₄ 1.0, pH 7.4 \pm 0.2) at a flow rate of 1.0 μ l/min through the mPFCx and 0.5 μ l/min through the DRN using CMA/100 pump (Carnegie Medicine, Stockholm, Sweden), while animals were awake and freely moving in their cage. One hour after the start of aCSF perfusion stabilization period, four fractions were collected (one every 25 min) to measure the basal extracellular serotonin (5-hydroxytryptamine, [5-HT]_{ext}) levels in the mPFCx and DRN by using a high-performance liquid chromatography (HPLC) system

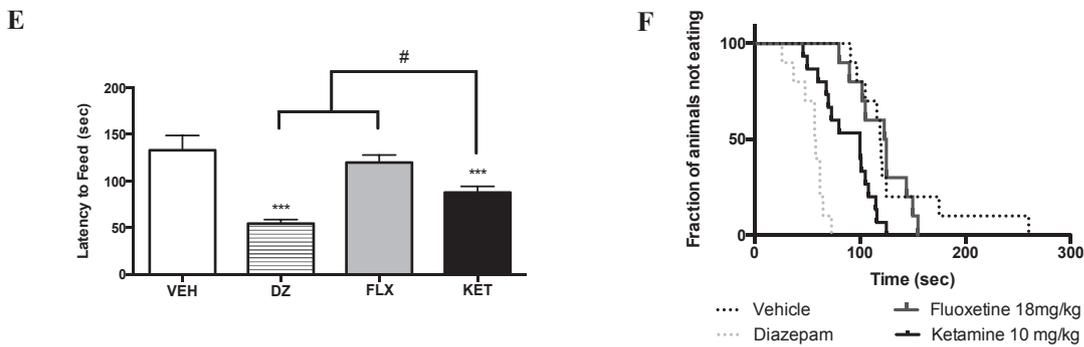
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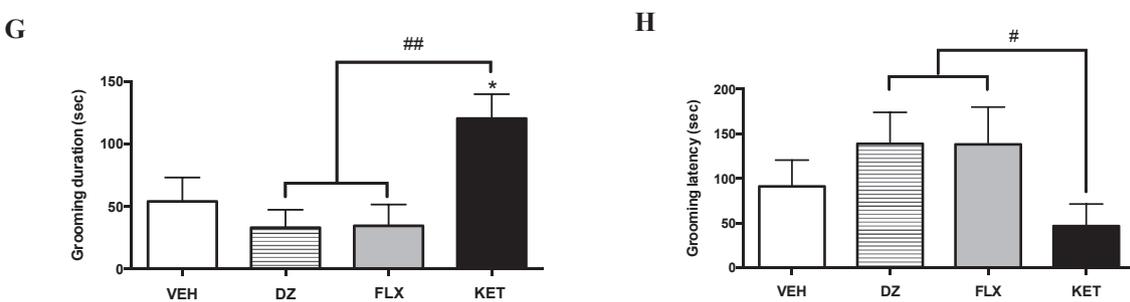
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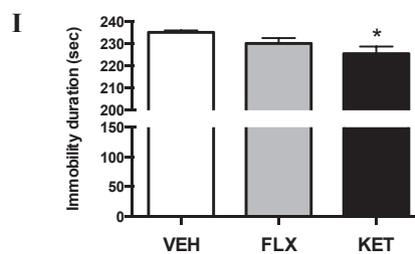
Novelty Suppressed Feeding



Splash Test



Forced Swim Test



(column Ultramex 3 μ C18, 75 \times 4.60 mm, particle size 3 μ m, Phenomenex, Torrance, CA) coupled to an amperometric detector (VT03; Antec Leyden, The Netherlands). AUC values (% of baseline) were also calculated during the sample collections as previously described (Nguyen et al., 2013). The limit of sensitivity for 5-HT was \approx 0.5 fmol/sample (signal-to-noise ratio = 2). At the end of the experiments, localization of microdialysis probes was verified histologically (Bert et al., 2004). To clarify the specific role of the mPFCx-DRN circuit, we also performed microdialysis and behavioral experiments in which ketamine (0.1 or 0.5 μ g, i.e., \approx 500 μ M or 2.5 mM, respectively) or fluoxetine (0.5 μ g) were dissolved in the aCSF and perfused locally at 0.2 μ l/min into the mPFCx (bilateral) or DRN for 2 min via a silica catheter glued to the microdialysis probe 24 h prior to the tests. The concentration of ketamine was chosen according to Lopez-Gil et al., 2012, showing that a bilateral perfusion of ketamine 3 mM into the mPFCx produced a significant increase in local extracellular 5-HT levels in rats. The FST was performed when the microdialysis procedure still continued.

2.5. In vivo electrophysiological recordings

Twenty-four hours after a single administration of ketamine (10 mg/kg, i.p.) or fluoxetine (18 mg/kg, i.p.), mice were anesthetized with chloral hydrate (400 mg/kg, i.p.) and placed in a stereotaxic frame (using the David Kopf mouse adaptor) with the skull positioned horizontally. The extracellular recordings were performed using single glass micropipettes (R&D Scientific Glass, USA) for recordings in the DRN. Micropipettes were preloaded with fibreglass strands to promote capillary filling with a 2 M NaCl solution. Recording of DRN 5-HT neurons: Single glass micropipettes pulled on a pipette puller (Narishige, Japan) with impedances ranging from 2.5 to 5 mV, were positioned 0.2–0.5 mm posterior to the interaural line on the midline and lowered into the DRN, usually reached at a depth of 2.5–3.5 mm from the brain surface. Spontaneously active DRN 5-HT neurons were then identified according to the following criteria: a slow (0.5–2.5 Hz) and regular firing rate and a long-duration, positive action potential. Neurons were recorded for 2 min and data were expressed as the mean \pm SEM of firing rate from all 5-HT neurons encountered during the different tracts. In Supplemental Fig. S1, we show an example of the electrophysiological effects of cumulative doses of ketamine (1–5 mg/kg, i.p.) in an anesthetized mouse on the spontaneous activity of 5-HT neurons as well as the effects of WAY 100635. After ensuring the stability of the recording, ketamine was injected, and the degree of change in firing was observed upon stabilization.

2.6. Statistics

All experimental results are given as the mean \pm SEM. Data were analysed using Prism 6 software (GraphPad). The analyses of the behavioral data, the comparisons between groups were performed using one-way ANOVA followed by Fisher's PLSD *post hoc* analysis. In the NSF test, we used the Kaplan–Meier survival analysis owing to the lack of normal distribution of the data. Mantel–Cox log rank test was used to evaluate differences between experimental groups. A summary of statistical measures is included in Supplementary Table S2. Statistical significance was set at $p \leq 0.05$. A two-way

ANOVA with pre-treatment (Vehicle vs NBQX) and treatment (Vehicle vs Ketamine) factors was also used followed by Fisher's PLSD *post hoc* test.

3. Results

3.1. Behavioral characterization of the anxiolytic/antidepressant-like activity of an acute ketamine dose in BALB/cj mice

The anxiolytic-like responses elicited by ketamine administered 24 hrs before testing, were assessed in the Open Field (OF) and Elevated Plus Maze (EPM) tests. In both tests, diazepam (1.5 mg/kg, i.p.) had a marked effect on all anxiety parameters, resulting in an increased time spent in the centre, total ambulatory distance in the OF (Fig. 1A, B), time in open arms and entries into open arms in the EPM (Fig. 1C, D). Fluoxetine (18 mg/kg, i.p.) had no significant effects in both tests, while ketamine (10 mg/kg, i.p.) had only a significant effect on the total ambulatory distance (* $p < 0.05$, ANOVA one-way) in the OF (Fig. 1B, C). These data suggest that ketamine is devoid of long lasting anxiolytic-like activity in BALB/cj mice.

We further examined the antidepressant-like activity of ketamine in these mice using the Novelty Suppressed Feeding (NSF) test, the Splash Test (ST) and the Forced Swim Test (FST). Unlike diazepam, the most effective drug decreasing the latency to feed in the NSF, unlike fluoxetine, ketamine significantly decreased the latency to feed (Fig. 1E, F). In the ST, ketamine induced a significant increase in grooming duration (Fig. 1G) and a decrease in grooming latency (Fig. 1H), compared to diazepam and fluoxetine, which had no effects. In the FST, ketamine significantly decreased the immobility duration, while fluoxetine did not (Fig. 1I). These results show that a systemic administration of a low dose of ketamine displays a long lasting antidepressant-like activity compared to fluoxetine in BALB/cj mice.

3.2. Serotonergic parameters of response to ketamine: increases in the swimming duration in the FST after ketamine correlated with changes in extracellular 5-HT levels in the mPFCx and firing rate of DRN 5-HT neurons in mice

3.2.1. Swimming 5-HT behavior

Activation of the brain serotonergic system in rodents is known to mediate increases in swimming duration in the FST (Dulawa et al., 2004). In the FST, ketamine, but not fluoxetine, induced a significant increase in the swimming duration (Fig. 2A, B). According to Page et al., 1999, it suggests that the antidepressant-like activity of ketamine on FST-induced immobility at this time point requires endogenous 5-HT in BALB/cj mice.

3.2.2. Dialysate 5-HT levels

To study the mechanism underlying this behavioral effect, changes induced by a systemic administration of ketamine (3 or 10 mg/kg) on [5-HT]_{ext} in the mPFCx and DRN were evaluated by using intracerebral *in vivo* microdialysis. Since ketamine (3 mg/kg) did not change mPFCx and DRN [5-HT]_{ext} (Table S1), we measured the serotonergic effects of the 10 mg/kg dose only. In the mPFCx (Fig. 2C, D), both drugs ketamine (144%) and fluoxetine (171%) increased mPFCx [5-HT]_{ext} compared to vehicle. In the DRN (Fig. 2E,

Fig. 1. Unlike fluoxetine, acute systemic administration of ketamine, 24 h before testing induced long lasting antidepressant-like activity but not anxiolytic-like effects. Anxiolytic/antidepressant-like activity of a single dose of ketamine (KET, 10 mg/kg, i.p.) compared to acute diazepam (DZ, 1.5 mg/kg, i.p.) or fluoxetine (FLX, 18 mg/kg, i.p.). Values plotted are mean \pm S.E.M. (n = 10 per group). Effects of drugs on: (A, B) time in the centre and total ambulatory distance in the OF; (C, D) time and entries in open arms in the EPM; mean of latency to feed in seconds \pm S.E.M. and (E) cumulative survival of mice that have not eaten over 10 min in the NSF; (G) grooming duration and (H) grooming latency in the ST; (I) immobility duration in the FST. Values plotted are mean \pm S.E.M. (n = 10 per group). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ significantly different from vehicle-treated group (VEH); # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ significantly different from diazepam- or fluoxetine-treated group (One-way ANOVA).

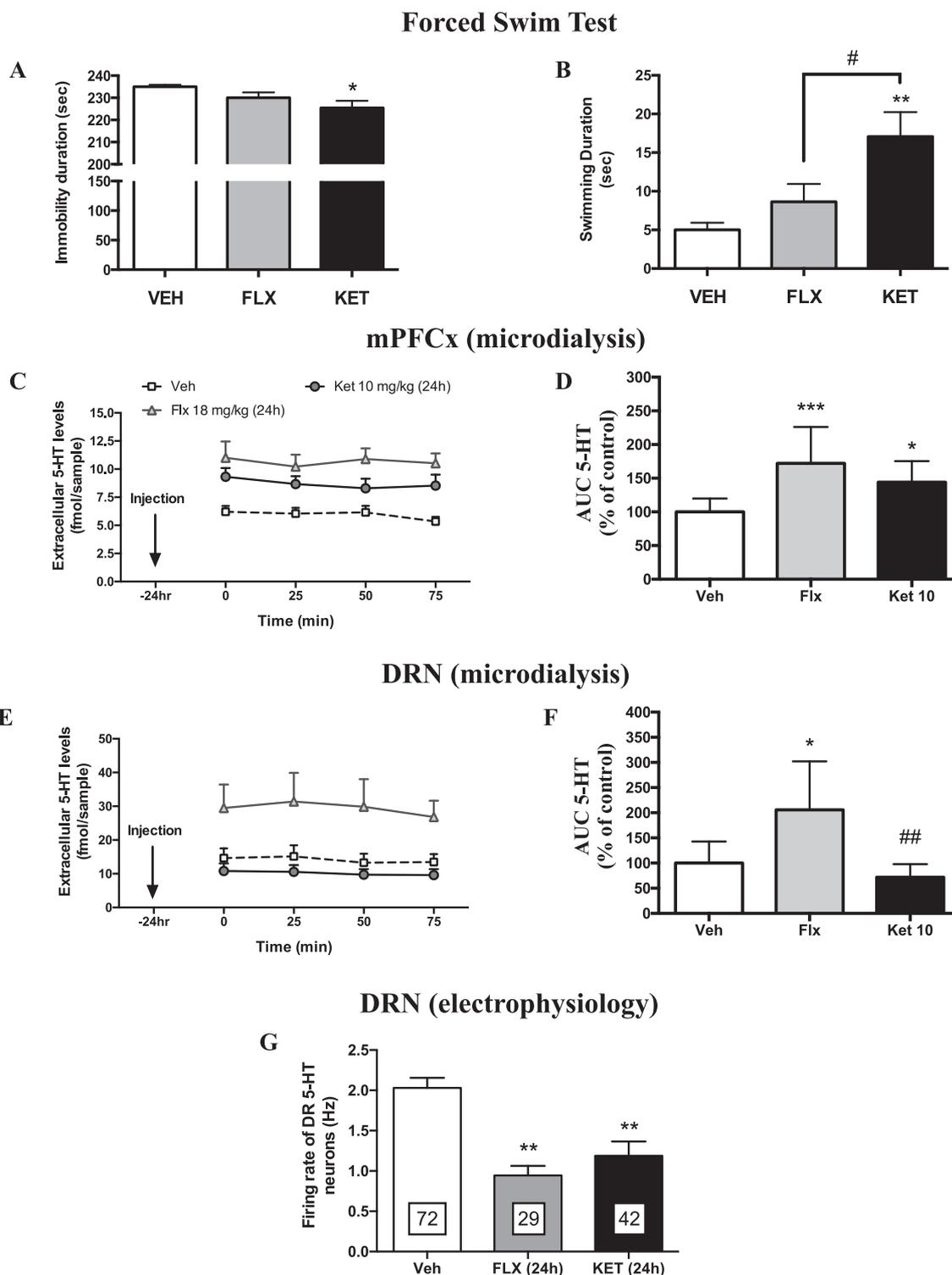


Fig. 2. Ketamine induced increase swimming behavior in the FST is related to enhanced extracellular 5-HT levels in the mPFCx in BALB/cj mice. Antidepressant-like activity of ketamine (10 mg/kg, i.p.) on (A) the immobility and (B) swimming duration in the FST, compared with the vehicle- and fluoxetine (18 mg/kg, i.p.)-treated group ($n = 10$ /group). (C, E) Time course. Values are mean \pm S.E.M. of [5-HT]_{ext} in the mPFCx and DRN expressed in fmol/sample following exposure to either vehicle, ketamine or fluoxetine. (D, F) Mean \pm S.E.M. of AUC values were calculated for the amount of 5-HT outflow collected during 0–75 min, and expressed as percentages of vehicle. (G) Frequency (Hz) of 5-HT neurons recorded in the DRN of mice administered ketamine (10 mg/kg, i.p.) or fluoxetine (18 mg/kg, i.p.). The numbers within the histograms indicate the number of neurons recorded. All the values are expressed as mean \pm S.E.M. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ significantly different from vehicle-treated group (Veh). # $p < 0.05$; ## $p < 0.01$ significantly different from fluoxetine-treated group (Flx).

F), fluoxetine (205%), but not ketamine, increased $[5\text{-HT}]_{\text{ext}}$ compared to vehicle. These results indicate that the increased serotonergic activity induced by ketamine and fluoxetine are similar in the mPFCx, but their effects are different in the DRN in BALB/cj mice.

3.2.3. Firing rate of DRN 5-HT neurons

To determine whether an acute administration of ketamine or fluoxetine can induce persistent changes in serotonergic activity, the firing rate of DRN 5-HT neurons was also measured (Fig. 2G). The DRN 5-HT neuronal activity was significantly reduced by 53% and 45% in fluoxetine and ketamine injected mice, respectively, compared to vehicle. Such decreases in DRN 5-HT cell firing suggest that, especially for ketamine, the antidepressant-like effect measured in the FST is not driven by an increased activity at the cell body level. The mPFCx contains different populations of serotonin neurons. Using whole-cell recordings in mPFCx slices, it was found that 5-HT dose-dependently increased the firing of fast spiking interneurons and decreased the firing of pyramidal neurons (Zhong and Yan, 2011). In addition, subanesthetic doses of ketamine selectively enhanced serotonergic neurotransmission in the mPFCx by inhibition of SERT activity (Yamamoto et al., 2013). Fluoxetine also induced a concentration-dependent increase in the excitability of interneurons, but had little effect on pyramidal neurons. These data suggest that the excitability of different neuronal populations in the mPFCx is tightly regulated by 5-HT. Thus, ketamine may also induce a global increase in the excitability of cortical neurons, but by a different mechanism of action than fluoxetine.

3.3. Effects of 5-HT depletion by PCPA on ketamine antidepressant-like activity in the FST

Depletion of serotonin by a pre-treatment with *p*-chlorophenylalanine (PCPA), a tryptophan hydroxylase (TPH) inhibitor, prevented SSRI-induced increases in swimming duration in the FST (Page et al., 1999). Pre-treatment with PCPA caused an average 79% decrease in the 5-HT content in the frontal cortex in mice, compared with a vehicle-treated group (Table S3). Ketamine significantly reduced the immobility time (Fig. 3A) and increased the swimming duration (Fig. 3B) in the FST in vehicle-pre-treated

group ($*p < 0.05$ vs vehicle, ANOVA two-way). Changes in these parameters were blocked by pre-treatment with PCPA, while PCPA alone did not affect the immobility time, nor the swimming duration. These data again suggest that the antidepressant-like activity of ketamine requires an activation of the serotonergic neurotransmission in BALB/cj mice.

3.4. Effects of local bilateral ketamine intra-mPFCx injection on extracellular 5-HT levels in the mPFCx and swimming behavior in the FST in the same BALB/cj mice

To reveal the specific role of the mPFCx in the antidepressant-like activity of ketamine, we injected ketamine or fluoxetine (0.25 μg each side) dissolved in the aCSF bilaterally into the mPFCx. Then, 24 h after bilateral intra-mPFCx drugs injection, we measured the swimming duration for 6 min (at t60) in the FST while microdialysis samples were collected for 120 min. Ketamine 0.1 μg had no effect (data not shown). At 0.5 μg , it decreased the immobility duration due to an increase in swimming duration (Fig. 4A, B), and increased $[5\text{-HT}]_{\text{ext}}$ (AUC values by 157%) in the mPFCx compared to vehicle (Fig. 4C, D). By contrast, fluoxetine failed to alter both swimming duration and mPFCx $[5\text{-HT}]_{\text{ext}}$.

In addition, ketamine-induced increases in mPFCx $[5\text{-HT}]_{\text{ext}}$ correlated with its antidepressant-like activity, i.e., with increases in swimming duration in the FST (Fig. 4E). Although a correlation was found in the fluoxetine-treated group, the pattern of the correlation observed for both group are completely different: the range of values of swimming duration following intra-mPFCx fluoxetine injection is lower than that found in the ketamine-treated animals. This could be related to the absence of efficacy of local acute fluoxetine treatment in the FST parameters and in the mPFCx $[5\text{-HT}]_{\text{ext}}$.

Since a single intra-mPFC ketamine injection increased the swimming duration and mPFCx $[5\text{-HT}]_{\text{ext}}$ (Fig. 4), we tried to dissect the responsible neural circuit for this response by using an AMPA receptor antagonist locally injected into the DRN. We found that NBQX prevented the effects of intra-mPFCx ketamine injection on both the immobility (Fig. 5A) and swimming duration (Fig. 5B) in the FST, and blunted the effects of ketamine on $[5\text{-HT}]_{\text{ext}}$ in the same mice (Fig. 5C). AUC 5-HT values increased by only 31% in the

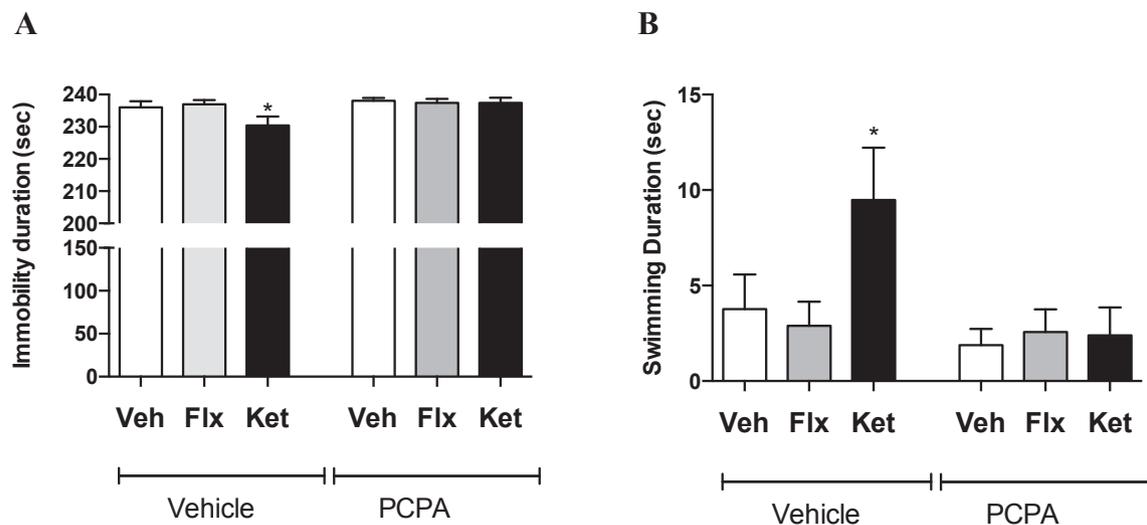
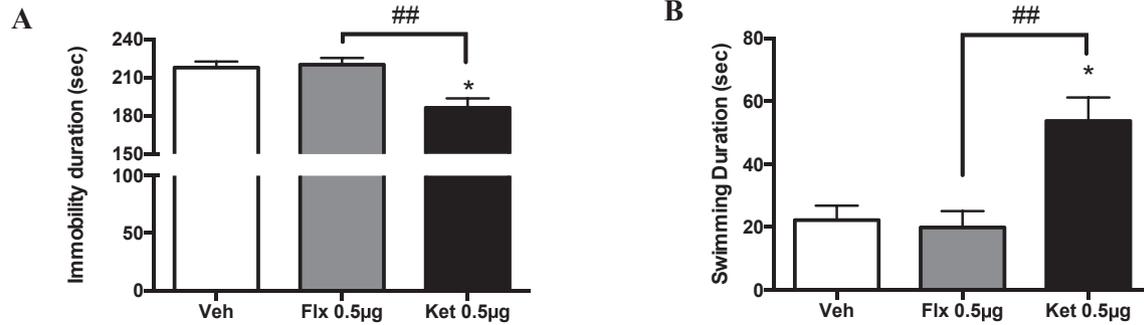
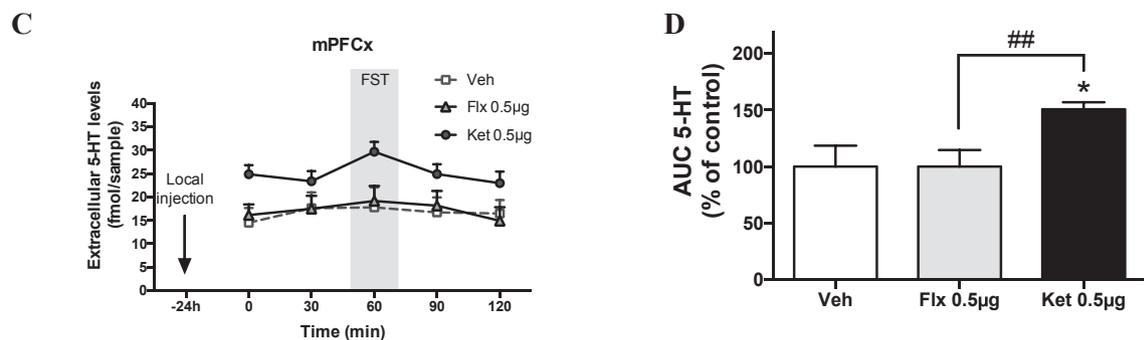


Fig. 3. Pre-treatment with PCPA abolished ketamine-induced antidepressant-like effect in the FST. Two groups of mice pre-treated with PCPA or vehicle prior to receiving ketamine (Ket, 10 mg/kg), fluoxetine (Flx, 18 mg/kg) or vehicle (Veh, NaCl 0.9%) were compared in the FST. In mice groups pre-treated with PCPA for 3 consecutive days, ketamine no longer exhibited its antidepressant-like effects, i.e., (A) decreases in the immobility duration and (B) increases in the swimming duration as it did in naïve groups pre-treated with the vehicle ($n = 6$ mice per group). $*p < 0.05$ different from Veh/Veh-treated group (two-way ANOVA).

Forced Swim Test



Microdialysis



Correlation between 5-HT content and swimming duration

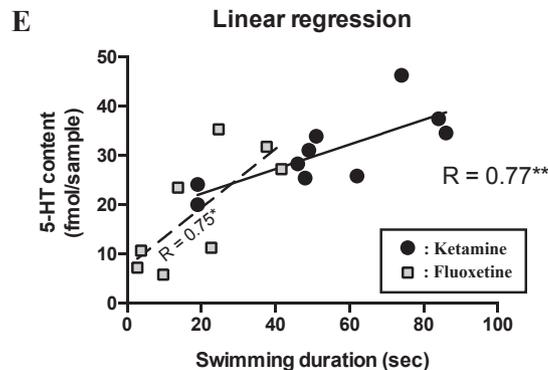


Fig. 4. Intra-mPFCx injection of ketamine-induced increase in swimming behavior in the FST, 24 h after drug injection, is correlated to increase in extracellular cortical 5-HT levels. Unlike fluoxetine (0.25 µg each side), bilateral intra-mPFCx ketamine (Ket) injection at dose 0.5 µg (0.25 µg each side) induced (A) a significant decrease in the immobility duration due to an increase in (B) the swimming duration, a serotonergic parameter in the FST. (C) A statistically significant increase in extracellular mPFCx 5-HT levels was observed in the same Ket-treated mice, but not in Flx-treated mice. The gray area indicates the duration of the FST (i.e., 6 min). (D) AUC values were calculated for the amount of 5-HT outflow collected during 0–120 min and expressed as percentages of baseline. (E) The correlation between extracellular mPFCx 5-HT levels at t60 min (i.e., during the FST) and the swimming duration is stronger in Ket-treated than in Flx-treated mice in regard to their respective *R* Pearson value. **p* < 0.05 vs Vehicle (Veh). ##*p* < 0.01 vs Flx-treated group (one-way ANOVA)., **p* < 0.05 and ***p* < 0.01 for the correlation of dialysate cortical 5-HT levels at t60 min with the swimming duration (*n* = 8–10 mice per group).

NBQX/Ketamine group compared with the NBQX/Veh group; Fig. 5D.

4. Discussion

In the present study, we performed a characterization of ketamine's anxiolytic/antidepressant-like effects in mice using five different behavioral tests in the highly anxious BALB/cj strain of

mice. We also conducted *in vivo* microdialysis after its systemic or intra-mPFCx administration to unveil putative associations between ketamine's behavioral activity and activation of the serotonergic neurotransmission. A dose of 10 mg/kg has been used in the present study and experiments were conducted 24 h after drugs' administration. It corresponds to conditions already described in other studies avoiding in particular hyperlocomotion observed with higher ketamine doses (Koike et al., 2013; Liu et al., 2012; Yang

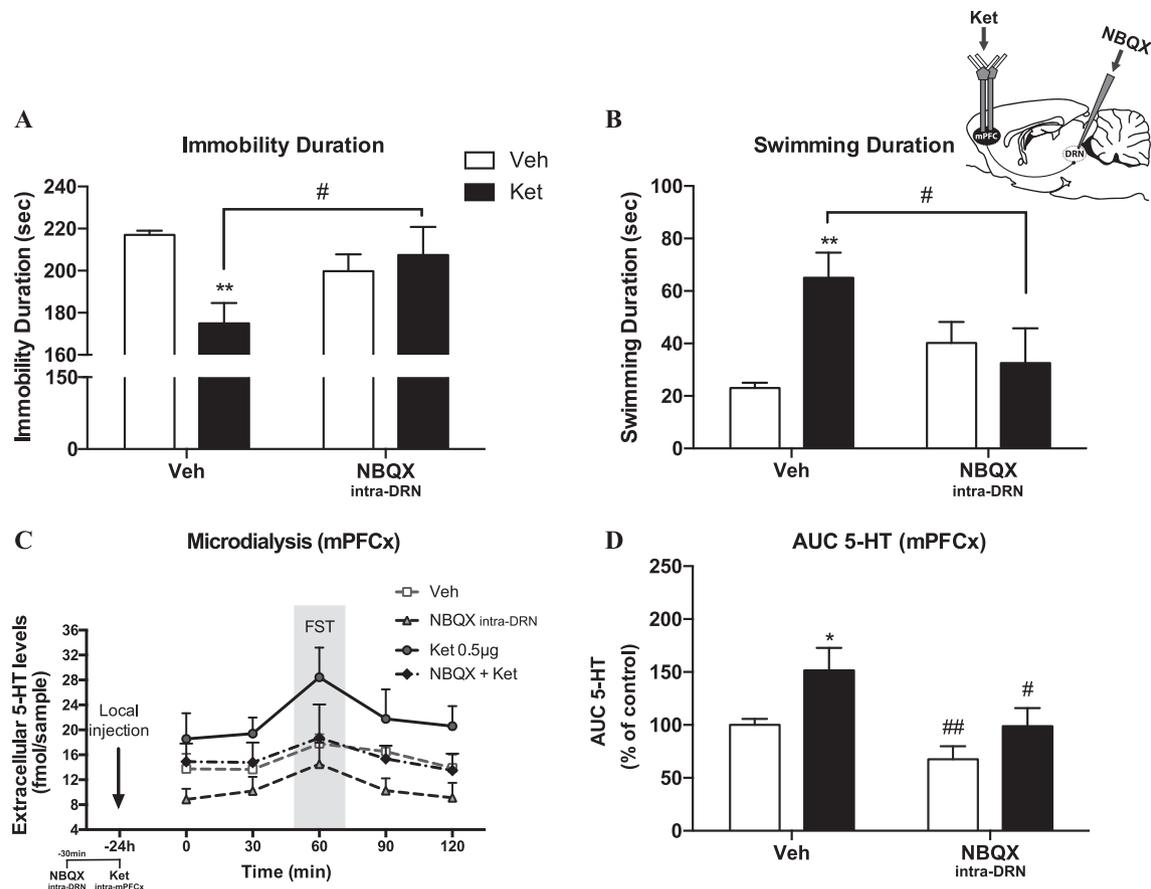


Fig. 5. Intra-DRN injection of NBQX, an AMPA receptor antagonist, abolished the effects of intra-mPFCx ketamine injection in both the FST and mPFCx [5-HT]_{ext} as measured in the same mice. Intra-DRN NBQX injection at dose 0.1 µg blunted the effects of bilateral intra-mPFCx ketamine (Ket) injection at dose 0.5 µg (0.25 µg each side) on (A) the immobility duration due to an increase in (B) the swimming duration in the FST. This abolishment was also observed in (C) the time course and (D) AUC values of mPFCx [5-HT]_{ext}. Both the FST and microdialysis technique were performed in the same mice. The gray area in Fig. 5C indicates the duration of the FST (i.e., 6 min). **p* < 0.05 and ***p* < 0.01 vs Vehicle/Vehicle (Veh). #*p* < 0.05 and ##*p* < 0.01 vs Veh/Ket (two-way ANOVA). *n* = 4–5 mice per group.

et al., 2012; Reus et al., 2011; Li et al., 2010) or when behavioral tests were performed immediately after its administration. By this time, ketamine no longer remained in the animals' circulatory system due to its short elimination half-life ($t_{1/2}$ –13 min in mice: Maxwell et al., 2006), thus no more alteration in locomotor activity was observed beyond 30 min (Lindholm et al., 2012). More precisely, here, a single ketamine dose had no anxiolytic-like activity in the OF and EPM. In addition, at doses ≥ 20 mg/kg, changes in [5-HT]_{ext} are not selective since ketamine induced an immediate increase in extracellular glutamate and dopamine (DA) levels (less than 140 min) in the rat nucleus accumbens and mPFCx (Moghaddam et al., 1997; Razoux et al., 2007).

By contrast, the three behavioral tests evaluating ketamine antidepressant-like activity yielded positive results. Ketamine effects in the NSF agree with the literature (Iijima et al., 2012), but here with a lower dose: 10 mg/kg vs 30 mg/kg. As expected, a single fluoxetine administration had no effects in the NSF and ST (David et al., 2009). In the FST, ketamine response on immobility time is mainly due to increases in swimming duration in BALB/cj mice, a parameter likely reflecting activation of the brain serotonergic system (Page et al., 1999). Recently with a similar protocol (drug doses, time of study), Zanos and colleagues showed that, distinct from fluoxetine, ketamine administration resulted in rapid and persistent antidepressant-like effects following a single treatment in mice (Zanos et al., 2015). However, these authors did not measure the swimming duration in the FST. Here, the swimming/

serotonergic parameter in the 24-h-FST gave new information compared to what was observed 30 min or several days after ketamine administration at similar doses (Koike et al., 2013). In the ST, ketamine increased grooming duration in BALB/cj mice similarly to C57BL/6j mouse strain exposed to chronic mild stress (Franceschelli et al., 2015). Overall, such a behavioral characterization revealed that ketamine principally exerts an antidepressant-like activity, via an activation of serotonergic synaptic transmission.

Increase in swimming duration in the FST after a systemic ketamine administration suggests an activation of serotonergic synaptic transmission. Thus, we evaluated, the potential contribution of the serotonergic system to the antidepressant-like effect of ketamine in the FST using a pre-treatment with PCPA. PCPA-induced decrease in frontal cortex 5-HT levels in BALB/cj mice (79%, see Supplementary Table S3) prevented the anti-immobility effects of ketamine, confirming a key role of the serotonergic system in its antidepressant-like effect. Interestingly, a serotonergic-dependent mechanism of ketamine was already observed following TPH inhibition by PCPA pre-treatment in rats (Gigliucci et al., 2013). We then investigated the brain regions involved in this activity.

Using both routes of administration, we found that systemic or intra-mPFCx ketamine injection increased mPFCx/[5-HT]_{ext}, but not DRN/[5-HT]_{ext}. Microdialysis studies already reported ketamine-induced increases in cortical [5-HT]_{ext}, but over the first hours after its acute administration, and at higher doses (Amargos-Bosch et al., 2006; Lopez-Gil et al., 2012). Here, fluoxetine increased

mPFCx and DRN [5-HT]_{ext} as it usually does after its immediate injection (David et al., 2003). Fluoxetine long $t_{1/2}$ (~5 h in rodents; Maxwell et al., 2006) may explain this persistent effect. At the electrophysiological level, acute fluoxetine still decreased discharge of 5-HT neurons confirming its ability to block SERT. Ketamine and fluoxetine can block the serotonin transporter, despite their differential inhibitory effects *in vitro* on SERT function ($K_i = 160 \mu\text{M}$ versus 1 nM, respectively; Owens et al., 2001; Zhao and Sun, 2008). Their effects after systemic administration on mPFCx/[5-HT]_{ext} are similar. By contrast, ketamine and fluoxetine had different effects on DRN/[5-HT]_{ext}. Autoradiographic studies indicated that the DRN contains a high number of SERT binding sites in rodents (Hrdina et al., 1990). Thus, unlike fluoxetine, ketamine effects on dialysate DRN/5-HT do not seem to involve a direct blocking effect on SERT (Malagie et al., 2002). However, a complex regulation of DRN/5-HT neurons by mPFCx afferents has been demonstrated: the stimulation of some DRN/5-HT neurons by descending excitatory fibers leads to 5-HT release (Celada et al., 2001). Thus, more experiments are required to understand the role of serotonin receptors, mPFCx-DRN circuit and signalling pathways in ketamine-induced antidepressant-like activity.

Interestingly, fluoxetine can bind to NMDA-R in rat cortex, but with a lower affinity than ketamine ($K_i = 10.5 \mu\text{M}$ and $K_i = 1.35 \mu\text{M}$, respectively; Gilling et al., 2009; Szasz et al., 2007). Here, fluoxetine increased mPFCx/[5-HT]_{ext} in the mPFCx and DRN, but only 24 h after its systemic administration and did not decrease immobility duration in the FST. These neurochemical effects were already reported in mice when assessed immediately after an acute SSRI i.p. injection and were linked to the selective inhibition of the serotonin transporter (Bortolozzi et al., 2004; Guiard et al., 2004).

A systemic administration of fluoxetine induced changes in [5-HT]_{ext} in the mPFCx and DRN, but failed to trigger an antidepressant-like effect in the FST (Fig. 2). However, intra-mPFCx injection of fluoxetine did not alter both mPFCx [5-HT]_{ext} and the antidepressant-like activity in the FST (Fig. 4). These different results may be due to the key difference between the protocol of Fig. 2 and that of Fig. 4: in the second one, mPFCx [5-HT]_{ext} was measured under stressful conditions, i.e., the FST was performed when collecting dialysate samples in the same animal. In this case, the effects of increasing mPFCx [5-HT]_{ext} are lost for fluoxetine, but not for ketamine, which still increased these cortical levels over 50%. It has been shown that, as a stressor, the FST did not modify [5-HT]_{ext} in the frontal cortex, but increased its levels in the striatum and decreased them in the amygdala (Kirby et al., 1995). Thus, the FST modulates brain circuits involving the serotonergic system in a region-specific manner. It is likely that antidepressant drugs induced changes in these 5-HT circuits, so that fluoxetine has lost its ability to increase mPFCx [5-HT]_{ext} at 24 h when microdialysis is performed during a stressful event as the FST, while ketamine has retained this ability. It is the increase in cortical [5-HT]_{ext} during the FST that induces the antidepressant-like activity of ketamine. In this case, the measurement of mPFCx [5-HT]_{ext} reflects this activity. By contrast, the increase in mPFCx [5-HT]_{ext} induced by fluoxetine is not sufficient to induce a marked antidepressant-like effect under stress conditions. The correlation we draw between swimming duration and mPFCx [5-HT]_{ext} when combining FST and microdialysis in the same mouse (Fig. 4) further confirms that there are clear differences between ketamine and fluoxetine-treated groups regarding their responses in both tests. Taken together, our data reveal that mPFCx plays a major role in promoting the [5-HT]_{ext}/FST responses to ketamine, and less for those of fluoxetine under stressful conditions.

To analyze these behavioral and neurochemical responses of ketamine possibly mobilizes neural circuits linking the mPFCx to the DRN, we studied the effects of an intra-DRN NBQX injection in

BALB/c mice. The antidepressant-like effect of ketamine on the immobility duration due to an increase in the swimming duration was totally blocked by NBQX (Fig. 5A, B). This blockade was associated with a blunted effect of ketamine on mPFCx [5-HT]_{ext} levels, which suggests that AMPA receptors located in mPFCx-DRN neural circuits. When given alone, intra-DRN NBQX decreased mPFCx [5-HT]_{ext} levels in comparison to the corresponding control group (Fig. 5D). Taken together, these results suggest that DRN AMPA receptors exert a tonic control on mPFCx 5-HT release, and activation of mPFCx/DRN circuitry may underlie at least in part, ketamine's antidepressant-like activity. Although the functional relationship between the DRN and mPFCx is well documented (Lopez-Gil et al., 2007), further optogenetic experiments may help to confirm this hypothesis.

NMDA-R is widely expressed in the mPFCx (Kamiyama et al., 2011; Sanz-Clemente et al., 2013). Ketamine's rapid antidepressant-like response may require an increase in mammalian Target of Rapamycin (mTOR)-dependent expression of BDNF, ultimately leading to increased synaptogenesis in rat mPFCx (Li et al., 2010). Indeed, clinical evidence showed a direct relationship between prefrontal cortex activities, synaptic plasticity, plasma BDNF levels, and the rapid antidepressant effect of ketamine in treatment-resistant depression (Cornwell et al., 2012; Haile et al., 2014). Moreover, it is well known that the mTORC1 signaling pathway regulates protein translation following alterations in neuronal activity contributing to synaptic plasticity (Gerhard et al., 2016 for review). Previous studies showed that ketamine increased the proportion of large-diameter, mushroom-like spines in the prefrontal cortex *in vivo* 24 h after its administration (Li et al., 2010). In addition, expression of the synaptic markers synapsin I and postsynaptic density 95 (PSD95) remains increased 24 h after ketamine. Therefore, the effect of ketamine in modulating cortical glutamate signaling and the expression of neuroplasticity markers may explain its long lasting behavioral effect.

Local injection of a drug in a specific brain region provides useful information on its mechanism of action: intra-mPFCx ketamine increased cortical [5-HT]_{ext} similarly to what was found after its systemic administration. It indicates that the NMDA-R responsible for serotonergic effects are located in the mPFCx. Our results stand out from those of the literature. For example, no changes in cortical [5-HT]_{ext} occurred immediately after a local ketamine perfusion of 100–1000 μM in naïve rats (Amargos-Bosch et al., 2006). Thus, differential dosage of ketamine and time of observation might have divergent actions depending on the site of blockade of NMDA-R, either inside the mPFCx or outside, e.g., in the ventral hippocampus (Lopez-Gil et al., 2012; Brown et al., 2015). Our data agree with the work of Gigliucci et al., 2013, which has also demonstrated a role of 5-HT in mediating sustained antidepressant-like activity of ketamine in the FST. We added here a mechanistic approach showing in particular a sustained effect of ketamine on cortical [5-HT]_{ext} after its local application.

Stimulation of the cortical serotonergic system may play, at least partially, a role in ketamine antidepressant-like activity here. However, this role may be either insufficient, or incomplete to explain this activity in the mPFCx because behavioral responses were different between ketamine and fluoxetine in the FST (swimming duration), ST and NSF, while they induced comparable increases in mPFCx/[5-HT]_{ext} at this time point. To explain its fast antidepressant-like activity, it was hypothesized that ketamine directly blocks NMDA-R located on GABA interneurons. As a consequence, it decreases the inhibitory GABA-ergic tone, thus increases excitatory synapses and glutamate release in the mPFCx (Moghaddam et al., 1997). This cascade of events might explain the greater and persistent increase in mPFCx/[5-HT]_{ext} induced by systemic and intra-mPFCx ketamine. In addition, ketamine may act

outside of the mPFCx to activate excitatory glutamatergic transmission, but within the mPFCx to release DA (Lorrain et al., 2003).

In conclusion, ketamine displays a more effective, a persistent more rapid antidepressant-like activity than fluoxetine in several behavioral tests. Unlike fluoxetine, acute ketamine reduced immobility duration at this time point in the FST by inducing a robust increase in swimming duration associated with mPFCx/[5-HT]_{ext} increases. Moreover, the depletion of 5-HT synthesis by PCPA abolished ketamine effects in the FST. Thus, ketamine, a non 5-HT compound, surprisingly requires cortical 5-HT system to induce its antidepressant-like effects. Differences with fluoxetine in neural adaptation of mPFCx-DRN circuits are likely to mediate their serotonergic characteristics.

Conflict of interest

D.J.D. serves as a consultant for Lundbeck, Roche, and Servier. BPG serves as a consultant for Lundbeck and Phodé laboratoires. AMG serves as a consultant for Lundbeck and Servier.

Author's contribution

Thu Ha Pham, Denis J David and Alain M Gardier contributed to the conception and design of the study; Thu Ha Pham, Indira Mendez-David, Céline Defaix, Bruno Guiard, Laurent Tritschler and Denis J David contributed to the acquisition of data. Thu Ha Pham and Alain M Gardier wrote the manuscript. All the authors contributed to analysis of data, drafting the article for key intellectual content.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.neuropharm.2016.05.010>.

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