New insights into methoxetamine mechanisms of action: focus on serotonergic 5HT-2 receptors in pharmacological and behavioral effects in the rat

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Running title: Effect of MXE on brain neurotransmission

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Abbreviations

aCSF	Artificial cerebro-spinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ATP	Adenosine triphosphate
DAT	Dopamine transporter
DSI	Depolarization-induced suppression of inhibition
ED50	Median effective dose
EDTA	Ethylenediaminetetraacetic acid
fEPSPs	Field excitatory postsynaptic potentials
GABA	Gamma-aminobutyric acid
HPLC	High-performance liquid chromatography
IP	Intraperitoneal (administration)
IV	Intravenous (administration)
ketamine	2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one
Ketanserine	3-[2-[4-(4-fluorobenzoyl)piperidin-1-yl]ethyl]-1H-quinazoline-2,4
LSD	Lysergic acid diethylamide
MXE	Methoxetamine ([2-(3-methoxyphenyl)-2-(ethylamino)-cyclohexanone])
MDL100907	R-(2,3-dimethoxyphenyl)-[1-[2-(4-fluorophenyl)ethyl]piperidin-4-yl]methanol
NAc	Nucleus accumbens
NBQX	2,3-dioxo-6-nitro-7-sulfamoyl-benzo[f]quinoxaline
NET	Noradrenaline transporter
NMDA	N-methyl-D-aspartate receptor
mPFC	Medial Prefrontal cortex
PCP	Phencyclidine
PPI	Prepulse inhibition
PTSD	Post-traumatic stress disorder
SERT	Serotonin transporter
sIPSC	Spontaneous inhibitory postsynaptic currents
VGCCs	Voltage-gated calcium channels
WDR	World Drug report
5-HT	Serotonin

Abstract

Methoxetamine (MXE) is a dissociative substance of the arylcyclohexylamine class that has been present on the designer drug market as a ketamine-substitute since 2010. We have previously shown that MXE possesses ketamine-like discriminative effects and positive rewarding effects in rats, affects brain processing involved in cognition and emotional responses, causes long-lasting behavioral abnormalities and neurotoxicity in rats, and induces neurological, sensorimotor and cardiorespiratory impairments in mice. To shed light on the mechanisms through which MXE exerts its effects, we conducted a multidisciplinary study to evaluate the neurotransmission systems more likely involved in its action on the brain. *In vivo* microdialysis study first showed that a single administration of MXE (0.25 and 0.5 mg/kg i.v.) increases serotonin levels in the rat prefrontal cortex (PFC) and nucleus accumbens with respect to basal values. Then, we observed that blockade of the serotonin 5-HT_2 receptors through two selective antagonists, ketanserin (0.1 mg/kg, i.p.) and MDL 100907 (0.03 mg/kg, i.p.), at doses not affecting animals behavior per sé, attenuated the facilitatory motor effect and the inhibition on visual sensory responses induced by MXE (3 mg/kg i.p.) and ketamine (3 mg/kg i.p.), and prevented MXE-induced inhibition of the prepulse inhibition in rats, pointing to the 5-HT_2 receptors as a key target for the recently described MXE-induced sensorimotor effects. Finally, *in-vitro* electrophysiological studies revealed that the GABAergic and glutamatergic systems are also likely involved in the mechanisms through which MXE exerts its central effects, since MXE inhibits NMDA-mediated field postsynaptic potentials and GABA-mediated spontaneous currents, in a dose dependent manner. Conversely, MXE failed to alter both the AMPA component of field postsynaptic potentials and presynaptic glutamate release, and seems to not interfere with the endocannabinoid-mediated effects on medial PFC (mPFC) GABAergic synapses. Altogether, our results confirm MXE as a NMDA receptor antagonist and shed further lights into the central mechanisms of action of this ketamine-substitute by pointing to serotonin 5-HT_2 receptors as crucial players in the expression of its sensorimotor altering effects and to the NMDA and GABA receptors as potential further important targets of action.

Keywords: Methoxetamine; Ketamine; Ketanserin; MDL100907; 5HT-2 receptor; sensorimotor responses; Prepulse inhibition (PPI); GABA; glutamate; endocannabinoid.

1. Introduction

In recent years, a new wave of Internet commerce for new illicit drugs has characterized the drug market (Miliano et al., 2018), with hundreds of newly synthetized substances with diverse and potent psychoactive effects advertised and marketed as "legal highs", i.e. as "legal" replacements to controlled drugs such as cocaine, heroin and cannabis (Shafi et al., 2020). The prevalence and use of these new psychoactive substances (NPS) has rapidly increased over the last 15 years, NPS number raising from 166 over the period 2005-2009 to 950 by the end of 2019 (WDR, 2020). According to the last World Drug Report (WDR, 2020), although NPS has declined after national legislations have been adopted, their use is still widespread and very popular among vulnerable population groups. At present, NPS are causing growing worldwide health-care concern and present clear challenges to toxicologists and medical staff that are called to face with new compounds with mostly unknown pharmacological profile (Schifano et al., 2021). Indeed, the majority of NPS remain unfamiliar to health care providers (Jolanta and Andrzejczak, 2015) but some of them have been recently shown to possess abuse potential (Riley et al., 2020) and to elicit toxic effects both in the brain and periphery (Costa et al., 2020).

Methoxetamine (MXE) is an arylcyclohexylamine derivative of ketamine which acute toxicity is associated with "dissociative" and "sympathomimetic" clinical features (Zawilska et al., 2014). Symptoms are consistent with ketamine-induced adverse effects and include profound agitation, confusion, stupor, tachycardia, hypertension, ataxia, mydriasis and nystagmus, which typically resolved with symptomatic treatment (Zanda et al., 2016). Proposed in the drug market as a legal and "bladder-friendly" alternative to ketamine, MXE rapidly appeared associated with worse side effects than ketamine (Corazza et al., 2013). Prolonged (3 months) exposure to MXE, for example, resulted in significant bladder and renal toxicity in mice (Dargan et al., 2014), but shorter periods of exposure can also result in bladder dysfunction and inflammation in rats (Wang et al., 2017). MXE-induced cardiotoxicity has also been reported recently by *in vitro* studies (Yoon et al., 2019). Recreational use of MXE is known since 2012 (Hofer et al., 2012; Misselbrook and Hamilton, 2012). Since then, acute fatal and non-fatal intoxications have been reported in young (25-31 years old) polydrug abusers (Adamowicz and Zuba, 2014; Chiappini et al., 2015; Sein Anand et al., 2012; Wiergowski et al., 2014; Wikström et al., 2013). The first analysis of online, non-peer reviewed, information material indicated that MXE is a dissociative drug related to ketamine but with more intense effects and much long lasting effect (Corazza et al., 2012). In line with these findings, first cases of intoxication associated with analytically confirmed MXE exposure revealed acute cerebellar toxicity (Shields et al., 2012) and induction of epileptic seizures (Imbert at al., 2014). Subsequent studies and case reports confirmed a MXE-induced toxidrome consisting of sympathetic activation, dissociation, delirium and

cerebellar symptoms (Craig and Loeffler, 2014). The notion that a single acute MXE intoxication may produce severe brain impairment is supported by the recent finding of an impaired dorsolateral prefrontal cortex (PFC) in MXE-induced psychosis (Moccia et al., 2019). A phenomenological study of the experiences induced by MXE and collected from public Internet fora confirmed that its effects are similar to those induced by classic hallucinogens (e.g., LSD, PCP) and dissociative drugs (e.g., ketamine) but also reported negative effects, such as anxiety and fear, and quite a high abuse potential (Kjellgren and Jonsson, 2013). Worryingly, MXE can also be used for self-medication purposes, as an analgesic (Maskell et al., 2016) or for alleviating posttraumatic stress disorder (PTSD) symptoms (Striebel et al., 2017).

In the last few years, animal studies have allowed a characterization of the effects induced by acute and repeated exposure to MXE at behavioral level. These studies revealed a behavioral profile similar to that of other psychotomimetic uncompetitive NMDA receptor antagonists, including locomotor hyperactivity and hypomotility at low and high doses, respectively, and disruption of prepulse inhibition (PPI) of acoustic startle (Halberstadt et al., 2016; Horsley et al., 2016; Zanda et al., 2017). MXE was also shown to (i) generalize to phencyclidine (PCP) (Berquist et al., 2018) and ketamine (Chiamulera et al., 2016) in a drug discrimination paradigm, (ii) substitute for ketamine in a self-administration model (Mutti et al., 2016), (iii) induce behavioral alterations in the marble burying and in the novel object recognition tests (Costa et al., 2019) and (iv) cause anxiety-like state and antidepressant-like effects in the elevated plus maze test and forced swim test, respectively (Zanda et al., 2017). Interestingly, the MXE-induced rapid and sustained antidepressant effect was shown to likely occur through glutamatergic and serotonergic mechanisms (Botanas et al., 2017). Comparative studies in mice aimed at evaluating the detrimental impact of a systemic administration of MXE on neurological, sensorimotor and cardiorespiratory parameters have showed that although MXE is behaviourally active at higher doses than ketamine and PCP, some effects are more intense or frequent (e.g., rotations) (Ossato et al., 2018). Similarly to PCP, and in a more selective manner than ketamine, MXE was found to interfere with working memory in the odour span task (Mathews et al., 2018). In light of its effects on the sensorimotor domain, MXE could be extremely dangerous when used by drivers, as its cardiovascular, respiratory and skeletal muscular effects can seriously impair driving (Elian and Hackett, 2014; Fassette and Martinez, 2016; Wille et al., 2018).

In rat primary cortical cells, MXE inhibited potently neuronal activity (Hondebrink et al., 2016) and increased the glutamate-evoked increase in $[Ca2^{+}]_{I}$, without affecting voltage-gated calcium channels (VGCCs) (Hondebrink et al., 2017). The group of Remco Westerink also showed that MXE slightly inhibits the K^{+}- and acetylcholine-evoked increase in $[Ca^{2+}]_{I}$, slightly reduces the ATP-evoked

increase in $[Ca^{2+}]_I$ and potently inhibits the uptake via monoamine transporters (DAT, NET and SERT) (Hondebrink et al., 2017). How MXE exerts its effects on brain and behavior, however, remains to be elucidated. MXE has been reported to possess high affinity for the PCP-site on the glutamate NMDA receptor and to display appreciable affinity for the serotonin transporter (Roth et al., 2013), which could explain its dissociative effects. Immunohistochemical analysis showed that behaviourally active doses of MXE acutely increase phosphorylation of ribosomal protein S6 in the rat mPFC and hippocampus (Zanda et al., 2017), while repeated exposure to MXE induces dopaminergic damage in the mPFC, nucleus accumbens, caudate-putamen, substantia nigra pars compacta, and ventral tegmental area, along with accumbal serotonergic damage (Costa et al., 2019). In a series of our previous studies, we have shown that activation of the mesolimbic dopamine system is likely responsible for MXE-induced positive rewarding effects (Chiamulera et al., 2016; Mutti et al., 2016), but other evidences have shown that other neurotransmitters are likely affected by MXE, including the serotoninergic system (Hondebrink et al., 2017; Roth et al., 2013). This multidisciplinary study was undertaken to explore whether the serotonin system may be responsible for the MXE-induced sensorimotor alterations (Ossato et al., 2016) and which other neurotransmission systems may also be affected by MXE use.

2. Materials and methods

2.1 Animals

Male Sprague-Dawley rats (Envigo, Italy) weighing 275-300 g were housed in groups of 4 per cage, at a constant temperature (22 ± 2 °C), humidity (60%), and light/dark cycle (lights on from 08:00 to 20:00 h). Tap water and standard laboratory rodent chow (Mucedola, Settimo Milanese, Italy) were provided *ad libitum* in the home cage. All animal experiments were carried out in accordance with the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research according to Italian (D.L. 116/92 and 152/06) and European Council directives (609/86 and 63/2010) and in compliance with the approved animal policies by the Ethical Committee for Animal Experiments (CESA, University of Cagliari) and the Italian Ministry of Health (Aut. n°162/2016-PR). All animals were handled once daily for 5 min for 5 consecutive d before experimentation began. We made every effort to minimize pain and suffering, and to reduce the number of animals used.

2.2 Drug Preparation and Dose Selection

MXE and ketamine were purchased from LGC Standards S.r.l. (Milan, Italy) and were dissolved in saline solution. Ketanserin (Sigma Aldrich, Milan, Italy) and MDL100907 (Tocris, Bristol, United

Kingdom) were dissolved in 2% EtOH, 2% Tween 80, and 96% saline. On the basis of our previous work (Mutti et al., 2016; Ossato et al., 2018; Zanda et al., 2017), MXE was administered intravenously (i.v.) at 0.125-0.5 mg/kg for *in vivo* microdialysis experiments, while MXE and ketamine were administered intraperitoneally (i.p.) at 0.3-3 mg/kg for behavioral testing. Ketanserin and MDL100907 were administered i.p. 20 minutes before MXE and ketamine administration. For in vitro experiments MEX and ketamine were perfused in brain fresh slice at the concentration of 1 – 50 mM

2.3 Neurochemical analysis: *in vivo* microdialysis

2.3.1 *Surgery*. Rats were anaesthetized with Fentanyl (0.06 mg/kg i.p.), placed in a stereotaxic apparatus, and implanted with homemade vertical dialysis probes (1.5 or 3 mm dialyzing portion for NAc or mPFC, respectively). Animals were implanted in the NAc shell (A: +2.2, L: +1.0 from bregma, V: -7.8 from dura), or in the mPFC (A: +3.7, L: +0.8 from bregma, V: -5.0 from dura), according to the rat brain atlas of Paxinos and Watson (1998). In order to perform intravenous (i.v.) drug administration, a catheter (Silastic, Dow Corning Corporation, Michigan, USA) was inserted in the right jugular vein as previously described (De Luca et al., 2015).

2.3.2 *Analytical Procedure*. On the day following surgery, animals were connected to an infusion pump and probes were perfused with Ringer's solution (in mM: 147 NaCl, 4 KCl, 2.2 $CaCl_2$) at a constant rate of 1 µl/min. After rinsing for at least 1 h, dialysate samples (20 µl) were collected every 20 minutes and injected into an HPLC equipped with a reverse phase column (C8 3.5 um, Waters, USA) and a coulometric detector (ESA, Coulochem II) to quantify serotonin. The electrodes of the analytical cell were set at –175 mV (oxidation) and +220 mV (reduction). The mobile phase, containing (in mM) 50 NaH_2PO_4, 0.1 Na_2EDTA, 0.5 n-octylsulfate and 22% (v/v) methanol (the pH of mobile phase was adjusted with Na_2HPO_4 to 5.7). The sensitivity of the assay was 5 fmol/sample. When the serotonin (5-HT) measurements did not differ more than 10% in three consecutive samples, the average value was considered as the basal levels and the animals were treated with either vehicle or MXE (0.125-0.5 mg/kg/i.v.), and serotonin levels were monitored for 3 hours following the treatment. At the end of the experiment, animals were sacrificed and their brains removed, stored in formalin (8%), and used for probe placement histological confirmation.

2.4 Behavioural Studies

Visual response was verified by two behavioural tests that evaluated the ability of the rat to capture visual information even when stationary (the visual *object* response) or when moving (the visual *placing* response). The effects of MEX and ketamine on spontaneous locomotion, visual object, visual

placing and startle/PPI responses were investigated using behavioural tests widely used in studies of safety pharmacology for the preclinical characterization of NPS in rodents (Bilel et al., 2019; Canazza et al., 2016, 2017; De Luca et al., 2015; Fantinati et al., 2017; Marti et al., 2019; Ossato et al., 2015, 2018; Vigolo et al., 2015). To reduce the number of animals used, the evaluation of spontaneous locomotion, visual object test and visual placing test were evaluated in the same rat in consecutive manner: recording of spontaneous locomotion, visual object and visual placing. Moreover, to reduce the stress induced by manipulation, and to confirm the stability and reproducibility over time of the responses of our tests, animals were trained twice per week for two weeks before the pharmacological treatment. All experiments were performed between 8:30 am and 2:00 pm. Experiments were conducted in blind by trained observers working in pairs (Ossato et al., 2016). The behaviour of rats (sensorimotor responses) was videotaped and analysed offline by a different trained operator that gives test scores.

2.4.1 Spontaneous locomotion

Spontaneous locomotor activity was investigated by using a camera (B/W USB Camera day&night with varifocal lens; Ugo Basile, Italy) and movies were analyzed off-line by a trained operator who did not know the drug treatments performed. The rat was placed in a square plastic cage (60x60 cm) located in a sound- and light-attenuated room and horizontal motor activity (in seconds) was monitored for 5 minutes in each time point (0, 10, 30, 60, 120 and 180 minutes post injection). To avoid rat olfactory cues, cages were carefully cleaned with a dilute (5%) ethanol solution and washed with water between animal trials (Marti et al., 2019).

2.4.2 Visual Object Response

The visual object response test was used to evaluate the ability of the rat to see an object approaching from the front or the side, inducing the animal to shift or turn its head or to retreat (Bilel et al., 2019; Marti et al., 2019). For the frontal visual response, a white horizontal bar was moved frontally to the rat's head; the manoeuvre was repeated three times. For the lateral visual response, a small dentist's mirror was moved into the rat's field of view in a horizontal arc until the stimulus was between the rat's eyes. The procedure was conducted bilaterally and was repeated three times. The score assigned was a value of 1 if there was a reflection in the rat movement or 0 if not. The total value was calculated by adding the scores obtained in the frontal with that obtained in the lateral visual object response (overall score 9). Evaluation of the visual object response was measured at 0, 10, 30, 60, 120, and 180 min post-injection.

2.4.3 Visual Placing Response

The visual placing response test was performed using a tail suspension-modified apparatus able to bring the rat towards the floor at a constant speed of 10 cm/sec (Bilel et al., 2019; Marti et al., 2019).

The downward movement of the rat was videotaped. Frame-by-frame analysis allowed us to evaluate the beginning of the reaction of the rat while it was close to the floor. When the rat started the reaction, an electronic ruler evaluated the perpendicular distance in millimetres between the eyes of the rat to the floor. The naïve rats perceived the floor and prepared for contact at a distance of about 27 ± 4.5 mm. Evaluation of the visual placing response was measured at 0, 15, 35, 65, 125, and 185 min post-injection.

2.4.4 Startle and Pre-Pulse Inhibition

As previously reported (Bilel et al., 2019; Marti et al., 2019), rats were tested for acoustic startle reactivity in startle chambers (Ugo Basile apparatus, Milan, Italy) consisting of a sound-attenuated, lighted, and ventilated enclosure holding a transparent non-restrictive Perspex® cage (modified version for rats 200×90×80 mm). A loudspeaker mounted laterally by the holder produced all acoustic stimuli. Peaks and amplitudes of the startle response were detected by a load cell. At the onset of the startling stimulus, 300-ms readings were recorded, and the wave amplitude evoked by the movement of the rat startle response was measured. Acoustic startle test sessions consisted of startle trials (pulse-alone) and pre-pulse trials (pre-pulse + pulse). The pulse-alone trial consisted of a 40-ms 120-dB pulse. Pre-pulse + pulse trial sequences consisted of a 20-ms acoustic pre-pulse, 80-ms delay, and then a 40-ms, 120-dB startle pulse (100-ms onset–onset). There was an average of 15 seconds (range = from 9 to 21 seconds) between the trials. Each startle session began with a 10-min acclimation period with a 65-dB broadband white noise that was present continuously throughout the session. The test session contained 40 trials composed by pulse-alone and pre-pulse + pulse trials (with three different pre-pulses of 68 dB, 75 dB, and 85 dB) presented in a pseudorandomized order. Rats were placed in the startle chambers 5 min after treatment with MEX or KET. The entire startle/PPI test lasted 20 min. The pre-pulse inhibition (PPI) was expressed as the percentage decrease in the amplitude of the startle reactivity caused by the presentation of the pre-pulse. MEX or ketamine (0.3-3 mg/kg) was administered intraperitoneally, and startle/PPI responses were recorded 15 min (including the 10-min acclimation period) after drug injections. Ketanserin (0.1 mg/kg i.p.) and MDL 100907 (0.03 mg/kg i.p.) were administered 20 minutes before MEX or ketamine (3 mg/kg i.p.) or saline (when we have investigated the effect of the antagonist alone on startle/PPI responses).

2.5 *In vitro* electrophysiology

2.5.1 Preparation of brain slices

Coronal brain slices containing the mPFC were prepared as previously described (Dazzi et al., 2014). Briefly, after reaching deep anesthesia with vapors of isoflurane (3%), rats were euthanized and brains removed rapidly from the skull and transferred to a beaker containing a modified artificial cerebro-

spinal fluid (aCSF) prepared as follow (in mM): 220 sucrose, 2 KCl, 0.2 $CaCl_2$, 6 $MgSO_4$, 26 $NaHCO_3$, 1.3 NaH_2PO_4, and 10 D-glucose (pH 7.4, set by aeration with 95% O2 and 5% CO2). In agreement with Bizon et al. (2012), coronal brain slices (thickness of 260 μm) containing the mPFC, which includes prelimbic, infralimbic, and anterior cingulate nuclei, were cut using a vibratome (Leica, Germany). Slices were then immediately transferred to a nylon net submerged, for at least 40 min, in a standard aCSF containing (in mM): 126 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, and 10 D-glucose (pH 7.4, set by aeration with 95% O_2 and 5% CO_2 and controlled temperature of 35 °C). After subsequent incubation for at least 1 h at room temperature, hemi-slices were transferred to the recording chamber with a constant flow rate of ~2 ml/min of aCSF at the controlled temperature of 33 °C. The effect of MXE and ketamine on different neurotransmission systems was evaluated by perfusion of both drugs in the bath for 10 min. In all experiments, no attempt was made to wash-out drugs due to their lipophilic properties.

2.5.2 Patch-clamp recordings

For patch-clamp recordings on mPFC layer 5 pyramidal neurons, spontaneous GABAergic inhibitory postsynaptic currents (sIPSC) were recorded with an Axopatch 200B amplifier, filtered at 2 kHz, and digitized at 5 kHz. Resistance of the pipettes ranged from 4.5 to 6.0 MΩ when they were filled with (in mM): 140 CsCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 EGTA, 10 HEPES, 2 ATP-Na, pH 7.3 with CsOH 5 N. GABA-mediated sIPSC were recorded in the presence of the non-selective glutamate receptor antagonist kynurenic acid (1 mM). Access resistance ranged from 15 to 30 MΩ and was monitored throughout the recording by injection of 10 mV hyperpolarizing pulses; in cases of changes > 20%, the cell was automatically discarded from analysis. Analysis of sIPSC was performed using Mini analysis software (Synaptosoft, Inc., version 6.0.2) with a noise amplitude threshold of 5 pA. Amplitude, decay time and frequency of sIPSC have been analyzed in the presence of 10 min of MXE at different concentrations (1-50 μM).

2.5.3 fEPSPs recordings

Recordings of extracellular field excitatory postsynaptic potentials (fEPSPs) were performed in the L1 layer of mPFC where proximal dendrites of pyramidal neurons are placed. Excitatory afferents were stimulated through a bipolar concentric electrode (FHC, ME) placed medially at 300 μm from recording site. Responses were recorded filling the recording electrode with KCl 3 mM and were triggered digitally every 20 s by application of a constant current pulse of 0.2-0.4 mA with a duration of 60 μs, which yielded a half-maximal response, and a stimulator (Digitimer Ltd, UK). Even AMPA and NMDA components of the fEPSP were different in the peak duration (5 ms and 20 ms from the stimulation, respectively) NMDA-mediated responses were isolated in the presence of the AMPA selective antagonist NBQX (5 μM).

2.5.4 Paired-pulse (PP) protocol

Paired-pulse protocol, used to evaluate the presynaptic effect of both MXE and ketamine on glutamatergic synapses, consisted of delivering two consecutive electrical stimuli with an inter-event interval of 100 ms, and the paired-pulse ratio of the amplitude of the second fEPSP and that of the first was calculated. The effect of MXE or ketamine was evaluated during the bath perfusion of both drugs for 10 min.

To assess whether MXE may affect the endocannabinoid system, the depolarization-induced suppression of inhibition (DSI) protocol was applied in mPFC pyramidal neurons. For DSI experiments, after a recording period of sIPSC for approximately 3 min, the membrane potential was stepped from −65 to 0 mV for 5 s under voltage clamp condition. The magnitude of DSI was calculated as the sIPSC amplitude and frequency after the depolarization, which in turn was calculated as percentage of variation with respect to the average observed during a 3-min pre-depolarization period. The effect of DSI was evaluated in the absence and presence of 10 μM MXE perfused in the bath for 10 min.

3. Statistical Analysis

For microdialysis study, all the numerical data are given as mean ± SEM. Data were analyzed using Prism software (GraphPad Prism, USA), by performing ANOVA (one-way or two-way) for repeated measures, followed by Tukey's *post hoc* test.

For behavioral studies, in sensorimotor response experiments data are expressed in arbitrary units (visual object response) and percentage of baseline (visual placing response and spontaneous locomotion). The amount of PPI was calculated as a percentage score for each pre-pulse + pulse trial type: % PPI = 100 − {[(startle response for prepulse + pulse trial) / (startle response for pulse-alone trial)] × 100}. Startle magnitude was calculated as the average response to all pulse-alone trials. All the numerical data are given as mean ± SEM. The statistical analysis of the effects of the individual substances in different concentrations over time and that of antagonism studies in histograms were performed by two-way ANOVA followed by Bonferroni's test for multiple comparisons. The unpaired Student's t-test (two-tailed) was used to determine statistical significance ($p < 0.05$) between two groups (see Table 1). The statistical analysis was performed using Prism software (GraphPad Prism, USA). The ED50 (Table 1) on mean or max effects induced by MXE or ketamine was calculated, when possible (visual object and visual placing), using the non-linear regression analysis of dose-response curves using Prism software (GraphPad Prism, USA).

For electrophysiology experiments, data are reported as average of pooled data ± ESM. Statistical comparisons of pooled data were performed by t test, one- or two-way ANOVA followed by Bonferroni *post-hoc* tests.

In all cases, a P value < 0.05 was considered statistically significant.

3. Results

3.1 *In vivo* microdialysis

Figure 1 illustrates the effect of the administration of MXE (0.125- 0.5 mg/kg i.v.) on extracellular 5-HT levels in mPFC (*left*) and NAc shell (*right*) of rats. Data show that only the highest dose tested was able to induce an early positive trend in the release of 5-HT the mPFC, i.e. +20% and +25% vs baseline at 20 and 40 min, respectively. One-way ANOVA for repeated measures showed a significant decrease of dialysate 5-HT in the mPFC after MXE 0.25 mg/kg revealing differences at the 120-160 min samples with respect to basal values. Two-way ANOVA (in PFC, giusto?) showed a main effect of time x treatment interaction ($F_{27, 126} = 1.67$; *$p < 0.05$).

Tukey's *post-hoc* tests revealed that in animals implanted in the NAc shell, all doses showed a different time-to-onset of the effect. One-way ANOVA for repeated measures showed a larger increase of dialysate 5-HT in the NAc shell after MXE 0.25 mg/kg revealing differences at the 140 min sample with respect to basal values. Two-way ANOVA (in NAc?) showed a main effect of time x treatment interaction ($F_{27, 144} = 1.9$; *$p < 0.01$). Tukey *post-hoc* test showed a larger increase of dialysate 5-HT in the NAc shell after MXE 0.25 mg/kg revealing differences the 120-140 min samples with respect to vehicle.

3.2 Behavioral studies

3.2.1 Evaluation of Spontaneous Locomotion

Spontaneous locomotion did not change in saline-treated rats over 180 min observation (Figure 2A,B). Intraperitoneal (i.p.) administration with MXE and ketamine (0.1-3 mg/kg) facilitated spontaneous locomotor activity in rats (Figure 2). In particular, MXE transiently facilitated spontaneous locomotor in rats at 1 mg/kg (up to 10 min) and at 3 mg/kg (up to 60 min), [Figure 2A; effect of treatment ($F_{4,150} = 24.17$, $p < 0.0001$), time ($F_{5,150} = 206.8$, $p < 0.0001$) and time × treatment interaction ($F_{20,150} = 3.605$, $p < 0.0001$)], while ketamine transiently facilitated spontaneous locomotor in rats only at the highest dose of 3 mg/kg and the effect persisted up to 60 min [Figure 2B; effect of treatment ($F_{4,150} = 14.69$, $p < 0.0001$), time ($F_{5,150} = 250.7$, $p < 0.0001$) and time × treatment interaction

($F_{20,150} = 3.003$, $p < 0.0001$)]. Spontaneous locomotion did not change in vehicle-treated rats over 180 min observation (Figure 2C). Administration of ketanserine (0.1 mg/kg i.p.) did not affect spontaneous locomotor activity in rats (Figure 2C) but partially prevented the facilitatory effect induced by MXE 3 mg/kg ($F_{3, 20} = 62.85$; $p <0.0001$) and ketamine 3 mg/kg ($F_{3,20} = 18.37$; $p<0.0001$). Similarly, MDL 100907 (0.03 mg/kg i.p.) did not affect per se spontaneous locomotor activity in rats (Figure 2C) but partially prevented the facilitatory effect induced by MXE 3 mg/kg ($F_{3,20} = 64.92$; $p<0.0001$) and ketamine 3 mg/kg ($F_{3,20} = 21.20$; $p<0.0001$).

3.2.2 Evaluation of the Visual Object Response

Visual object response did not change in saline-treated rats over 180 min observation (Figure 3A,B). Acute administration of MXE (0.1-3.0 mg/kg i.p.) dose-dependently reduced the visual object response in rats. At 1 mg/kg, the effect was transient (up to 60 min), while the effect caused at the highest dose (3.0 mg/kg) persisted up to 180 min [Figure 3A; effect of treatment ($F_{4,150} = 143.3$, $p < 0.0001$), time ($F_{5,150} = 20.82$, $p < 0.0001$) and time × treatment interaction ($F_{20,150} = 7.995$, $p < 0.0001$)]. Systemic administration of ketamine (0.1-3.0 mg/kg i.p.) dose-dependently reduced the visual object response in rats. The effect was transient both at 1 mg/kg (persisted up to 30 min) and at 3 mg/kg (persisted up to 60 min) [Figure 3B; effect of treatment ($F_{4,150} = 60.74$, $p < 0.0001$), time ($F_{5,150} = 11.01$, $p < 0.0001$) and time × treatment interaction ($F_{20,150} = 6.159$, $p < 0.0001$)]. As illustrated in Table 1, the inhibitory effect caused by MXE (ED_{50} ~ 4.09 mg/kg) appeared to be greater than that induced by ketamine (ED_{50} ~ 7.01 mg/kg). MXE induced a greater maximal inhibitory effect on visual object response (ED_{50} ~ 2.30 mg/kg) than that induced by ketamine (ED_{50} ~ 3.69 mg/kg; Table 1). In particular, at 3 mg/kg dose, MXE is more effective than ketamine ($t = 2.947$, $df = 10$; $P = 0.0146$) and induces a greater maximum inhibitory effect ($t = 2.392$, $df = 10$; $P = 0.0378$; Table 1). Visual object response did not change in vehicle-treated rats over 180 min observation (Figure 3C). Administration of ketanserine (0.1 mg/kg i.p.) did not affect visual object response in rats (Figure 3C) but partially prevented the inhibitory effect induced by MXE 3 mg/kg ($F_{3,20} = 77.68$; $p < 0.0001$) and ketamine 3 mg/kg ($F_{3,20} = 20.92$; $p < 0.0001$). Similarly, MDL 100907 (0.03 mg/kg i.p.) did not affect per se visual object response in rats (Figure 3C) but partially prevented the inhibitory effect induced by MXE 3 mg/kg ($F_{3,20} = 104.1$; $p < 0.0001$) and ketamine 3 mg/kg ($F_{3,20} = 26.89$; $p < 0.0001$).

3.2.3 Evaluation of the Visual Placing Response

Visual placing response did not change in saline-treated rats over 180 min observation (Figure 3D,E). Acute administration of MXE (0.1- 3.0 mg/kg i.p.) dose-dependently reduced the visual placing response in rats at all doses tested and effects persisted up to 180 min [Figure 3D; effect of treatment ($F_{4,150} = 143.9$, $p < 0.0001$), time ($F_{5,150} = 77.65$, $p < 0.0001$) and time × treatment interaction ($F_{20,150} = 7.141$, $p < 0.0001$)]. Acute administration of ketamine (0.1-3.0 mg/kg i.p.) dose-dependently

reduced the visual placing response in rats. The effect was transient both at 0.1 mg/kg and 0.3 mg/kg (persisted up to 30 min), while persisted up to 185 min at 1 and 3 mg/kg [Figure 3E; effect of treatment ($F_{4,150}$ = 63.11, $p < 0.0001$), time ($F_{5,150}$ = 42.15, $p < 0.0001$) and time × treatment interaction ($F_{20,150}$ = 3.730, $p < 0.0001$)]. As shown in Table 1, the inhibitory effect caused by MXE (ED_{50} ~ 0.57 mg/kg) appeared to be greater than that induced by ketamine (ED_{50} ~ 1.45 mg/kg). MXE induced a greater maximal inhibitory effect on visual placing response (ED_{50} ~ 0.31 mg/kg) than that induced by ketamine (ED_{50} ~ 0.56 mg/kg; Table 1). In particular, at 3 mg/kg dose, MXE is more effective than ketamine (t = 2.325, df = 10; P = 0.0424) and induces a greater maximum inhibitory effect (t = 2.280, df = 10; P = 0.0458; Table 1).

Visual placing response did not change in vehicle-treated rats over 180 min observation (Figure 3F). Administration of ketanserine (0.1 mg/kg i.p.) did not affect visual placing response in rats (Figure 3F) but partially prevented the inhibitory effect induced by MXE 3 mg/kg ($F_{3,20}$ = 33.76; $p < 0.0001$) and ketamine 3 mg/kg ($F_{3,20}$ = 23.33; $p < 0.0001$). Similarly, MDL 100907 (0.03 mg/kg i.p.) did not affect per se visual object response in rats (Figure 3F) but partially prevented the inhibitory effect induced by MXE 3 mg/kg ($F_{3,20}$ = 41.38; $p < 0.0001$) and ketamine 3 mg/kg ($F_{3,20}$ = 24.65; $p < 0.0001$).

3.2.4 Startle and Pre-Pulse Inhibition

Saline injection did not change startle/PPI response in rats, and the effect was similar in naïve untreated animals (data not shown). Startle amplitude in rats was not modified by the administration of MXE (0.3-3 mg/kg; Figure 4A; $F_{3,14}$ = 0.3149; P = 0.8143) and ketamine (0.3-3 mg/kg; Figure 4A; $F_{3,14}$ = 0.8305; P = 0.4990).

MXE at the highest dose of 3 mg/kg inhibited the PPI in rats at 68 ($F_{3,14}$ = 17.30; $P < 0.0001$), 75 dB ($F_{3,14}$ = 11.70; P = 0.0004) and 85 dB ($F_{3,14}$ = 9.222; P = 0.0013) of pre-pulse intensity (Figure 4B) while lower doses tested (0.3 and 1 mg/kg) were ineffective. Similarly, ketamine at the highest dose of 3 mg/kg inhibited the PPI in rats at 68 ($F_{3,14}$ = 11.25; P = 0.0005) and 75 dB ($F_{3,14}$ = 8.223; P = 0.0021) but not at 85 dB ($F_{3,14}$ = 2.565; P = 0.0963) of pre-pulse intensity (Figure 4D) while lower doses tested (0.3 and 1 mg/kg) were ineffective.

Vehicle injection did not change startle/PPI response in rats, and the effect was similar in naïve untreated animals (data not shown). Systemic administration of ketanserine (0.1 mg/kg i.p.) did not affect startle amplitude in rats (data not shown) but prevented the inhibition of PPI induced by MXE 3 mg/kg at 68 ($F_{3,14}$ = 19.09; $P < 0.0001$), 75 dB ($F_{3,14}$ = 13.91; P = 0.0002) and 85 dB ($F_{3,14}$ = 11.79; P = 0.0004) of pre-pulse intensity (Figure 5A). Ketanserin also prevented the inhibitory effect of ketamine 3 mg/kg on PPI in rats at 68 ($F_{3,14}$ = 12.37; P = 0.0003) and 75 dB ($F_{3,14}$ = 10.27; P = 0.0008) of pre-pulse intensity (Figure 5B). Similarly, systemic administration of MDL 100907 (0.03 mg/kg

i.p.) did not affect startle amplitude in rats (data not shown) but prevented the inhibition of PPI induced by MXE 3 mg/kg at 68 ($F_{3,14} = 18.16$; $P < 0.0001$), 75 dB ($F_{3,14} = 12.79$; $P = 0.0003$) and 85 dB ($F_{3,14} = 15.11$; $P = 0.0001$) of pre-pulse intensity (Figure 5C). MDL 100907 also prevented the inhibitory effect of ketamine 3 mg/kg on PPI in rats at 68 ($F_{3,14} = 12.40$; $P = 0.0003$) and 75 dB ($F_{3,14} = 11.04$; $P = 0.0006$) of pre-pulse intensity (Figure 5D).

3.3 *In vitro* electrophysiology

3.3.1 MXE inhibits glutamatergic NMDA-mediated postsynaptic potentials in a dose dependent manner: comparison with ketamine

In a first set of experiments we evaluated the effect of MXE perfusion at different concentrations (1-50 µM) on the NMDA component of extracellular post synaptic excitatory potentials (fEPSP) recorder in the dendritic layer of mPFC pyramidal neurons. After a control period, during which we observed a stable response using an electric stimulation (0.3-0.5 mA) that induced a 50% of the maximal response, we applied MXE at various concentration and observed its effect for a period of 10 min. Perfusion of MXE caused a concentration dependent inhibition of NMDA fEPSP with a significant effect ($F_{3,19} = 1.07$, $p < 0.001$) at concentrations as low as 5 µM (Figure 6A,D). On the other hand ketamine showed a significant ($F_{3,17} = 0.07$, $p < 0.01$) effect at concentration as low as 10 µM (Figure 6B,D). In order to further compare the effect of both drugs, the two-way ANOVA with Bonferroni *post-hoc* test revealed a lower IC50 showed by MXE (9.2 µM) and a higher maximal effect (81.42 ± 6.5%) ($F_{1,35} = 6.59$, $p < 0.05$) compared with ketamine (IC50, 11.7 µM; max eff, 59 ± 9.7%) (Figure 6C).

3.3.2 Both MXE and ketamine failed to alter AMPA component of field postsynaptic potentials and presynaptic glutamate release

In order to assess the effect of MXE on AMPA receptors we performed recordings of fEPSP from mPFC pyramidal neurons in the presence of the NMDA antagonist AP5 (50 µM) and applied both MXE and ketamine at the concentration where we have seen a significant effect on NMDA-mediated potentials. Ten minutes of drugs perfusion at the concentration of 10 µM, failed to alter the AMPA component of fEPSPs (Figure 7A-C). Through the paired pulse protocol, we then evaluated whether MXE or ketamine may affect the presynaptic release of glutamate. This protocol consists in a change in the second postsynaptic response cause by two electrical stimulations of equal intensity applied at presynaptic sites (at an interpulse interval of 100 msec). It has been widely reported that changes in the paired pulse ratio is related to transmitter release (Mennerick and Zorumski 1995). In our experimental condition, perfusion of MXE or ketamine, at the concentration that induces significant inhibition at the NMDA component, failed to alter the AMPA-mediated fEPSP paired

pulse ratio when applied for 10 min, suggesting a lack of effect on the probability of presynaptic release of glutamate (Figure 7D-F).

3.3.3. Methoxetamine inhibits GABA-mediated spontaneous currents in a dose dependent manner

With the aim to explore whether MXE might affect other neurotransmitter systems we evaluated the potential effect of MXE on GABA-mediated currents recorded from mPFC pyramidal neurons. Whole cell patch clamp recordings in the presence of the non-selective glutamate receptors antagonist kynurenic acid (1 mM) revealed an evaluable amount of GABA-mediated spontaneous currents (sIPSCs) recorded from mPFC principal neurons (average: amplitude 30.9 ± 4.8 pA; decay time 35.4 ± 0.7; event frequency 2.2 ± 0.7 Hz) (Figure 8A CTRL). The perfusion of different concentrations of MXE (1-10 mM) caused a dose-dependent reduction of sIPSCs frequency without any effect on event amplitude or decay time suggesting an action in the probability of neurotransmitter release at presynaptic level with no effect on postsynaptic GABAA receptors. One-way ANOVA revealed that both 5 and 10 but not 1 mM of MXE were effective in modulating sIPSCs frequency [$F(3, 28) = 4,09$, $p = 0.015$].

3.3.4 Methoxetamine fails to alter endocannabinoid-mediated effects on mPFC GABAergic synapses

The latest results obtained on GABAergic synapses in mPFC principal neurons led us to examine also the possible interactions between MXE and endocannabinoid signaling at this level since presynaptic cannabinoid CB1 receptors are strongly implicated in the control of neurotransmitter release (Augustine and Lovinger, 2018). In order to assess this aspect, we used the DSI protocol before and 10 min after MXE (10 mM) perfusion. As expected, DSI protocol caused a fast but short-term (only during the first 15 sec after depolarization) decrease ($t = 10.31$, $df = 8$, $p = 0.011$) in sIPSCs frequency (Figure 9A,B) with no change in amplitude (Figure 9A,C), in agreement with the activation of presynaptic cannabinoid CB1 receptors by the DSI-induced release of endocannabinoids from post synaptic compartments (Augustine and Lovinger, 2018). After 10 min of MXE perfusion, the amount of DSI on sIPSCs frequency was indistinguishable to that obtained before drug perfusion (Figure 9A,B).

4. Discussion

The present study was undertaken to expand our previous investigation on the neurochemical, behavioral and electrophysiological properties of MXE in rats with the ultimate goal to increase our knowledge on the brain neurotransmitter systems involved in the action of this ketamine-like NPS. We have previously demonstrated that MXE is able to induce positive reinforcing and discriminative stimulus effects in rats likely increasing the mesolimbic dopaminergic neurotransmission (Chiamulera et al., 2016; Mutti et al., 2016). However, the wide variety of behavioral effects induced by a single administration of MXE in both rats (Zanda et al., 2017) and mice (Ossato et al., 2018) suggested the involvement of other neurotransmission systems. In light of previous evidence of (i) the stimulation of serotonin release induced in mice by a very high dose of MXE (Fuchigami et al., 2015), (ii) the serotonergic damage observed in repeatedly MXE-exposed rats (Costa et al., 2019) and (iii) the reversal of MXE-induced antidepressant effects by serotonin 5-HT2 receptors blockade (Botanas et al., 2017), we started by evaluating the role of the serotoninergic system in modulating the neurochemical and behavioral effects of acute administration of MXE in rats.

In vivo microdialysis experiments showed a small increase in serotonin concentration after injections of MXE, which was immediate and transient but not significant in the PFC and significant but of late-onset in the NAc shell. Accordingly, its referent compound, ketamine, has been consistently reported to significantly stimulate serotonin release in the rat PFC (López-Gil et al., 2019, Kinoshita et al., 2018; Gasull-Camós et al., 2018; Nishitani et al., 2014). In light of a previous study showing MXE as able to induce a robust increase in the extracellular concentrations of serotonin in the mPFC of mice (Fuchigami et al., 2015), the small increase observed in the present study was quite unexpected. Yet, it is possible that the low dose used in our study (MXE 0.125-0.5 mg/kg), that resulted in a positive trend only, was not enough to increase the extracellular concentration of serotonin in the rat PFC to the same level observed by Fuchigami et al. after administration of 20 mg/kg of MXE Conversely, the significant and late onset increasing effect of MXE observed in the NAc shell could be responsible, at least in part, of the long-lasting dissociative effects reported by MXE users (Van Hout and Hearne, 2015). We thus decide to investigate further the role of the serotonin system in MXE-induced effects and, in light of previous findings linking the 5-HT2 receptor to the behavioral effects of MXE (Botanas et al., 2017) and MXE analogs (Sayson et al., 2019), we examined the role of this class of serotonin receptors in the expression of MXE-induced sensorimotor alterations.

As we expected in light of our recent study in mice (Ossato et al. 2018), we found that both MXE and ketamine significantly increased spontaneous motor activity in rats, with MXE showing overall a more potent motor effect than ketamine. Our finding that both 5-HT2 receptor antagonists,

ketanserin and MDL 100907, were able to significantly attenuate MXE- and ketamine-induced hypermotility is in line with a recent study showing that ketanserin prevents ketamine-evoked development of locomotor sensitization in mice (Galvanho et al., 2020). Visual sensory responses were also affected in a dose-dependent manner by acute administration with either MXE or ketamine, confirming our previous observations of MXE-induced sensorimotor deficit in mice (Ossato et al., 2018) and providing a solid rationale for the severe MXE-induced impairment in driving ability (Elian and Hackett, 2014). Interestingly, even in the visual object response and the visual placing response test, MXE induced more potent effects than ketamine and reduced animals' responses for a longer period of time. In both tests, pretreatment with ketanserin or MDL 100907 significantly reduced MXE- and ketamine-induced deficit in visual sensory responses, although animals' performance did not fully recover to controls level. Finally, in line with our previous observation in mice (Ossato et al., 2018), we observed a dramatic impairment of the prepulse inhibition (PPI) of the acoustic startle reflex, an operational measure of the sensory gating, which confirmed the ability of MXE to induce sensory and information processing deficits that could explain the dissociative/psychotic effects reported by users. Notably, the remarkable reduction of the PPI induced by MEX was completely prevented by the pretreatment with either ketanserin or MDL 100907, in a manner very similar to that observed for ketamine, suggesting that also the detrimental effects of MEX on information processing and attention functions are mediated through the 5-HT2 receptors. These findings could explain the severe dissociative symptoms, the impairment of sensory functions and the attention deficit reported in MXE-intoxicated patients (Moccia et al., 2019) and described in MXE-treated mice (Ossato et al., 2019). On the other hand, the inability of the two serotonin antagonists to fully prevent MXE-induced hyperlocomotion and visual sensory responses deficit in rats suggests that MXE-induced effects on motor and sensory functions are only in part mediated by the 5-HT2 receptors and that other receptors and/or neurotransmitter systems are likely involved.

We therefore conducted *in vitro* electrophysiological experiments in brain slice to further explore whether other neurotransmitters system are probably affected by MEX exposure. Extracellular field potential recordings in the glutamatergic synapses of the rat mPFC showed that MXE significantly and dose-dependently reduces the postsynaptic potentials mediated by the activation of NMDA receptors. This finding strengthens the notion that MXE, like ketamine, is a NMDA antagonist/negative modulator (Roth et al., 2013), but with a potency and efficacy higher than ketamine. Intriguingly, the effects of MXE seem to be selective for NMDA receptors, as it is ineffective in modulating AMPA responses. Lack of effect of MXE on AMPA receptors was quite unexpected, since its rapid antidepressant effect observed in the forced swim test was reported to be fully prevented in mice by pre-treatment with the AMPA receptor antagonist NBQX (Botanas et al.,

2017), suggesting that our experimental conditions were not appropriate to reveal such an effect. Importantly, although our paired pulse stimulation and patch clamp experiments revealed, respectively, that MXE failed to alter glutamate release at the presynaptic level and is unlikely to impact on the endocannabinoid system, we found that MXE is able to alter GABA neurotransmission in the mPFC. Specifically, MXE reduces the probability of GABA presynaptic release, as evidenced by the reduction of the frequency of spontaneous sIPSCs currents, without affecting the current amplitude and the decay time, thus excluding an effect on postsynaptic GABAA receptors. This latter result confirms previous observations showing that MXE is able to decrease neuronal activity *in vitro* and confirms the multiple modes of action of MXE (Hondebrink et al., 2017).

Conclusions

Altogether, findings of the present study extend the current knowledge about the effect of a single exposure to MXE on brain neurotransmission systemsby (i) pointing to the serotonin 5-HTs receptors as major players of its effects on motor activity, sensory responses and sensorimotor gating, (ii) confirming its selective action on the glutamatergic system as NMDA, and not AMPA, receptor antagonist without affecting glutamate release, (iii) revealing an inhibitory effect of MXE on GABA release, and (iv) excluding, at least in our experimental conditions, an involvement of the endocannabinoid system in the action of MXE. The use of animal models as well as multidisciplinary studies may help to better understand the pharmacology of new ketamine-like dissociative compound like MXE, which are among the most frequent NPS detected in intoxicated patients (Hondebrink et al., 2015; Papa et al., 2021), will provide clinicians and emergency staff new strategies to recognize symptoms and manage intoxicated patients presenting at emergency centers.

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Figures

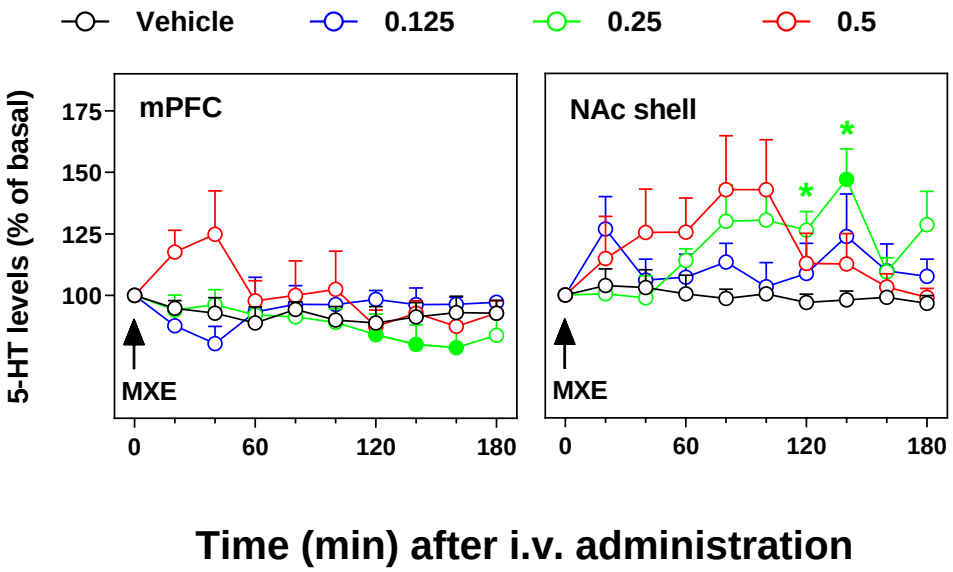
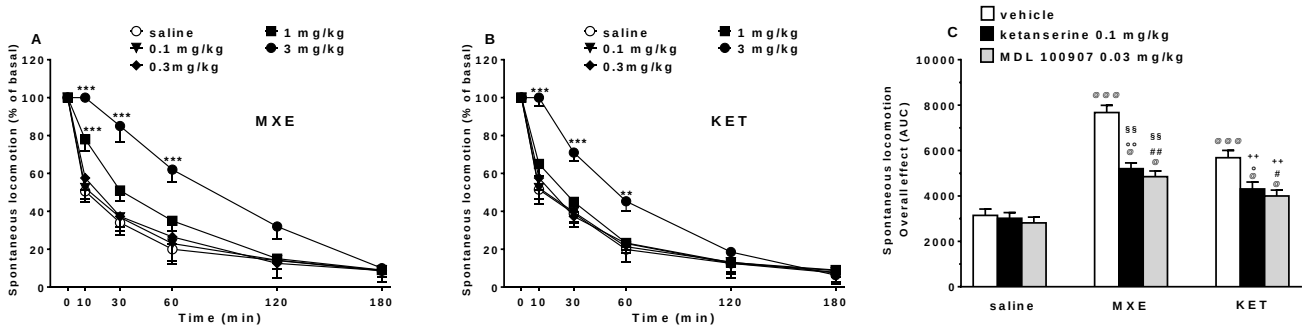


Figure 1.



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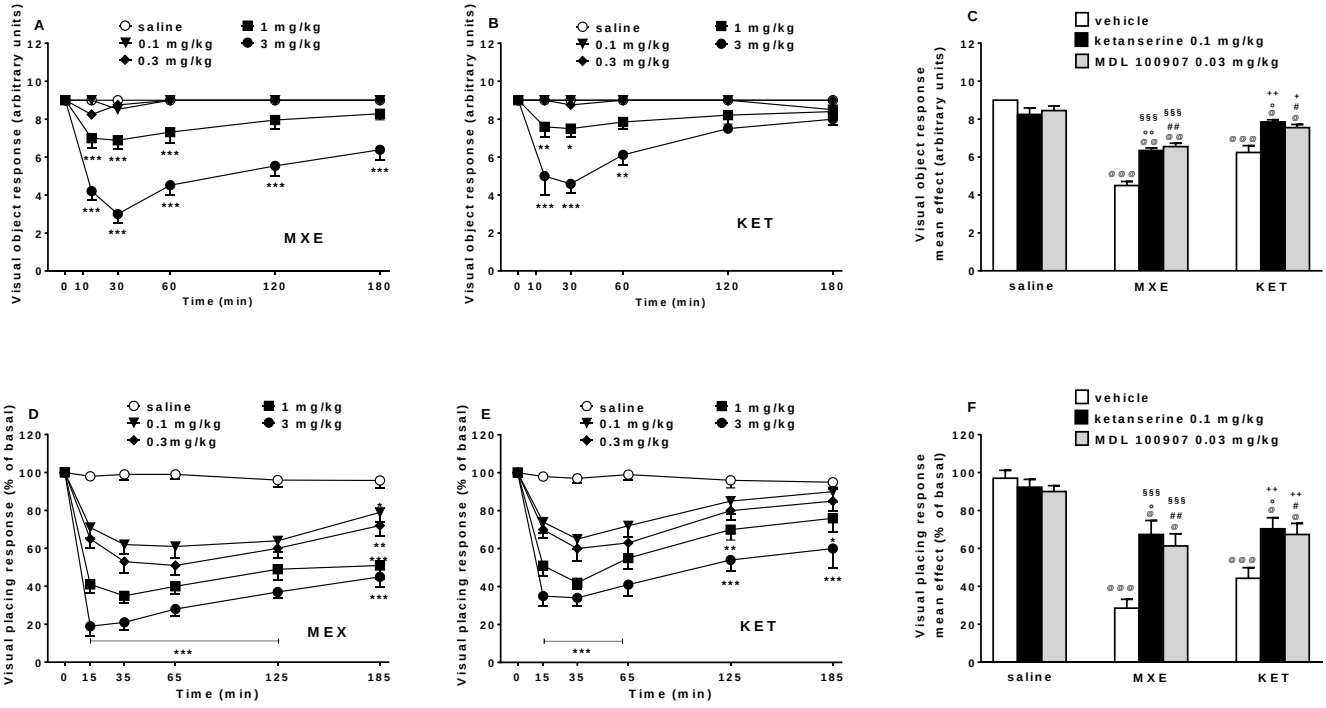

Figure 3

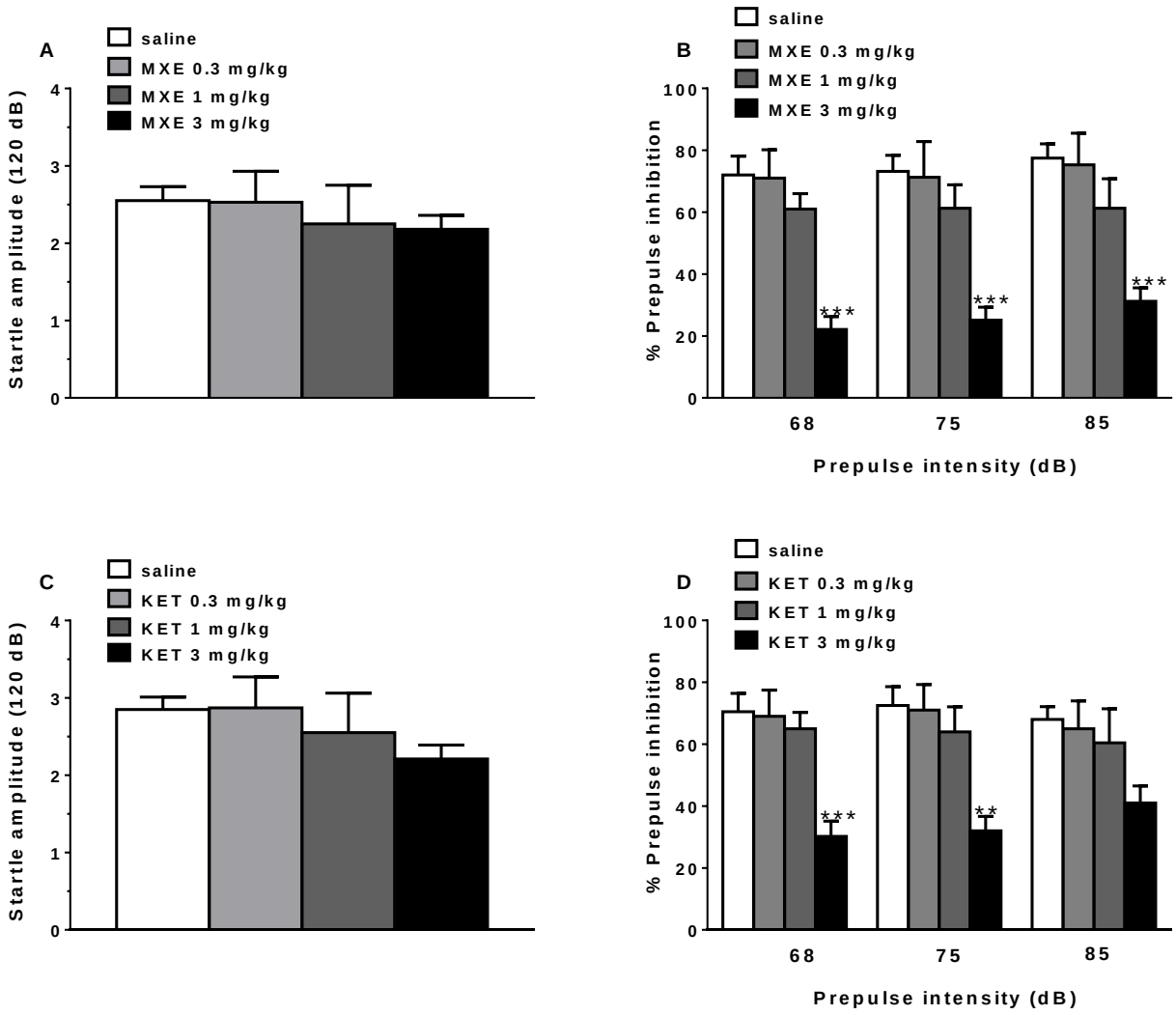
A
saline
MXE 0.3 mg/kg
MXE 1 mg/kg
MXE 3 mg/kg
Startle amplitude (120 dB)
0
1
2
3
4
B
saline
MXE 0.3 mg/kg
MXE 1 mg/kg
MXE 3 mg/kg
% Prepulse inhibition
0
20
40
60
80
100
***
***
***
68
75
85
Prepulse intensity (dB)
C
saline
KET 0.3 mg/kg
KET 1 mg/kg
KET 3 mg/kg
Startle amplitude (120 dB)
0
1
2
3
4
D
saline
KET 0.3 mg/kg
KET 1 mg/kg
KET 3 mg/kg
% Prepulse inhibition
0
20
40
60
80
100
***
**
68
75
85
Prepulse intensity (dB)

Figure 4

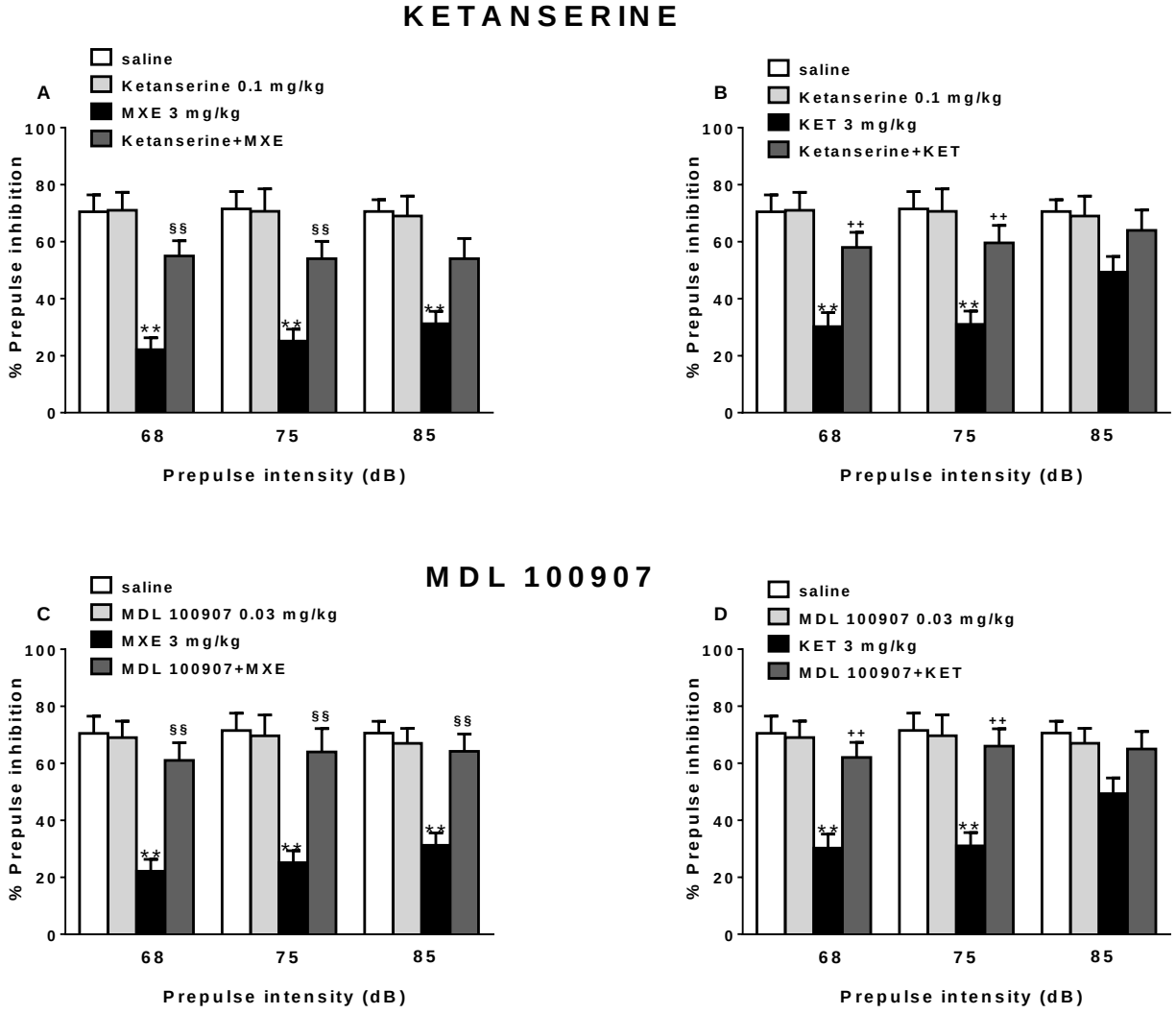
KETANSERINE
A
saline
Ketanserine 0.1 mg/kg
MXE 3 mg/kg
Ketanserine+MXE
B
saline
Ketanserine 0.1 mg/kg
KET 3 mg/kg
Ketanserine+KET
MDL 100907
C
saline
MDL 100907 0.03 mg/kg
MXE 3 mg/kg
MDL 100907+MXE
D
saline
MDL 100907 0.03 mg/kg
KET 3 mg/kg
MDL 100907+KET
% Prepulse inhibition
Prepulse intensity (dB)
68
75
85
0
20
40
60
80
100
**
§§
++

Figure 5

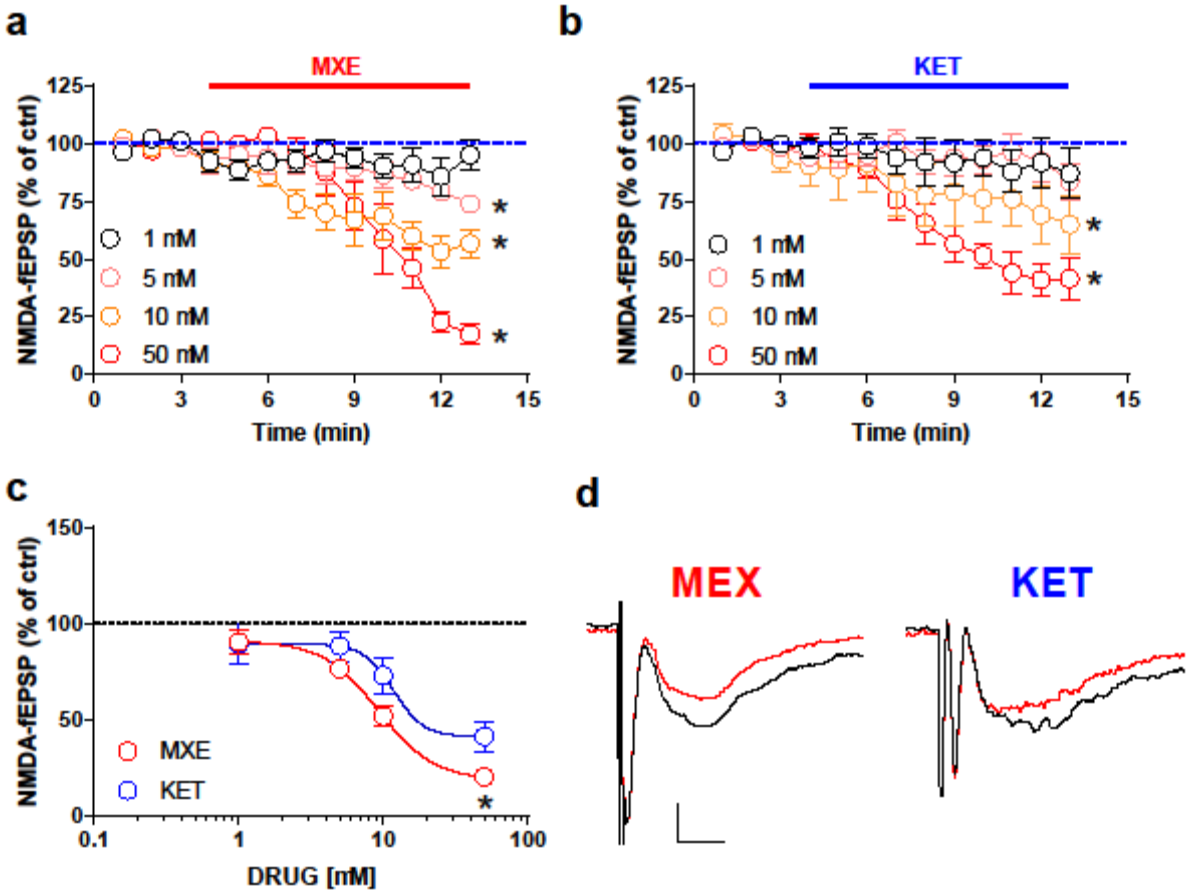
a
MXE
NMDA-fEPSP (% of ctrl)
125
100
75
50
25
0
1 mM
5 mM
10 mM
50 mM
*
*
*
0
3
6
9
12
15
Time (min)
b
KET
NMDA-fEPSP (% of ctrl)
125
100
75
50
25
0
1 mM
5 mM
10 mM
50 mM
*
*
0
3
6
9
12
15
Time (min)
c
NMDA-fEPSP (% of ctrl)
150
100
50
0
MXE
KET
*
0.1
1
10
100
DRUG [mM]
d
MEX
KET

Figure 6

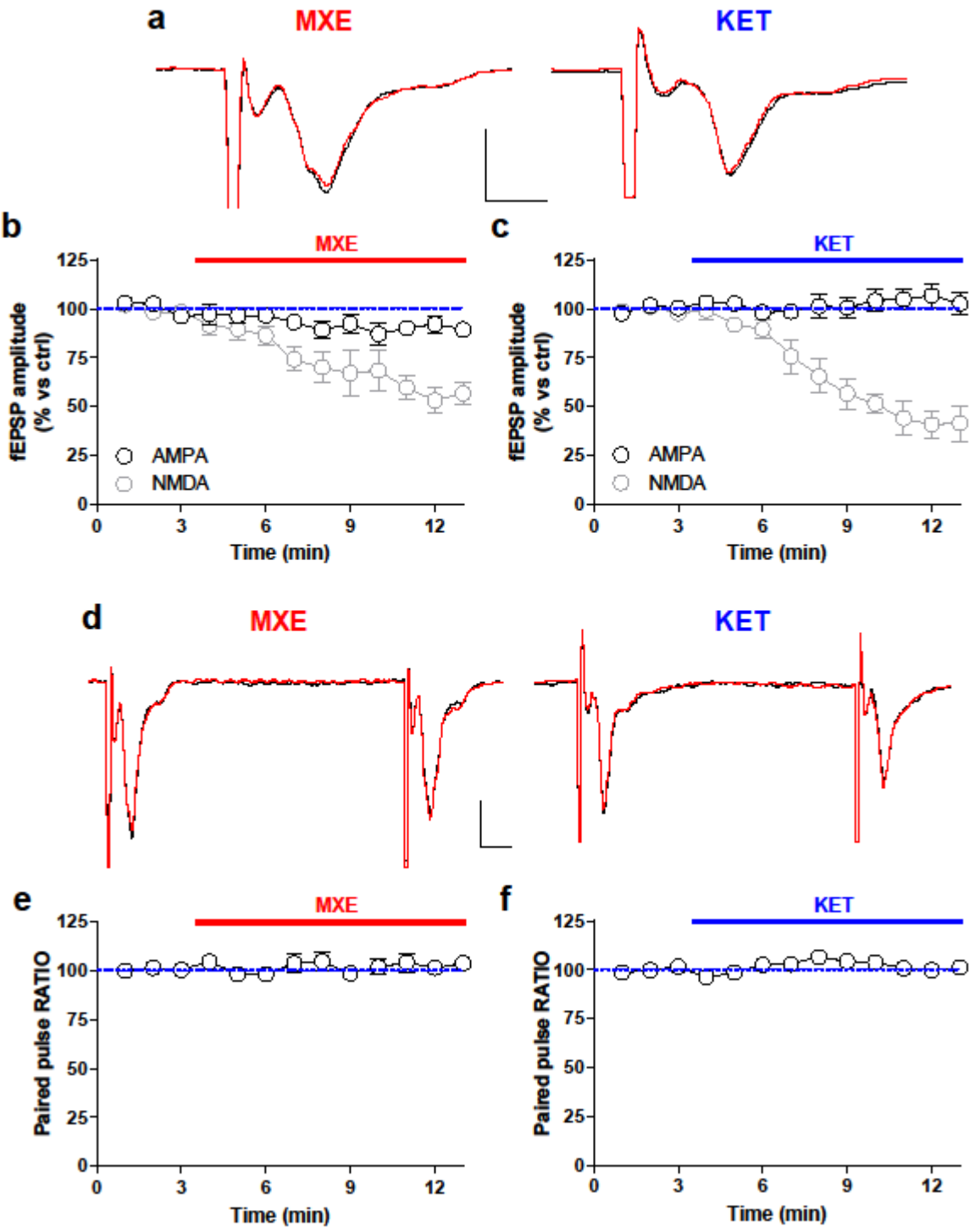
a
MXE
KET
b
MXE
fEPSP amplitude (% vs ctrl)
AMPA
NMDA
Time (min)
c
KET
fEPSP amplitude (% vs ctrl)
AMPA
NMDA
Time (min)
d
MXE
KET
e
MXE
Paired pulse RATIO
Time (min)
f
KET
Paired pulse RATIO
Time (min)

Figure 7

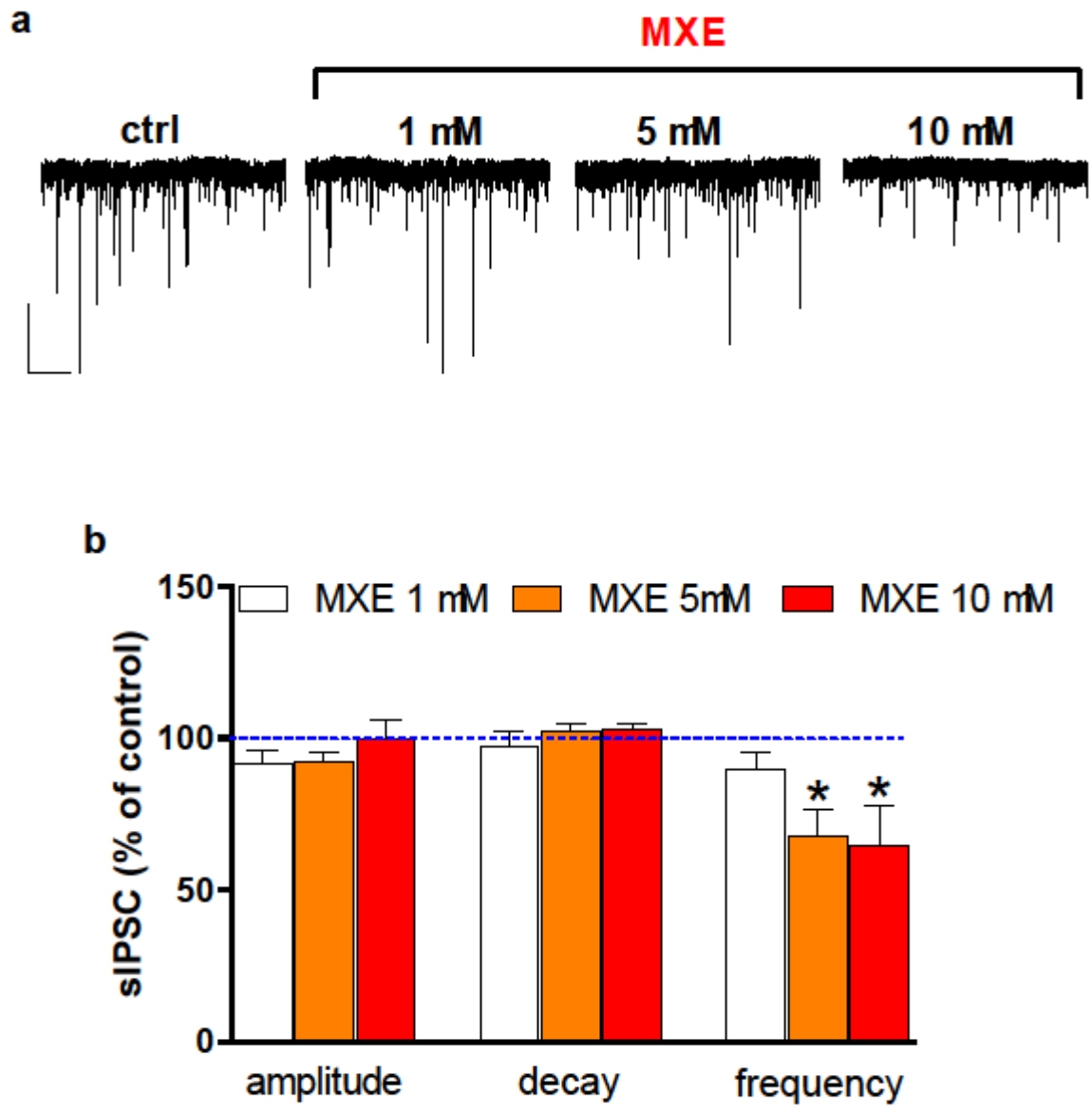
a
MXE
ctrl
1 mM
5 mM
10 mM
b
MXE 1 mM
MXE 5mM
MXE 10 mM
sIPSC (% of control)
150
100
50
0
*
*
amplitude
decay
frequency

Figure 8

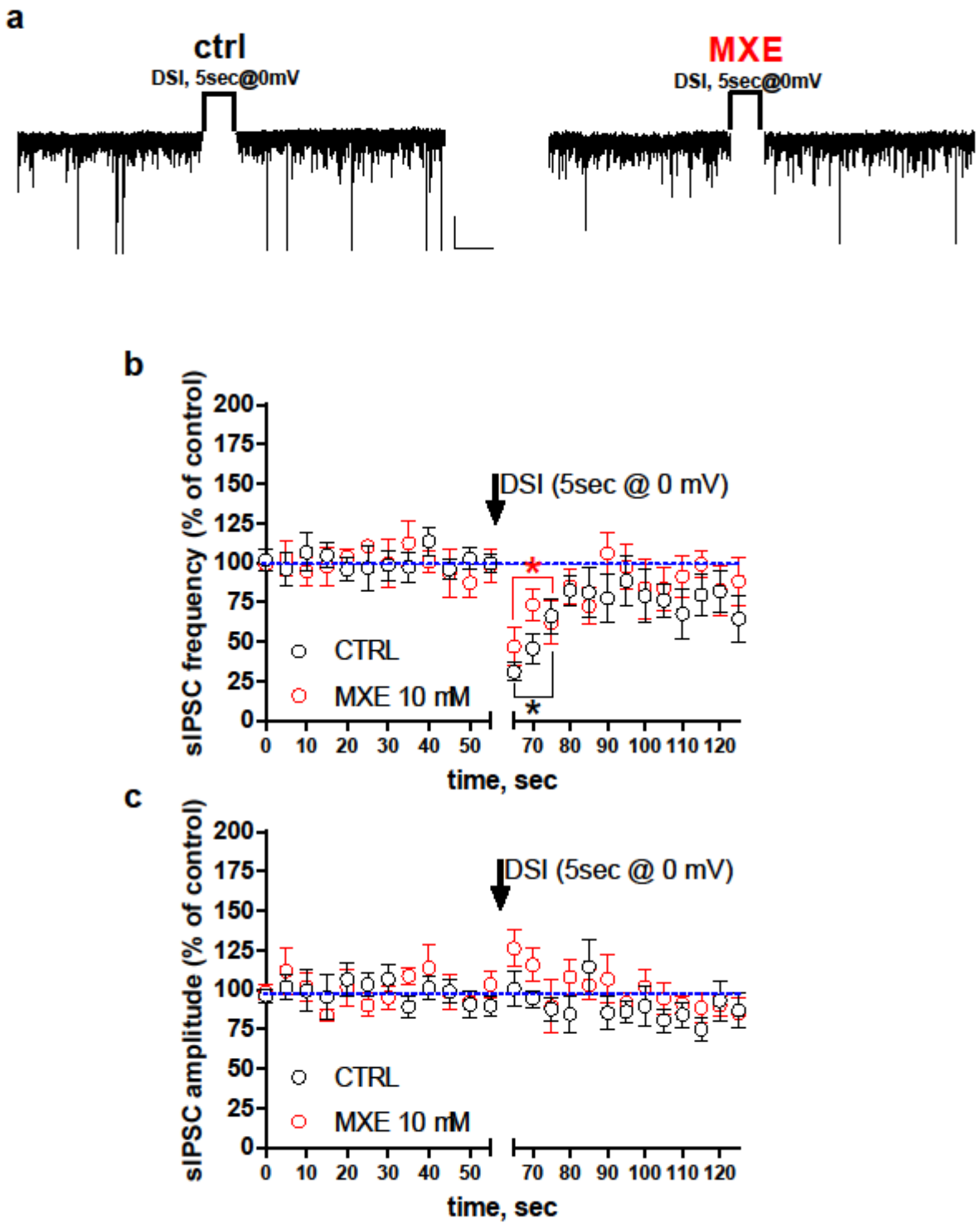
a
ctrl
DSI, 5sec@0mV
MXE
DSI, 5sec@0mV
b
sIPSC frequency (% of control)
DSI (5sec @ 0 mV)
CTRL
MXE 10 mM
time, sec
c
sIPSC amplitude (% of control)
DSI (5sec @ 0 mV)
CTRL
MXE 10 mM
time, sec

Figure 9

Table 1

Visual Object Test					
	Mean effect *arbritary units; (% of basal)*			*Max effect* *arbritary units; (% of basal)*	
	MEX	**KET**		**MEX**	**KET**
ED50 (mg/kg)	4.09 (3.75 - 4.48)	7.01 (6.61 -7.47)		2.30 (2.06 - 2.58)	3.69 (3.33 - 4.09)
Dose (mg/kg) :					
0.1	8.8 ±0.12; (97.7 ±0.12)	8.90 ±0.1; (98.8 ±0.1)		8.52 ±0.21; (94.6 ±0.21)	8.5 ±0.12; (94.4 ±0.12)
0.3	8.9 ±0.13; (98.8 ±0.13)	8.95 ±0.05; (99.4 ±0.05)		8.25 ±0.24; (91.6 ±0.24)	8.75 ±0.23; (97.2 ±0.23)
1	7.49 ±0.27; (83.2 ±0.27)	7.90 ±0.17; (87.7 ±0.17)		6.89 ±0.45; (76.5 ±0.45)	7.50 ±0.45; (83.3 ±0.45)
3	4.5 ±0.46*****; (52.4 ±0.46)	6.24 ±0.37; (69.3 ±0.67)		3.01 ±0.47*****; (34.5 ±0.67)	4.59 ±0.47;(51 ±0.67)
Visual Placing Test					
	Mean effect *(% of basal)*			*Max effect* *(% of basal)*	
	MEX	**KET**		**MEX**	**KET**
ED50 (mg/kg)	0.57 (0.40 - 0.82)	1.45 (0.98 - 2.16)		0.31 (0.22 - 0.46)	0.56 (0.36 - 0.87)
Dose (mg/kg) :					
0.1	67.4 ±3.4	77.2 ±4.5		61 ±6.3	65.3 ±4.5
0.3	60.2 ±3.9	71.6 ±4.8		51.1 ±4.5	60 ±6.3
1	43.2 ±3.1	58.8 ±6.2		34.9 ±3.6	42.1 ±3.4
3	28.5 ±4.7*****	44.8±5.2		19.0 ±5.2*****	34.1±4.1

Figure Captions

Figure 1. Effect of intravenous (i.v.) MXE administration (0.125- 0.5 mg/kg) on serotonin transmission in the rat mPFC and nucleus accumbens shell. Results are expressed as mean ± SEM of change in 5-HT extracellular levels expressed as the percentage of basal values. The arrow indicates the time of injection of vehicle (*black*) or MXE 0.125 mg/kg (*blue*), 0.25 mg/kg (*green*) or 0.5 mg/kg (*red*). Statistical analysis was performed by two-way ANOVA followed by the Tukey's HSD *post-hoc* test for multiple comparisons. Solid symbol: $p<0.05$ with respect to basal values. *$p<0.05$ MXE vs vehicle (mPFC: n=4-5; NAc shell: n=5).

Figure 2. Effect of intraperitoneal injection (i.p.) of MXE (0.1-3 mg/kg; panel A) and ketamine (KET, 0.1-3 mg/kg; panel B) on spontaneous locomotion in the rat. Interaction of MXE (3 mg/kg) and KET (3 mg/kg) with the selective 5-HT_2 receptor antagonist ketanserine (0.1 mg/kg, i.p.) and MDL 100907 (0.03 mg/kg, i.p.) were reported in panel C. Data are expressed as mean ± SEM (n=4-6/group). Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose-response curve of each compound at different times (panels A and B), while the statistical analysis of the interaction with 5-HT_2 antagonists (panel C) was performed with one-way ANOVA followed by the Bonferroni's test for multiple comparisons. **$p<0.01$, ***$p<0.001$ versus saline; $^{@}p<0.05$, $^{@@@}p<0.001$ versus vehicle; $^{+}p<0.05$, $^{++}p<0.01$ versus KET; $^{§§§}p<0.001$ versus MXE; $^{\circ}p<0.05$, $^{\circ\circ}p<0.01$ versus ketanserine; $^{\#}p<0.05$, $^{\#\#}p<0.01$ versus MDL 100907.

Figure 3. Effect of intraperitoneal injection (i.p.) of MXE (0.1-3 mg/kg; panels A, D) and ketamine (KET, 0.1-3 mg/kg; panels B, E) on the visual object (panels A, B) and placing response (panels D, E) test in the rat. Interaction of MXE (3 mg/kg) and KET (3 mg/kg) with the selective 5-HT_2 receptor antagonist ketanserine (0.1 mg/kg, i.p.) and MDL 100907 (0.03 mg/kg, i.p.) were reported in panels C and F. Data are expressed as mean±SEM (n=4-6/group). Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose-response curve of each compound at different times (panels A, B, D, E), while the statistical analysis of the interaction with 5-HT_2 antagonists (panels C, F) was performed with one-way ANOVA followed by the Bonferroni's test for multiple comparisons. *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus saline; $^{@}p<0.05$, $^{@@}p<0.01$, $^{@@@}p<0.001$ versus vehicle; $^{+}p<0.05$, $^{++}p<0.01$ versus KET; $^{§§§}p<0.001$ versus MXE; $^{\circ}p<0.05$, $^{\circ\circ}p<0.01$ versus ketanserine; $^{\#}p<0.05$, $^{\#\#}p<0.01$ versus MDL 100907.

Figure 4. Effect of intraperitoneal injection (i.p.) of MXE (0.3-3 mg/kg; panels A, B) and ketamine (KET, 0.1-3 mg/kg; panels C, D) on startle amplitude (panels A, C) and pre-pulse inhibition (PPI;

panels B, D) in the rat. Effects on PPI are shown for the three prepulse intensities (68, 75 and 85 dB), 15 minutes after treatment. Data are expressed (see material and methods) as absolute values (dB; panels A, C) and percentage decrease in the amplitude of the startle reactivity caused by presentation of the pre-pulse (% PPI; panels B, D) and values represent mean ± SEM of 5 animals for saline, MXE and KET (1 and 3 mg/kg) treatment group, while n=3 rats were used for MXE and ketamine at the ineffective dose of 0.3 mg/kg. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's test for multiple comparisons. **p<0.01 and ***p<0.001 versus vehicle.

Figure 5. Effect of ketanserine (0.1 mg/kg i.p.; panels A, B) and MDL 100907 (0.03 mg/kg i.p.; panels C and D) on MXE (3 mg/kg; panels A, C) and ketamine (KET, 3 mg/kg; panels B, D) effects on pre-pulse inhibition (PPI; panels B, D) in the rat. Effects on PPI are shown for the three prepulse intensities (68, 75 and 85 dB), 15 minutes after MXE and KET. Ketanserine and MDL100907 were administered 20 min before MXE and KET injection. Data are expressed as percentage decrease in the pre-pulse (% PPI; panels A-D) and values represent mean ± SEM of 5 animals for vehicle, MXE and KET (3 mg/kg) treatment group, while n=4 rats were used for ketanserine, MDL 100907, ketanserine +MXE/KET, MDL 100907+MXE/KET. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's test for multiple comparisons. **p<0.01 versus saline. $^{++}$p<0.01 versus KET; §§p<0.01 versus MXE.

Figure 6. Effect of MXE (1-50 nM) on glutamatergic NMDA-mediated field postsynaptic potentials and comparison with ketamine (KET, 1-50 nM). A) Scatter plot indicating the effect of 10 min bath perfusion of increasing concentrations of MEX (1-50 μM) in the NMDA-mediated fEPSPs recorded in layer 1 of mPFC pyramidal neurons (n = 5-8). $p < 0.05$ vs baseline, one-way ANOVA. B) Scatter plot indicating the effect of 10 min bath perfusion of increasing concentrations of KET (1-50 μM) in the NMDA-mediated fEPSPs recorded in layer 1 of mPFC pyramidal neurons (n = 5-6). $p < 0.05$ vs baseline, one-way ANOVA. C) Comparison between MEX and KET on their effects at all concentration tested in panels A and B. $p < 0.05$ vs KET, two-ways ANOVA. D) Representative traces of NMDA-mediated component before (*black trace*) and after (*red trace*) 10 min of drug perfusion. Recordings were obtained in the presence of the AMPA selective antagonist NBQX (5 μM). Scale-bar 1 mV/10 ms.

Figure 7. Effect of MXE on AMPA component of field postsynaptic potentials and presynaptic probability of glutamate release in comparison with ketamine (KET). A) Representative traces of AMPA-mediated component before (*black trace*) and after (*red trace*) 10 min of drug perfusion. Scale-bar 2 mV/10 ms. B) Scatter plot indicating the effect of 10 min bath perfusion of MEX (10 μM) in the AMPA-mediated fEPSPs recorded in layer 1 of mPFC pyramidal neurons (n=9). For

comparison, we added, in gray, the effect of MXE on NMDA component as shown in Figure 1A using the same concentration of the drug. C) Scatter plot indicating the effect of 10 min bath perfusion of KET (10 µM) in the AMPA-mediated fEPSPs recorded in layer 1 of mPFC pyramidal neurons (n=8). For comparison, we added. in gray. the effect of KET on NMDA component as shown in Figure 1B using the same concentration of the drug. D) Representative traces of AMPA-mediated component before (black trace) and after (red trace) 10 min of drug perfusion using the protocol of paired pulse with an interpulse interval of 100 msec. Scale-bar 2 mV/10 ms. E) Scatter plot indicating the effect of 10 min bath perfusion of MEX (10 µM) (red traces) in the ratio between the amplitude of the second response and the amplitude of the first using the paired pulse protocol (n=9). F) Scatter plot indicating the effect of 10 min bath perfusion of KET (10 µM) (red traces) in the ratio between the amplitude of the second response and the amplitude of the first using the paired pulse protocol (n=8).

Figure 8. Effect of MXE on GABA-mediated spontaneous postsynaptic currents. A) Representative traces of GABA-mediated sIPSCs recorded from pyramidal neurons of mPFC in the absence (CTRL) and presence of increasing concentrations (1-10 µM) of MXE perfused for 10 min at each concentration. Scale-bar 40 pA/20 s. b) Bar graph representing the average of sIPSC amplitude, decay time and frequency in the presence of increasing concentrations of MXE. $p < 0.05$ vs baseline, one-way ANOVA, (n = 8).

Figure 9. Effect of MXE on endocannabinoid-mediated effects on mPFC GABAergic synapses . A) Representative traces of GABA-mediated sIPSCs recorded from pyramidal neurons of mPFC in the absence (CTRL) and presence of MXE (10 µM) perfused for 10 min during the application of DSI protocol. Scale-bar 30 pA/20 s. B) Scatter plot representing the change in event frequency (bin 5 sec) before and after de depolarization of membrane potential up to 0 mV for 5 s. The protocol of DSI have been applied before (CTRL) and after the perfusion of the drug for 10 min. $p < 0.05$ vs baseline, one-way ANOVA, (n = 8). C) Scatter plot representing the change in event amplitude (bin 5 sec) before and after de depolarization of membrane potential at 0 mV for 5 sec.

Table 1. Mean and maximal effect of MXE (0.1-3 mg/kg i.p.) and KET (0.1-3 mg/kg) on the visual object and visual placing test in the rat. Data are expressed as arbitrary units (visual object test) or percentage of basal values (visual object and visual placing test) and represent the mean ± SEM of 6 animals for each treatment. Unpaired Student's t-test (two-tailed) was used to determine statistical significance ($P<0.05$) between two groups. *$p<0.05$, versus KET at the same dosage.

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