

# CHARACTERIZATION OF THE NEUROCHEMICAL AND BEHAVIORAL EFFECTS OF THE PHENETHYLAMINE 2-CI-4,5-MDMA IN ADOLESCENT AND ADULT MALE RATS

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## Significance statement

The advent of Novel Psychoactive Substances (NPS) has contributed to a new ‘drug scenario’ characterized by an increased number of drug users among youth and consumption of drugs with unknown effects and safety profiles. NPS may produce effects comparable to those of ‘classical’ psychoactive drugs, such as Cannabis, heroin, cocaine and MDMA, however, with more severe consequences. In this alarming context, this study was aimed at characterizing the pharmacological profile of the NPS 2-Cl-4,5-MDMA.

2-Cl-4,5-MDMA differentially affected DA and 5-HT transmission at adulthood and adolescence. It more markedly increased dialysate DA in the NAc shell and mPFC of adult than adolescent rats. The opposite was observed on dialysate 5-HT in the NAc shell, with adolescents being more responsive than adults. The behavioral effects of 2-Cl-4,5-MDMA also showed some differences in the two age groups.

Data will be helpful to understand and prevent health damage in 2-Cl-4,5-MDMA users of different ages.

## ABSTRACT

### Background

The proliferation of Novel Psychoactive Substances (NPS) in the drug market raises concerns about uncertainty on their pharmacological profile and the health hazard linked to their use. Within the category of synthetic stimulant NPS, the phenethylamine 2-Cl-4,5-methylenedioxymethamphetamine (2-Cl-4,5-MDMA) has been linked to severe intoxication requiring hospitalization. Thereby, the characterization of its pharmacological profile is urgently warranted.

### Methods

By *in vivo* brain microdialysis in adolescent and adult male rats we investigated the effects of 2-Cl-4,5-MDMA on dopamine (DA) and serotonin (5-HT) neurotransmission in two brain areas critical for the motivational and reinforcing properties of drugs, the nucleus accumbens (NAc) shell and the medial prefrontal cortex (mPFC). Moreover, we evaluated the locomotor and stereotyped activity induced by 2-Cl-4,5-MDMA, and the emission of 50-kHz ultrasonic vocalizations (USVs) to characterize its affective properties.

### Results

2-Cl-4,5-MDMA increased dialysate DA and 5-HT in a dose-, brain area-, and age-dependent manner. Notably, 2-Cl-4,5-MDMA more markedly increased dialysate DA in the NAc shell and mPFC of adult than adolescent rats, while the opposite was observed on dialysate 5-HT in the NAc shell, with adolescent rats being more responsive. Furthermore, 2-Cl-4,5-MDMA stimulated locomotion and stereotyped activity in both

adolescent and adult rats, although to a greater extent in adolescents. Finally, 2-Cl-4,5-MDMA did not stimulate 50-kHz USV emissions.

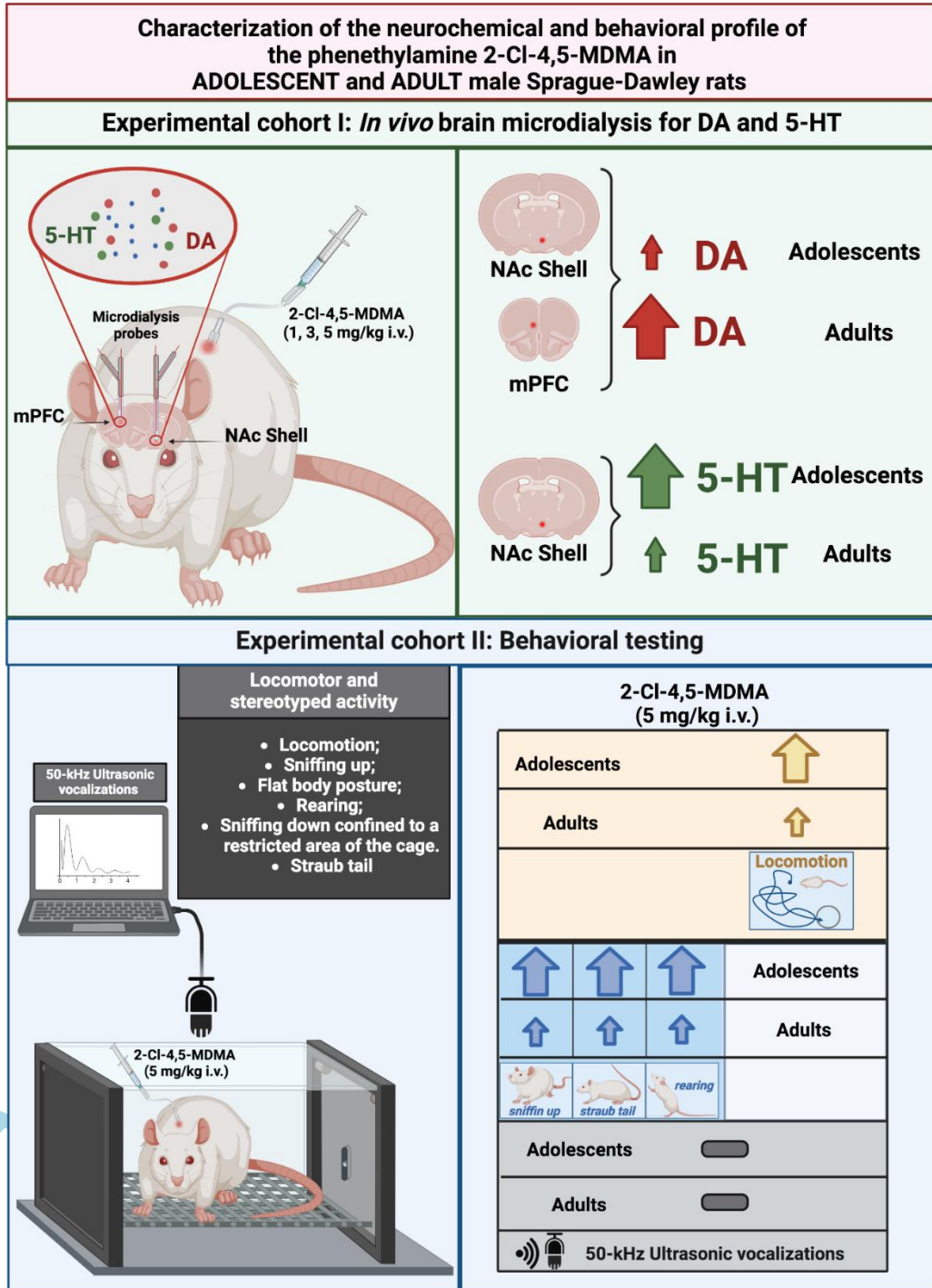
### **Conclusions**

This is the first pharmacological characterization of 2-Cl-4,5-MDMA demonstrating that its neurochemical and behavioral effects may differ between adolescence and adulthood. These preclinical data could help understanding the central effects of 2-Cl-4,5-MDMA, by increasing awareness on possible health damage in users.

**Key words:** addiction, dopamine, Novel Psychoactive Substances, serotonin, ultrasonic vocalizations.

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Graphical abstract



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## INTRODUCTION

Novel psychoactive substances (NPS) are a group of substances not controlled under the international drug conventions (UNODC, 2013, 2022). NPS can be either analogous to existing controlled drugs or new chemicals synthesized to mimic the effects of controlled drugs, thus evading drug control policy. The proliferation of NPS in the global market poses serious health hazard and raises concerns over uncertainty on their toxicity and clinical treatment of NPS-induced intoxication (Al-Banaa et al., 2020; Costa et al., 2020; Dinis-Oliveira and Magalhães, 2020). NPS have been divided into four main classes, based on their psychopharmacological effects: cannabinoids, depressants, hallucinogens, and stimulants (Tracy et al., 2017). Alternatively, NPS can be categorized into six groups based on their chemical structure: alkylindoles, arylcyclohexylamines, phenethylamines, piperazines, synthetic cathinones, and tryptamines (Miliano et al., 2016).

At the end of 2021, the EU Early Warning System was monitoring around 880 NPS, 106 of which were phenethylamines (EMCDDA, 2022). Phenethylamines may act as either stimulants or hallucinogens, but also possess entactogenic effects (Schifano et al., 2007; 2015). Among the recently emerged phenethylamines is 2-chloro-4,5-methylenedioxymethamphetamine (2-Cl-4,5-MDMA), also known as 6-Cl-MDMA, which was firstly detected in seizures of MDMA tablets (Lewis et al., 2000). Afterward, a case report of a 29-year-old male polydrug user transported to hospital in a state of unconsciousness and hypoxia, and displaying bradycardia and hypoventilation, raised concerns since toxicological analysis detected 2-Cl-4,5-MDMA in his urine (Maresova et al., 2005). Although 2-Cl-4,5-MDMA is hypothesized to be a residual impurity in the

synthesis of MDMA by illicit laboratories (Plummer et al., 2016), it is nonetheless circulating in the NPS market as an individual substance. Considering the bulk of preclinical studies that demonstrate the existence of severe neurotoxic effects of MDMA (Lyles and Cadet, 2003; Costa and Golembioskwa, 2022), and brain dysfunctions, serotonin syndrome, and hepatotoxicity linked to the use and misuse of MDMA (Liechti, 2003; Patel et al., 2004), the lack of knowledge on the pharmacological and toxicological properties of 2-Cl-4,5-MDMA poses serious risks to users. MDMA and analogs are usually consumed for their ability to increase empathy, sociability, perceptions of sounds and colors, and to induce mild hallucinations (Green et al., 2003), and are most commonly used by adolescents. Adolescence is a critical period of brain development, highly sensitive to the rewarding effects of drugs (Corongiu et al., 2020) but also to the harmful consequences that the use of drugs may have on neural circuitries that are still under development (Keshavan et al., 2014; Richmond-Rakerd et al., 2017), such as the dopamine (DA) and serotonin (5-HT) pathways (Lazenka et al., 2017). This study was performed to investigate the changes in DA and 5-HT neurotransmission elicited by 2-Cl-4,5-MDMA in the nucleus accumbens shell (NAc shell) and medial prefrontal cortex (mPFC). We followed this approach because DA transmission in the NAc shell and mPFC crucially mediates the rewarding and addictive properties of drugs of abuse (Di Chiara et al. 2004; De Luca et al., 2011), whereas 5-HT transmission in the same areas shapes the entactogenic effects of MDMA-like substances (Green et al., 2003; Kehr et al., 2011; Heal et al., 2023) to which the phenethylamine 2-Cl-4,5-MDMA belongs. In order to characterize the neurochemical effects induced by 2-Cl-4,5-MDMA and the associated behavioral manifestations, we performed: i) *in vivo* microdialysis experiments to assess

the levels of DA and 5-HT in the NAc shell and mPFC (see Miliano et al., 2019; Valentini et al., 2013) after 2-Cl-4,5-MDMA administration; ii) behavioral testing for locomotor and stereotyped activity, instrumental to better clarify the dopaminergic and serotonergic profile of 2-Cl-4,5-MDMA (see Cadoni et al., 2001); and iii) recording of 50-kHz ultrasonic vocalizations (USVs) to characterize the effects of 2-Cl-4,5-MDMA on arousal and emotional state (see Simola, 2015). This study also aimed to identify possible differences between the responsiveness of adolescent and adult subjects to the neurochemical and behavioral effects of 2-Cl-4,5-MDMA.

## METHODS

### Animals

A total of 37 adolescent (5-7 weeks) and 40 adult (10-12 weeks) male Sprague-Dawley rats (Envigo, San Pietro al Natisone, Italy) were used for *in vivo* microdialysis or behavioral tests. Rats were housed 4 per cage, at temperature of  $22 \pm 2^\circ\text{C}$  and 60 % humidity and under a 12-h light/dark cycle (lights on from 7:00 a.m.). Tap water and standard laboratory chow were provided *ad libitum*. All animal experiments were carried out in accordance with the Guidelines for Care and Use of Mammals in Neuroscience and Behavioral Research according to Italian (D.Lgs 26/2014) and European Council Directive (2010/63/UE) and to the guidelines issued by the Committee for Animal Wellbeing (OPBA) at the University of Cagliari. All efforts were made to minimize pain and suffering and to reduce to the lesser extent the number of animals used.



## Drugs

2-chloro-4,5-methylenedioxymethamphetamine (2-Cl-4,5-MDMA) hydrochloride was synthesized by Dr. Tocco (see supplementary materials for details) and its purity was assessed by LC/MS using the analytical reference standard (Cayman Chemicals, Ann Arbor, Michigan, USA). 2-Cl-4,5-MDMA was dissolved in saline (0.9% NaCl) solution, and administered intravenously (i.v.) at the dose of 1, 3, 5 mg/kg (volume of 1ml/kg) for microdialysis experiments, in order to determine the lowest effective dose. The dose of 5 mg/kg i.v. was selected for behavioral tests since in microdialysis experiments it was the most effective in both adolescent and adult rats.

## In vivo microdialysis

### Surgery and Analytical Procedure

Rats (N=25 adolescents and N=28 adults) were anesthetized with 4% isoflurane gas (Merial, Milano, Italy), implanted with an intra-jugular vein catheter for drug administration, and placed in a stereotaxic apparatus for vertical microdialysis dual-probe implant in the NAc shell and mPFC of each individual rat, as previously described (De Luca et al., 2015). Twenty-four hours after surgery, microdialysis probes were perfused with Ringer's solution (pH 7.3-7.4) at a flow rate of 1 $\mu$ l/min. Dialysate samples (20  $\mu$ l) were split and analyzed for DA or 5-HT immediately after collection by HPLC. Quantification was made by comparison of a standard curve performed for each neurotransmitter. At the end of experiments, rats were sacrificed and their brains removed and stored in formalin (10%) for histological examination of probe placement

(see Supplementary Information for details).

### **Behavioral testing**

To evaluate locomotor and stereotyped activity stimulated by 2-Cl-4,5-MDMA, a separate cohort of rats (N=12 adolescents and N=12 adults), implanted the day before with an intrajugular vein catheter for drug administration, were individually placed in Plexiglas cages (L 47cm × H 19cm × W 27cm) having a metal grid floor and equipped with infrared photocell emitters and detectors situated along their long axis (Opto-Varimex, Columbus Instruments, Columbus, OH, USA). After placement in the cages, animals were allowed to habituate for 30 min and then administered with vehicle (i.v.) or 2-Cl-4,5-MDMA (5 mg/kg i.v.). Locomotor activity was scored by counting the number of single or total number of beam interruptions. A further behavioral analysis (i.e., stereotypies) was performed in the same rats simultaneously to recording of locomotor activity by an observer unaware of drug treatment. Scoring was performed by recording the percentage of time spent in each behavioral category considered in 10 min intervals for the total time of observation (60 min). Behavioral items observed were: locomotion, rearing, sniffing up, repetitive and confined sniffing down, gnawing, flat body posture, Straub tail, and hind limb abduction.

### **Recording of ultrasonic vocalizations**

The emission of 50-kHz USVs was recorded simultaneously to locomotor/behavioral evaluation by placing on the lid of each motility cages an ultrasonic microphone (CM16/CMPA, Avisoft, Berlin, Germany) connected to an ultrasound recording device (UltraSoundGate116 Hb, Avisoft, Berlin, Germany), according to Simola et al. (2012). Intensity gain was always kept at a constant level throughout recordings. The emission

of 50-kHz USVs was recorded every 10 min for a total of 60 min and recording started immediately after the administration of 2-Cl-4,5-MDMA.

## Statistics

All the numerical data are given as mean  $\pm$  SEM. Data were tested for normal distribution using Shapiro-Wilk's test. The effect of treatment (e.g., dose of 2-Cl-4,5-MDMA) on DA and 5-HT levels within each age group (adolescents, adults) and brain areas (NAc shell, mPFC) were analyzed by using repeated measures (RM) two-way ANOVA (dose  $\times$  time) followed by Tukey's multiple comparisons. For RM tests a Geisser-Greenhouse correction was carried out by Prism 9 software (GraphPad, La Jolla, CA, USA) whenever we could not assume sphericity. To evaluate the effect of age on DA or 5-HT response within each brain areas and 2-Cl-4,5-MDMA dose, an overall analysis of DA and 5-HT levels data obtained from each rat during the microdialysis experiment was conducted by calculating the area under the curve (AUC), obtained by plotting the values of the levels of DA or 5-HT vs time with the classical trapezoidal rule and then comparing the obtained values by Holm-Sidak corrected multiple paired t-tests. Possible pre-existing group differences in DA and 5-HT levels were analyzed by using one-way ANOVA, followed by Dunnett's multiple comparisons. The effect of treatment on locomotor counts and USVs within each age group was analyzed by using RM two-way ANOVA (dose  $\times$  time) followed by Tukey's multiple comparison. Behavioral scores were analyzed by factorial ANOVA for each behavioral item, with treatment and age as independent factors. Results showing significant effects following ANOVA were subjected to Tukey's post hoc test. Possible pre-existing group differences in locomotor activity and USVs emission were analyzed by using unpaired Student's t-test. Post hoc

tests were conducted only when a significant main effect and/or interaction were detected. Differences were considered significant at  $p < 0.05$ .

## RESULTS

### In vivo brain microdialysis studies

#### Effects of 2-Cl-4,5-MDMA on dopamine transmission in the NAc shell and mPFC of adolescent and adult rats

At first, we studied the effects of three i.v. doses of 2-Cl-4,5-MDMA (1, 3, 5 mg/kg), or vehicle (1 ml/kg) on dialysate DA in the NAc shell and mPFC of adolescent and adult male rats. As shown in Figure 1, the administration of 2-Cl-4,5-MDMA increased dialysate DA in a dose- and age- dependent manner. DA basal levels, expressed as fmol/10 $\mu$ l sample (mean  $\pm$  SEM), were in adolescents: NAc shell 49 $\pm$ 9, mPFC 20 $\pm$ 4; in adults: NAc shell 51 $\pm$ 5, mPFC 21 $\pm$ 3. One-way ANOVA showed no differences in DA basal outputs among groups.

#### *Adolescent rats*

Two-way ANOVA of NAc shell DA levels showed a main effect of dose [ $F_{(3,18)}=4.96$ ;  $p < 0.05$ ], time [ $F_{(1.947,35.05)}=10.76$ ;  $p < 0.001$ ], and a significant dose  $\times$  time interaction [ $F_{(27,162)}=3.07$ ;  $p < 0.0001$ ]. Tukey's post hoc test showed an increase of dialysate DA in the NAc shell after 1, 3, and 5 mg/kg of 2-Cl-4,5-MDMA compared to vehicle (1 mg/kg: 20 min; 3 mg/kg: 20 min; 5 mg/kg: 20 and 60 min), and after 3 and 5 mg/kg compared to 1 mg/kg (3 mg/kg: 20 min; 5 mg/kg: 20 and 60 min) and to basal values (3 and 5 mg/kg: 20 min; Fig. 1 a). Two-way ANOVA of mPFC DA levels showed a main effect of time [ $F_{(2.55,48.5)}=2.96$ ;  $p < 0.05$ ] and a significant dose  $\times$  time interaction [ $F_{(27,171)}=2.15$ ;

$p < 0.01$ ]. Tukey's post hoc test showed an increase of dialysate DA in the mPFC after 3 mg/kg of 2-Cl-4,5-MDMA with respect to vehicle (20 min, Fig. 1c).

### ***Adult rats***

Two-way ANOVA of NAc shell DA levels of adult rats showed a main effect of dose [ $F_{(3,24)}=3.33$ ;  $p < 0.05$ ], time [ $F_{(1.74,41.84)}=6.47$ ;  $p < 0.01$ ], and a significant dose  $\times$  time interaction [ $F_{(27,216)}=1.85$ ;  $p < 0.01$ ]. Tukey's post hoc test showed an increase of dialysate DA in the NAc shell after 3 and 5 mg/kg of 2-Cl-4,5-MDMA with respect to vehicle (3 and 5 mg/kg: 40 min), and after 5 mg/kg of 2-Cl-4,5-MDMA with respect to 1 mg/kg and to basal values (40 min, Fig. 1b). Two-way ANOVA of mPFC DA levels of adult rats showed a main effect of dose [ $F_{(3,18)}=7.01$ ;  $p < 0.0001$ ], time [ $F_{(1.44,25.96)}=19.12$ ;  $p < 0.0001$ ], and a significant dose  $\times$  time interaction [ $F_{(27,162)}=7.47$ ;  $p < 0.0001$ ]. Tukey's post hoc test showed an increase of dialysate DA in the mPFC after 1, 3, and 5 mg/kg of 2-Cl-4,5-MDMA with respect to vehicle (1 mg/kg: 20 min; 3 mg/kg: 20-40 min; 5 mg/kg: 20-120 min), after 5 mg/kg with respect to 1 and 3 mg/kg (1 mg/kg: 20-80 min; 3 mg/kg: 80-120 min), and after 3 and 5 mg/kg with respect to basal values (3 mg/kg: 20-40 min; 5 mg/kg: 20-180 min; Fig 1d).

### ***Comparison between adolescent and adult rats***

To evaluate how the overall DA response varied among rats of different age according to the dose of 2-Cl-4,5-MDMA administered, data were analyzed by multiple unpaired t-tests of AUCs that revealed a greater DA release after 1 mg/kg of 2-Cl-4,5-MDMA in the NAc shell of adult rats with respect to adolescent rats [ $t(8.23)= 4.19$ ;  $p < 0.05$ ], Fig. 2a, and after 5 mg/kg of 2-Cl-4,5-MDMA in both the NAc shell and mPFC of adult rats with respect to adolescent rats [NAc shell:  $t(6.82)= 3.41$ ,  $p < 0.05$ ; mPFC:  $t(4.07)= 6.27$ ,

$p < 0.05$ ], Fig. 2b-c). Data of DA levels, as determined from AUCs are also indicated (Fig. 2d).

### **Effects of 2-Cl-4,5-MDMA on serotonin transmission in the NAc shell and mPFC of adolescent and adult rats**

In parallel, we studied the effect of an i.v. challenge with 2-Cl-4,5-MDMA (1, 3, 5 mg/kg), or vehicle (1 ml/kg) on dialysate 5-HT in the NAc shell and mPFC of adolescent and adult male rats. As shown in Figure 3, the administration of 2-Cl-4,5-MDMA increased dialysate 5-HT in a dose- and age-dependent manner. 5-HT basal levels, expressed as fmol/10 $\mu$ l sample (mean  $\pm$  SEM), were in adolescents: NAc shell 10 $\pm$ 2, mPFC 20 $\pm$ 4; in adults: NAc shell 15 $\pm$ 2, mPFC 17 $\pm$ 4. One-way ANOVA showed no differences in DA basal outputs among groups.

#### ***Adolescent rats***

Two-way ANOVA of NAc shell 5-HT levels showed a main effect of dose [ $F_{(3,19)}=30.27$ ;  $p < 0.0001$ ], time [ $F_{(2,35,44,62)}=41.66$ ;  $p < 0.0001$ ] and a significant dose  $\times$  time interaction [ $F_{(27,171)}=26.46$ ;  $p < 0.0001$ ]. Tukey's post hoc test showed an increase of dialysate 5-HT in the NAc shell after 5 mg/kg of 2-Cl-4,5-MDMA with respect to all the other groups (vehicle: 20-100 min; 1 mg/kg: 20-80; 3 mg/kg: 20-60 min) and to basal values (20-100 min), as well as after 3 mg/kg of 2-Cl-4,5-MDMA compared to 1 mg/kg (60 min) (Fig. 3a).

Two-way ANOVA of mPFC 5-HT levels showed a main effect of dose [ $F_{(3,16)}=29.75$ ;  $p < 0.0001$ ], time [ $F_{(2,05,32,73)}=85.70$ ;  $p < 0.0001$ ], and a significant dose  $\times$  time interaction [ $F_{(27,144)}=27.76$ ;  $p < 0.0001$ ]. Tukey's post hoc test showed an increase of dialysate 5-HT

in the mPFC after 3 and 5 mg/kg of 2-Cl-4,5-MDMA compared to vehicle (3 mg/kg: 20-60, and 100 min; 5 mg/kg: 20-60), 1 mg/kg (3 mg/kg: 20-40 min; 5 mg/kg: 20-60 min) and to basal values (20-60 min), as well as after 5 mg/kg of 2-Cl-4,5-MDMA with respect to 3 mg/kg (20 min) (Fig. 3c).

### **Adult rats**

Two-way ANOVA of NAc shell 5-HT levels showed a main effect of time [ $F_{(2,34,42.15)}=10.16$ ;  $p<0.001$ ] and a significant time  $\times$  dose interaction [ $F_{(27,162)}=1.88$ ;  $p < 0.01$ ]. Tukey's post hoc test showed an increase of dialysate 5-HT in the NAc shell after 3 mg/kg of 2-Cl-4,5-MDMA compared to vehicle and to basal values (20-40 min; Fig 3b).

Two-way ANOVA of mPFC 5-HT levels showed a main effect of dose [ $F_{(3,18)}=15.29$ ;  $p<0.0001$ ], time [ $F_{(1.47,23.51)}=41.38$ ;  $p<0.0001$ ], and a significant dose  $\times$  time interaction [ $F_{(27,144)}=16.71$ ;  $p<0.0001$ ]. Tukey's post hoc test showed an increase of dialysate 5-HT in the mPFC after both 3 mg/kg and 5 mg/kg of 2-Cl-4,5-MDMA compared to vehicle (3 mg/kg: 20-40, and 80 min; 5 mg/kg: 20-80 min) and to basal values (3 mg/kg: 20-40, and 100 min; 5 mg/kg: 20-60 min), and after 5 mg/kg compared to both 1 mg/kg (20-60 min) and 3 mg/kg (40-60 min) (Fig. 3d).

### **Comparison between adolescent and adult rats**

To evaluate how the overall 5-HT response varied among rats of different age according to the dose of 2-Cl-4,5-MDMA administered, data were analyzed by multiple unpaired t-test of AUCs that revealed a greater 5-HT release only after 5 mg/kg of 2-Cl-4,5-MDMA in the NAc shell of adolescent with respect to adult rats [ $t(6.55)= 5.18$ ;  $p < 0.01$ ], Fig.4a . Data of 5-HT levels, as determined from AUCs are also indicated (Fig. 4b).

## Locomotor and stereotyped activity

Analysis of locomotor activity counts following 5 mg/kg i.v. (Fig. 5 a, b) by RM two-way ANOVA applied within each age group showed a significant effect of treatment [adolescents:  $F_{(1,10)}=25.52$ ,  $p<0.001$ ; adults:  $F_{(1,10)}=5.48$ ,  $p<0.05$ ], a significant effect of time [adolescents:  $F_{(3,30)}=7.23$ ,  $p<0.001$ ; adults:  $F_{(3,30)}=48.19$ ,  $p<0.00001$ ], and a significant treatment  $\times$  time interaction [adolescents:  $F_{(3,30)}=2.9$ ,  $p<0.05$ ; adults:  $F_{(3,30)}=3.74$ ;  $p<0.05$ ]. Tukey's post hoc test revealed an increased locomotor activity at 10 and 30 min post injection in adolescent rats and at 10 min post injection in adult rats ( $p<0.05$ ). No significant differences between vehicle and 2-Cl-4,5-MDMA groups were observed in locomotor activity during the 30 min time of habituation to the motility cages (Fig. 5 c, d).

Factorial ANOVA applied to each behavioral item during the 60 min of observation (Fig. 5 e, f) following challenge with 5 mg/kg i.v. confirmed that 2-Cl-4,5-MDMA stimulated locomotion in both age groups [F treatment $_{(1,20)}=30.42$   $p<0.0001$ ] but to a greater extent in adolescent rats [F age  $\times$  treatment $_{(1,20)}=4.62$   $p<0.05$ ]. Moreover, 2-Cl-4,5-MDMA significantly increased the time spent in sniffing up [F treatment  $\times$  age $_{(1,20)}=4.89$   $p<0.05$ ], rearing [F treatment  $\times$  age $_{(1,20)}=5.01$   $p<0.05$ ] and Straub tail [F treatment  $\times$  age $_{(1,20)}=6.74$   $p<0.01$ ] more in adolescent than adult rats (Fig. 5 e, f). Furthermore, 2-Cl-4,5-MDMA increased the time spent performing hind limb abduction and gnawing stereotypies in both adolescent and adult rats [hind limb abduction: F treatment $_{(1,20)}=62.03$   $p<0.00001$ ; gnawing: F treatment $_{(1,20)}=37.48$   $p<0.00001$ ] with no age differences [hind limb abduction: F treatment  $\times$  age $_{(1,20)}=1.83$   $p=0.19$ ; gnawing: F



treatment  $\times$  age<sub>(1,20)</sub>=62.03  $p$ <0.00001]. Flat body posture was only occasionally observed in adult rats with a non-significant overall effect [ $F_{(1,20)}$ =3.27,  $p$ =0.08].

### Emission of 50-kHz USVs

As shown in Figure 6 (a, b), 2-Cl-4,5-MDMA administration (5 mg/kg, i.v.) did not significantly stimulate the emission of 50-kHz USVs in adolescent and adult rats, compared with vehicle administration. Similarly, no significant differences in calling behavior of the 50-kHz USVs type were observed between vehicle and 2-Cl-4,5-MDMA groups during habituation to the motility cages (Fig. 6 d, e).

### DISCUSSION

In the present study we report that the phenethylamine 2-Cl-4,5-MDMA differentially stimulated DA and 5-HT transmission in the NAc shell and mPFC of male rats in a dose-, brain area-, and age-dependent manner. Notably, the effects of 2-Cl-4,5-MDMA differed between adolescent and adult rats. In particular, 2-Cl-4,5-MDMA more markedly increased the extracellular levels of DA in both the NAc shell and mPFC of adult than adolescent rats, while the opposite was observed on extracellular levels of 5-HT in the NAc shell, with a more marked increase in adolescent than adult rats. In addition, 2-Cl-4,5-MDMA stimulated locomotor and stereotyped activity in both adolescent and adult rats, although adolescent rats spent higher percentages of time performing locomotion, sniffing up and Straub tail than adult rats. Finally, 2-Cl-4,5-MDMA failed to stimulate the emission of 50-kHz USVs in both adolescent and adult rats.

The main results of this study were obtained by *in vivo* brain microdialysis that allowed to simultaneously evaluate in the same animal the changes of DA and 5-HT transmission in two brain areas that critically interact to encode motivated behaviors and responsiveness to motivational stimuli (De Luca, 2014) and whose interplay may differ between adolescence and adulthood. The quantification of extracellular DA from the NAc shell and mPFC of adolescent rats indicated no clear dose-response relationship following acute intravenous administration of 2-Cl-4,5-MDMA, since the doses of 3 and 5 mg/kg i.v. elicited a comparable DA release in both the NAc shell and mPFC. However, DA transmission in the NAc shell and mPFC of adult rats responded to 2-Cl-4,5-MDMA in a dose-dependent manner, with a more pronounced DA outflow in the mPFC than in the NAc shell. Notably, the difference between adolescents and adults appeared more evident when considering the effects of the dose of 5 mg/kg i.v. that yielded a higher increase of DA outflow in both the NAc shell and mPFC of adult rats, compared to adolescent rats, as clearly indicated also by the analysis of the AUCs. The dose-response curve of 2-Cl-4,5-MDMA on DA transmission in the NAc shell of adult rats acquires relevance when compared to that elicited by MDMA in adult rats, as demonstrated by previous studies. Thus, MDMA has been reported to dose-dependently stimulate DA release in both the shell and core of the NAc at doses ranging from 0.32 to 3.2 mg/kg i.v., being the increase more pronounced in the shell than in the core (Cadoni et al., 2005). Moreover, in our previous study the increase of DA levels observed in the NAc shell after the highest dose of MDMA tested (3.2 mg/kg) was about 200%. On these bases, our data showing an increase of about 90% of DA in NAc shell of both adolescents and adults after the dose of 5 mg/kg i.v. of 2-Cl-4,5-MDMA may indicate

that this phenethylamine has either a reduced DA-releasing property when compared to MDMA, or is at least less potent than MDMA in stimulating DA release. The ability to increase DA in the NAc shell is a common feature of substances with abuse potential and is considered an index of the reinforcing and addictive properties of a drug (Carlezon and Wise, 1996; Di Chiara et al., 2004; Ikemoto and Bonci, 2014). Considering previous data on MDMA, and the fact that we administered 5 mg/kg of 2-Cl-4,5-MDMA intravenously, the scarce elevation of DA in the NAc shell observed here may account for low rewarding properties of 2-Cl-4,5-MDMA when compared to MDMA, and possibly other drugs of abuse. This is in line with other data indicating that 2-Cl-4,5-MDMA does act as a reinforcer in an intravenous self-administration paradigm in adolescent rats (Pisanu et al., 2022), while MDMA is able to sustain intravenous self-administration responding (Schenk, 2009).

The quantification of 5-HT from the NAc shell showed a higher responsiveness of adolescent than adult rats, while no age-dependent differences were observed in the mPFC. Thus, the increase of 5-HT after the highest dose of 2-Cl-4,5-MDMA tested (5 mg/kg i.v) reached a mean of about 300% over basal values in the NAc shell of adolescents and a mean of about 50% in the same brain region of adults, as confirmed by the analysis of the AUC. However, the release of 5-HT from the mPFC was similar in adolescent and adult rats, displaying a time- and dose-dependent response peaking from 20 min post injection after both 3 and 5 mg/kg i.v. of 2-Cl-4,5-MDMA. The predominant effect on 5-HT transmission may indicate that 2-Cl-4,5-MDMA acts as MDMA-like psychostimulant most likely through the inhibition of the 5-HT (SERT) and other monoaminergic transporters (Iravani et al., 2000; Liechti et al., 2000; Simmler

and Liechti, 2018), and possibly by stimulating the release of 5-HT and other monoamines (Green et al., 2003). Notably, the peculiar response of 5-HT transmission in the NAc shell of adolescent rats displayed a prolonged and a four-fold higher increase in 5-HT release than adult rats, whilst no age-related differences in this response were observed in the mPFC. Reasons for the higher responsiveness of the 5-HT system in adolescent rats may be searched in the light of several factors. It should be considered that 5-HT is one of the first neurotransmitter systems to develop in the mammalian brain and that it plays an important role in brain development (Lauder 1990; 1993; Rubenstein, 1998). Animal studies have shown that 5-HT content, SERT levels, and 5-HT binding sites are all generally higher in the developing brain as compared to the adult brain, and that before puberty they all decline to levels similar to those found in the adult brain (Murrin et al., 2007). Nonetheless, although the 5-HT system appears to be mature early in life as regard fibre density and 5-HT synthesis, each of 5-HT receptors and enzymes have a unique pattern of development, with some stabilizing before puberty (Galineau et al., 2004; Murrin et al., 2007), and others not before adulthood, depending on the brain area considered (Booij et al., 2015). Serotonergic innervation of the rat cerebral cortex begins to show patterns characteristic of the adult cortex by the end of the third postnatal week (Dori et al., 1996). While the latter finding is consistent with our results showing no differences in 5-HT release in the mPFC following 2-Cl-4,5-MDMA, the early development of 5-HT neuronal system does not explain the striking differences in 5-HT responsiveness in the NAc shell between adolescent and adult rats. While not neglecting the several changes occurring in the adolescent brain in neuronal systems other than the 5-HT system, which might interact

with and affect 5-HT neurotransmission, one likely explanation for the greater increase of 5-HT observed in the NAc shell of adolescent rats might be linked to the contribution of oxytocin (OT). The OT system is implicated in social behavior (Sanna and De Luca, 2021) and, as other neuronal systems, it undergoes changes during brain development with a significant phase of transition following puberty (Sannino et al., 2017). OT has been shown to exert its prosocial effects through an action on OT receptors in the NAc and by increasing the 5-HT input from dorsal raphe nuclei to the NAc (Dolen et al., 2013). Moreover, increased sociability through enhanced 5-HT release seems to be mediated by 5-HT<sub>1B</sub> receptors (Walsh et al., 2018). Given that MDMA has been shown to increase OT levels both in rodents and humans (Wolff et al., 2006), it may be speculated that 2-Cl-4,5-MDMA increased 5-HT transmission more markedly in adolescent than in adult rats not only by a direct action on 5-HT terminals but also through the contribution of released OT, likely induced by 2-Cl-4,5-MDMA, which could further increase 5-HT levels in the NAc. Remarkably, a similar mechanism has been suggested for the pro-social effect of MDMA (Nardou et al., 2019).

In the present study, the concurrent analysis of DA and 5-HT in the same dialysate sample from the NAc shell and mPFC of each individual rat allowed studying the relationships between DA and 5-HT transmission in these regions. In fact, 5-HT projections from the raphe nuclei to the ventral tegmental area (VTA), NAc and mPFC might be involved in the influences of 5-HT on DA functions (Kalivas, 1993; White et al., 1996). Recent studies indicate a facilitatory influence of 5-HT transmission on DA release, as revealed by the fact that the electrical stimulation of the dorsal raphe nucleus increases the levels of DA in the NAc (De Deurwaerdere et al., 1996), that 5-

HT infusion in the NAc increases the concentration of DA in the same area (Hallbus et al., 1997), and that endogenous 5-HT facilitates DA release in the striatum (Benloucif et al., 1993; Yadid et al., 1994). Other in vivo studies showed that systemic (Parsons et al., 1999) or intra-VTA infusion (O'Dell and Parsons, 2004) of 5-HT<sub>1B</sub> agonists potentiated the increase of dialysate DA in the NAc. Of note, repeated treatment with cocaine sensitizes both 5-HT and DA increase in the NAc and dorsal raphe nucleus in response to cocaine challenge (Parsons and Justice, 1993a,b; De Luca et al., 2018). Indeed, the NAc is a brain region where DA/5-HT interaction may take place and code for reward-related information (Bouyer et al., 1984; Beal and Martin, 1985; Van Bockstaele and Pickel, 1993; Phelix and Broderick, 1995; Sasaki-Adams and Kelley, 2001; Miliano et al., 2019). Taken together, the above evidence could suggest that an interplay exists between the marked increase of 5-HT outflow and the low increase of DA outflow in the NAc shell of adolescent rats observed here after the administration of 2-Cl-4,5-MDMA. Thus, these findings are consistent with the evidence indicating that adolescence may be characterized by differences in the maturation of rewarding/motivational and inhibitory brain systems that may contribute to adolescent novelty seeking/impulsivity (Takeuchi et al., 2000; Wahlstrom et al., 2010; Burke and Miczek, 2014; Thorpe et al., 2020; Peters and Naneix, 2022). Indeed, adolescence is the phase of neurodevelopment when the PFC matures, being PFC a key regulator of superior brain functions that are altered in psychiatric disorders (Renard et al., 2014; Beckmann et al., 2020), including substance use disorders (Squeglia and Cservenka, 2017). For these reasons and considering the differential ability of 2-Cl-4,5-MDMA to increase DA and 5-HT outflow in the mPFC and in the NAc shell, it might be possible

that the use of 2-Cl-4,5-MDMA during adolescence affects the development of motivational DA systems and of inhibitory 5-HT systems. Furthermore, since *in vivo* studies have demonstrated the neurotoxicity of molecules structurally-correlated to 2-Cl-4,5-MDMA (Cadoni et al., 2017) and *in vitro* studies have reported the occurrence of 2-Cl-4,5-MDMA-induced toxicity (Sogos et al., 2021), the results here obtained may also become of toxicological interest.

Our results on the behavioral effects of 2-Cl-4,5-MDMA have shown that it stimulates locomotor activity and induces the appearance of stereotypies in both adolescent and adult rats. Regarding locomotor activity, it is well known that activation of DA transmission in the NAc stimulates locomotion through an action on D1 and D2 receptors (Sharp et al., 1987; Campbell et al., 1997). Similarly, increased 5-HT transmission by indirect 5-HT agonists is able to stimulate locomotor activity through a 5-HT<sub>1B</sub> mediated mechanism (Geyer, 1996). Following 5 mg/kg i.v. of 2-Cl-4,5-MDMA we observed that adolescent rats had a longer lasting increase in overall locomotor activity. Moreover, adolescent rats displayed a significantly greater increase in the time spent performing locomotion, rearing, and sniffing up compared to adult rats. Regarding stereotypies linked to 5-HT release (Straub tail, hind limb abduction, and flat body posture) we observed a comparable hind limb abduction in adolescent and adult rats and a more marked Straub tail in adolescents as compared to adults.

The administration of 2-Cl-4,5-MDMA failed to stimulate the emission of 50-kHz USVs in adolescent and adult rats. This effect is similar to what was observed in previous studies after both acute and repeated administration of MDMA (Sadananda et al., 2012; Simola et al., 2012, 2014). Earlier investigations have shown that the emission

of 50-kHz USVs may be initiated by the activation of DA transmission in the NAc shell (Burgdorf et al., 2001; Thompson et al., 2006; Simola and Brudzynski, 2018). At the same time, only drugs that markedly activate DA transmission in the NAc shell (i.e., dopaminergic psychostimulants or the dopaminergic agonist apomorphine) consistently stimulate the emission of 50-kHz USVs in rats (Williams and Undieh, 2010; Simola et al., 2012, 2014; Simola and Costa, 2018). The emission of 50-kHz USVs is considered a behavioral marker of the positive effects that psychoactive drugs may elicit on arousal, affect, and motivation in rats (Simola, 2015). Nevertheless, it is noteworthy that several drugs that increase the affective and motivational state of rats only modestly increase the emission of 50-kHz USVs (Wright et al., 2012; Costa et al., 2019), likely because they activate DA transmission in the NAc shell to an extent that is insufficient to trigger the emission of appetitive 50-kHz USVs (Simola and Brudzynski, 2018). On these bases, the negligible emission of 50-kHz USVs observed here may depend on the fact that 2-Cl-4,5-MDMA only modestly increased DA levels in the NAc shell. Accordingly, the lack of effect of 2-Cl-4,5-MDMA on calling behavior should not be simply considered a result indicating that this NPS has no influence on the affective state of rats. Conversely, it may rather support the assumption suggested by microdialysis data that 2-Cl-4,5-MDMA has a psychopharmacological profile different from that of other psychostimulants of abuse.

This study has some limitations since it focused mainly on neurochemical and behavioral effects induced by the acute administration of 2-Cl-4,5-MDMA, without addressing the underlying molecular mechanisms, as well as the possible involvement of active metabolites in the findings observed. Moreover, we used only male rats in our



study and, therefore, we do not know if our findings can be extended to female rats. Nevertheless, this study completes previous investigations on the pharmacological and toxicological properties of 2-Cl-4,5-MDMA (Sogos et al., 2021; Pisanu et al., 2022) and demonstrates that 2-Cl-4,5-MDMA affects DA and 5-HT transmission in a dissimilar way at adolescence and adulthood, highlighting the existence of differences in the responsiveness to psychoactive drugs between individuals of different ages. This feature may influence the pattern of use and misuse of 2-Cl-4,5-MDMA, such as the frequency of ingestion, but also the psychoactive acute effects of this NPS that appear dissimilar from those of other psychostimulant drugs (Miliano et al. 2016; Loi et al., 2020) expanding concern about the short- and long-term consequences of 2-Cl-4,5-MDMA use in both adolescents and adults.

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## **INTEREST STATEMENT**

The authors have no conflicts of interest to disclose.

## **DATA AVAILABILITY STATEMENT**

The data underlying this article will be shared on reasonable request to the corresponding author.

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## Figure legends

### Figure 1. Effects of 2-Cl-4,5-MDMA on NAc shell and mPFC dopamine transmission

Data are presented as mean  $\pm$  SEM of change in extracellular DA in the NAc shell (a,b) and mPFC (c,d) of adolescent and adult rats, expressed as the percentage of basal values. The arrow indicates the 2-Cl-4,5-MDMA i.v. injection. Solid symbols:  $p < 0.05$  vs basal values; \*  $p < 0.05$  5 and 1 mg/kg vs Vehicle; ^  $p < 0.05$  3 mg/kg vs Vehicle; §  $p < 0.05$  5 mg/kg vs 1 mg/kg; °  $p < 0.05$  3 mg/kg vs 1 mg/kg; #  $p < 0.05$  5 mg/kg vs 3 mg/kg (RM two-way ANOVA, Tukey's test). Adolescents: NAc shell (Veh: N= 5; 1 and 3 mg/kg: N= 5 per group; 5 mg/kg: N= 7), mPFC (Veh: N= 5; 1 and 5 mg/kg: N= 5 per group; 3 mg/kg: N=8); Adults: NAc shell (Veh: N= 6; 5 mg/kg: N= 6; 1 and 3 mg/kg: N=8 per group), mPFC (Veh: N= 5; 5 mg/kg: N= 5; 1 and 3 mg/kg: N=6 per group). DA: dopamine; NAc: nucleus accumbens; mPFC: medial prefrontal cortex.

### Figure 2. Comparison between adolescents and adults of the NAc shell and mPFC DA response after 1, 3, and 5 mg/kg of 2-Cl-4,5-MDMA.

Data are presented as mean  $\pm$  SEM of change in extracellular dopamine (DA) in the NAc shell (a,b) and mPFC (c) of adolescent and adult rats, expressed as the percentage of basal values, or as mean  $\pm$  SEM of (d) overall DA levels as measured by areas under the curves (AUCs), calculated from data shown in Figure 1. The arrow indicates the 2-Cl-4,5-MDMA iv injection. \*  $p < 0.05$  adolescents vs adults (Holm-Sidak corrected multiple unpaired t tests). N= 5-8 per group.

### Figure 3. Effects of 2-Cl-4,5-MDMA on NAc shell and mPFC serotonin transmission

Data are presented as mean  $\pm$  SEM of change in extracellular 5-HT in the NAc shell (a,b) and mPFC (c,d) of adolescent and adult rats, expressed as the percentage of basal



values. The arrow indicates the 2-Cl-4,5-MDMA i.v. injection. Solid symbols:  $p < 0.05$  vs basal values; \*  $p < 0.05$  5 mg/kg vs Vehicle; ^  $p < 0.05$  3 mg/kg vs Vehicle; §  $p < 0.05$  5 mg/kg vs 1 mg/kg; #  $p < 0.05$  5 mg/kg vs 3 mg/kg, °  $p < 0.05$  3 mg/kg vs 1 mg/kg (RM Two-way ANOVA, Tukey's test). Adolescents: NAc shell (Veh: N= 5; 1, 5 mg/kg: N= 5 per group; 3 mg/kg: N= 8), mPFC (Veh, 1, 3, 5 mg/kg: N= 5 per group); Adults: NAc shell (Veh: N= 5; 1, 3 mg/kg: N=6 per group; 5 mg/kg: N= 5), mPFC (Veh, 1, 3, 5 mg/kg: N= 5 per group). 5-HT: serotonin; NAc: nucleus accumbens; mPFC: medial prefrontal cortex.

**Figure 4. Comparison between adolescents and adults of the NAc shell and mPFC 5-HT response after 1, 3, and 5 mg/kg of 2-Cl-4,5-MDMA.** Data are presented as mean  $\pm$  SEM of change in extracellular serotonin (5-HT) in the NAc shell (a) of adolescent and adult rats, expressed as the percentage of basal values, or as mean  $\pm$  SEM of (b) overall 5-HT levels as measured by areas under the curves (AUCs), calculated from data shown in Figure 3. The arrow indicates the 2-Cl-4,5-MDMA iv injection. \*\*  $p < 0.01$  adolescents vs adults (Holm-Sidak corrected multiple unpaired t tests). N= 5-8 per group.

**Figure 5. Behavioral response following 5 mg/kg i.v. of 2-Cl-4,5-MDMA**

Upper panels (a, b) show locomotor activity counts time-course following vehicle and 2-Cl-4,5-MDMA administration. Inset panels (c, d) show activity counts during the 30 min of habituation to the motility cages. \*  $p < 0.05$ , vs vehicle by two-way ANOVA followed by Tukey's post hoc test. Lower panels (e, f) show the percentage of time spent in each behavioral item during the total time of observation (60 min). Data are expressed both as means  $\pm$  SEM and individual values (N=6 per group). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , vs vehicle and § $p < 0.05$ , §§ $p < 0.01$  adolescents vs adults by

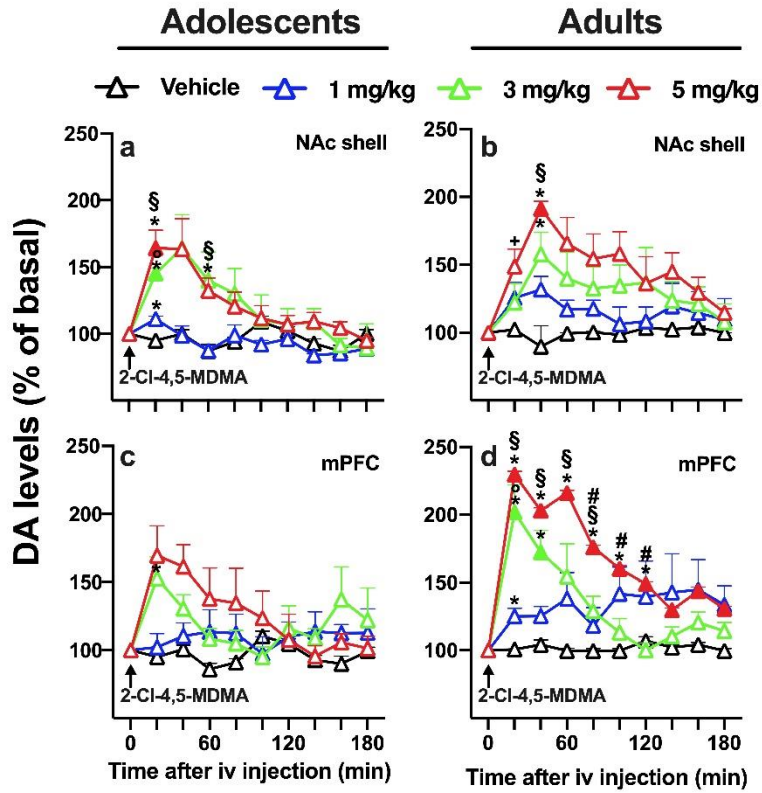
factorial ANOVA followed by Tukey's test. L: locomotion; SU: sniffing up; FBP: flat body posture; R: rearing; SD: sniffing down confined to a restricted area of the cage; GN: gnawing; ST: Straub tail; HLA: hind limb abduction.

**Figure 6. Effects of 2-Cl-4,5-MDMA on the emission of 50-kHz ultrasonic vocalizations**

Emission of 50-kHz ultrasonic vocalizations (USVs) in adolescent and adult rats treated with 2-Cl-4,5-MDMA (5 mg/kg, i.v.) recorded concomitantly to behavioral evaluation in motility cages. Rats that received vehicle or 2-Cl-4,5-MDMA emitted comparable numbers of 50-kHz USVs both after treatment (a, b, c) and during habituation to motility cages (d, e, f); N=6 per group. USVs: ultrasonic vocalizations

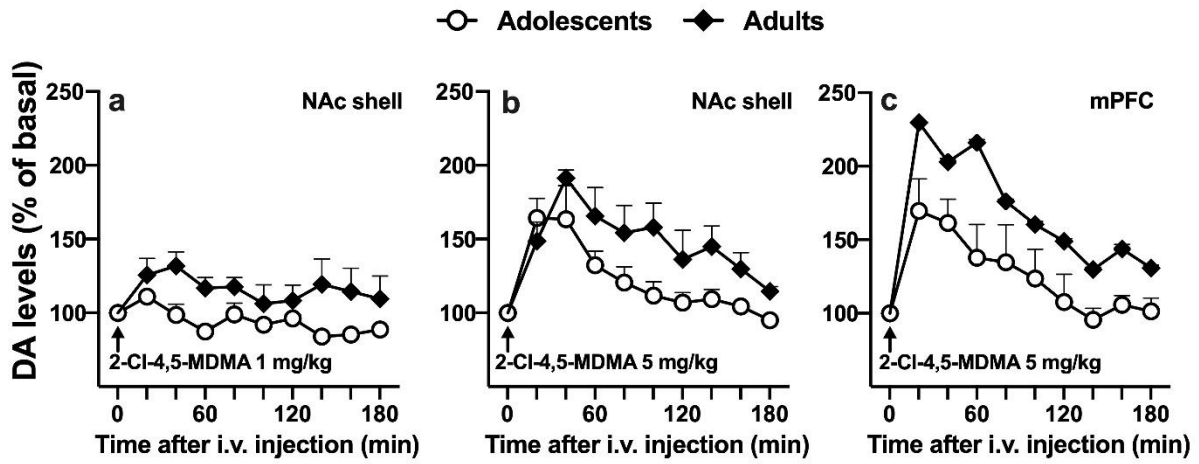
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Figure 1



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Figure 2

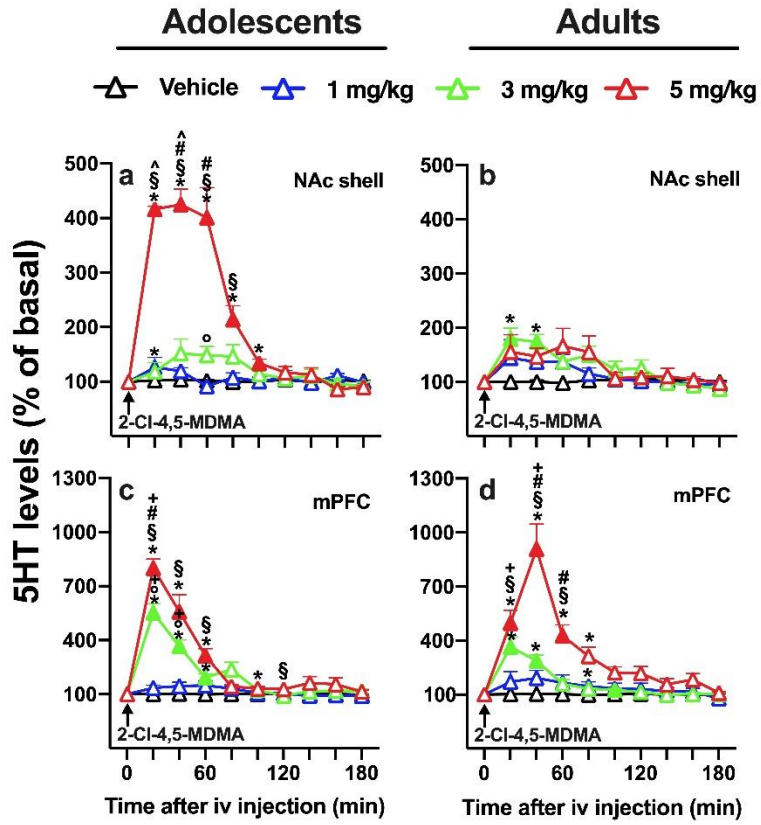


**d**

DA Levels (as Determined from AUCs)			
Brain area	Treatment	Age	
		Adolescents	Adults
NAc shell	2-Cl-4,5-MDMA 1mg/kg	16678 ± 485	19800 ± 2014 *
	2-Cl-4,5-MDMA 3 mg/kg	20508 ± 2603	22838 ± 4056
	2-Cl-4,5-MDMA 5mg/kg	20204 ± 1330	24550 ± 2869 *
mPFC	2-Cl-4,5-MDMA 1mg/kg	19788 ± 2202	23665 ± 3550
	2-Cl-4,5-MDMA 3 mg/kg	20130 ± 2962	21700 ± 2600
	2-Cl-4,5-MDMA 5mg/kg	20766 ± 2859	28821 ± 277,7 *

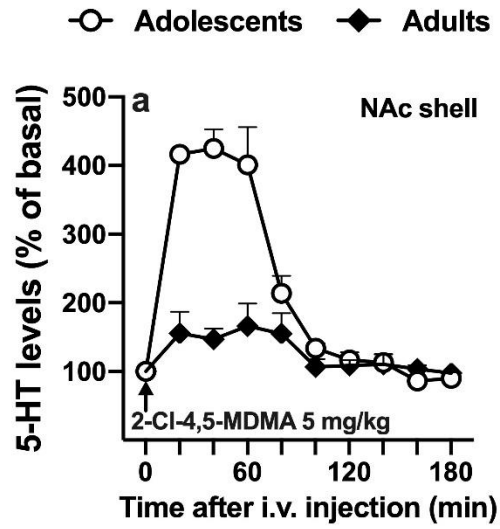
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Figure 3



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Figure 4

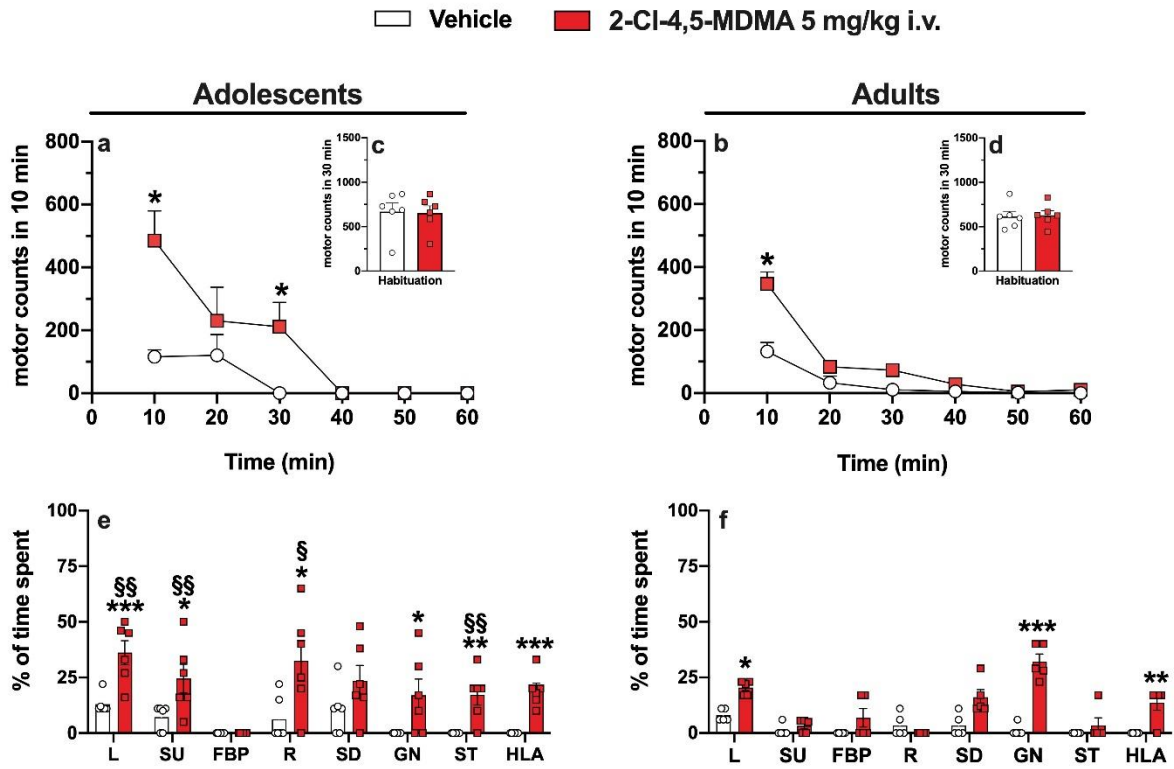


b

5-HT Levels (as Determined from AUCs)			
Brain area	Treatment	Age	
		Adolescents	Adults
NAc shell	2-Cl-4,5-MDMA 1mg/kg	17895 ± 1326	20130 ± 2459
	2-Cl-4,5-MDMA 3 mg/kg	21000 ± 2359	21320 ± 2459
	2-Cl-4,5-MDMA 5mg/kg	36762 ± 5311 **	22418 ± 3190
mPFC	2-Cl-4,5-MDMA 1mg/kg	20918 ± 2556	23028 ± 5327
	2-Cl-4,5-MDMA 3 mg/kg	23640 ± 1997	22500 ± 2529
	2-Cl-4,5-MDMA 5mg/kg	32963 ± 3838	44940 ± 6763

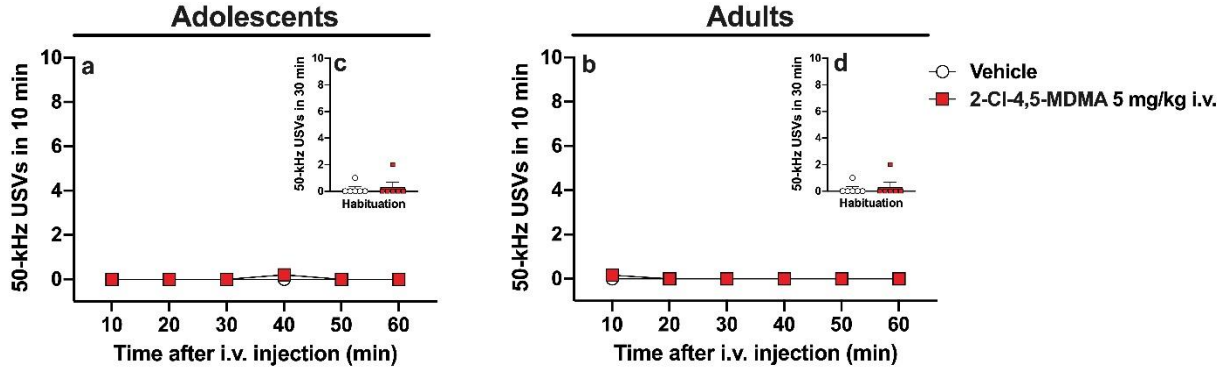
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Figure 5



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Figure 6



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