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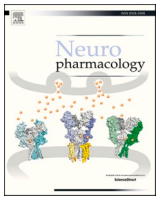

Repeated administration of the synthetic cannabinoid AKB48 induces serotonergic neuroadaptation in male and female mice: behavioural and immunohistochemical evidence

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ABSTRACT

Background and purpose: In the last years, Synthetic Cannabinoids (SCBs) have established themselves as one of the largest and most popular groups of Novel Psychoactive Substances (NPS), being frequently detected in biological samples of patients involved in intoxication and death cases. To date, compelling evidence on the potential interaction between SCBs and non-cannabinoid neurotransmission systems has emerged, with reference to a high-level overlap between endocannabinoid and serotoninergic system. Their long-term effect on serotoninergic pathways is still to be elucidated.

Experimental approach: Using a behavioural and immunohistochemical approach, we investigated the neuroplasticity at 5-HT_{2A} serotoninergic receptors and serotonin transporter in the cerebellum and cortex induced by repeated AKB48 administration in male and female mice. Further, pharmacological response to the serotoninergic compounds 2C-I or 25I-NBOMe has been studied.

Key results: The repeated exposure to AKB48 results in a worsening effect on the visual sensorimotor, sensory gating, and motor reactivity response to synthetic hallucinogens, that appears to be generally more prolonged in male with respect to AKB48-treated female mice. Interestingly, the effect has been related to cerebellar and cortical neuroplasticity at serotoninergic neurotransmission system, that involves both 5-HT_{2A} and SERT, that occurs more markedly and rapidly in female with respect to male mice.

Conclusion and implications: This data highlight the interaction between SCBs and psychedelic drugs that may be relevant to their long-term effects and psychiatric sequelae potentially related to their consumption.

1. Introduction

Since their first appearance in the illicit drug market, Synthetic Cannabinoids (SCBs) have established themselves as one of the largest and most popular groups of Novel Psychoactive Substances (NPS). New SCBs appear every year and are soon detected in biological samples of patients involved in intoxication and death cases (EMCDDA, 2024). These compounds are marketed and consumed with the aim of mimicking the effects of the main psychoactive ingredients of *Cannabis sativa*-based products (Luethi and Liechti, 2020). However, scientific literature has clarified that SCBs consumption can result in severe and atypical adverse effects (reviewed in Le Boisselier et al., 2017). Among these, a particular mention should be given to the exacerbation of psychiatric symptoms (i.e., agitation, visual and auditory hallucinations, psychosis, and paranoid behaviour) in vulnerable individuals and in

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Abbreviations

SCBs	Synthetic Cannabinoids
NPS	Novel Psychoactive Substances
AKB48	N-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide
2C-I	2,5-Dimethoxy-4-iodophenethylamine
25I-NBOMe	4-iodo-2,5-dimethoxy-N-[(2-methoxyphenyl) methyl]-benzeneethanamine
SERT	Serotonin Transporter
5-HT2A	Serotonin 2A receptor
DLPFC	Dorsolateral Prefrontal Cortex
OD	Optical Density
PyrNs	Pyramidal neurons
IGL	Internal Granular Layer

patients with no previous psychiatric history (van Amsterdam et al., 2015; Fattore, 2016; Kak et al., 2017; Graddy et al., 2018; Udow et al., 2018; Bassir Nia et al., 2019).

To date, preclinical research has investigated their pharmaco-toxicological profile demonstrating that they can induce short- (Wilson et al., 2019; Bilel et al., 2020; Corli et al., 2024a) and long-term (Bilel et al., 2023; Corli et al., 2024a) effects in rodents, via acting as full agonists on CB_1 cannabinoid receptors. However, compelling evidence on the potential interaction between SCs and non-cannabinoid neurotransmission systems has emerged, suggesting a high-level overlap between endocannabinoid and serotoninergic system. Evidence pointing out that SCBs administration can influence serotoninergic functions has emerged (Nakazi et al., 2000; Moranta et al., 2004; Balázsa et al., 2008). Accordingly, a recent study has demonstrated the role of 5-HT_{2A} receptors in the behavioural and physiologic acute responses to SCBs (Corli et al., 2024b). Despite the effect of a repeated exposure to SCBs on endocannabinoid and non-cannabinoid systems (i.e., dopaminergic and glutamatergic systems) has been recently pointed out (Bilel et al., 2023), their long-term effect on serotoninergic pathways is still to be elucidated.

It is worthy to note that several clinical and preclinical evidence has associated a sex-specific rate to the onset of the symptoms evoked by SCBs administration. While epidemiological (Castellanos et al., 2011; Bush and Woodwell, 2014; ESPAD, 2019) and preclinical (Wiley et al., 2019) data states that male are more at risk of consumption and intoxication by SCBs, some studies suggest that female may be more sensitive to specific effects induced by these compounds (Fattore et al., 2010; Craft et al., 2013; Bassir Nia et al., 2019). Thus, the need of clarifying the rationale behind a sex-tailored clinical approach to the intoxications has been outlined. Along these lines, we have previously demonstrated that repeated exposure to the synthetic cannabinoid N-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide (AKB48) results in behavioural and neuroadaptive changes at CB_1 receptors, that are influenced by both sex and repeated treatment (Corli et al., 2024a). This has led us to hypothesize that similar alterations may affect also serotoninergic pathways, previously linked to acute SCBs effects (Corli et al., 2024b). Thus, to evaluate the long-term impact of repeated exposure to synthetic cannabinoids (SCBs) on serotoninergic pathways we have considered the immunohistochemical assessment of 5-HT_{2A} serotoninergic receptors and serotonin transporter (SERT). Taking into account the potential relevance of this data for public health and safety, we verified whether these neuroadaptive changes have a translational impact on the risk associated with the observed abuse pattern among young adults (ESPAD, 2019). This allowed us to explore shared vulnerability pathways across drug classes in relation to psychiatric events. In fact, previous epidemiological studies have shown that young adults who use SCBs also report using hallucinogens (e.g. phenethylamines) (Caviness et al., 2015). Furthermore, a more recent systematic review showed that, alongside synthetic cannabinoids (SCBs), hallucinogens were among the top three classes of substance involved in novel psychoactive substance (NPS)-related intoxication cases in which acute or chronic psychiatric symptoms were reported (Taflaj et al., 2024). Thus, further male and female mice have been injected with the serotoninergic compounds 2,5-Dimethoxy-4-iodophenethylamine (2C-I) or 4-iodo-2,5-dimethoxy-N-[(2-methoxyphenyl)methyl]-benzeneethanamine (25I-NBOMe) as they act as fully efficacious agonists at 5-HT_{2A} receptors (Elmore et al., 2018). Sensorimotor, motor, and sensory gating changes have been evaluated in these groups of animals, to evaluate a potential variated sensitivity of $5HT_{2A}$ receptors in vivo after the same treatment regimen with SCBs.

2. Materials and methods

2.1. Animals

One hundred and twenty-eight adult (3–4 months aged) ICR (CD-1 ®) mice (32 for behavioural studies, 96 for blood and tissue collection) weighing 30–35 g (Centralized Preclinical Research Laboratory, University of Ferrara, Italy) were group housed (four per cage; floor area:80 cm 2 per mouse; minimum enclosure height: 12 cm), exposed to a12:12-h light–dark cycle (light on at 6:30 a.m.) at a temperature of 20–22 °C and humidity of 45 %–55 % and were provided with ad libitum access to food (Diet 4RF25 GLP; Mucedola, Settimo Milanese, Milan, Italy) and water. Comparison of the pharmaco-toxicological effects induced by AKB48 was studied in groups of male and female mice from the same litter (i.e. siblings). All experimental protocols were in accordance with the U.K. Animals (Scientific Procedures) Act of 1986 and associated guidelines and the new European Communities Council Directive of September 2010 (2010/63/EU) and approved by the Italian Ministry of Health (licence n. 223/2021-PR, CBCC2.46) and the Animal Welfare Body of the University of Ferrara. According to the ARRIVE guidelines, all possible efforts were made to minimize the animals' pain and discomfort and to reduce the number of experimental subjects. Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the British Journal of Pharmacology (Lilley et al., 2020).

2.2. Drug preparation and dose selection

AKB48, 2C-I, and 25I-NBOMe were purchased from LGC Standards S. r.L. (Milan, Italy). Drugs were dissolved in Tween 80 (2 %) and ethanol (5 %), brought to the final volume with saline (0.9 % NaCl), and administered by intraperitoneal (i.p.) injection at a volume of 4 μL/gr. The solution made by Tween 80 (2 %), ethanol (5 %), and saline were also used as the vehicle. The dose of AKB48 was chosen based on the previous pharmaco-toxicological studies performed in mice (Canazza et al., 2016; Ossato et al., 2017; Bilel et al., 2020; Corli et al., 2024a), while, the dose of 2C-I and 25I-NBOMe was chosen based on the pharmaco-toxicological effects observed in previous preclinical studies (Tirri et al., 2022) and reported by users on internet fora (psychonautwiki.org; erowid.org).

2.3. Experimental design

Male and female mice have been injected with AKB48 (6 mg/kg, i.p.) or its vehicle once a week for 3 consecutive weeks, for a total of three injections (D1, D7, and D13) with a 6-day period of washout between each consecutive injection, and have then been assigned to behavioural experiments (n = 8 per group), executed at D20 after the injection of 2C-I (1 mg/kg, i.p.) or 25I-NBOMe (1 mg/kg, i.p.), or tissue analyses (n = 5 per group). The treatment regimen was based on our previous study on the same molecule (Corli et al., 2024a) and mimic the frequency of use estimated by surveys on human SCBs consumption (Gunderson et al., 2014; Palamar and Acosta, 2015; Baweja et al., 2024). Behavioural

experiments have been performed at D20 (six days after the last AKB48 injection) to explore shared vulnerability pathways across drug classes in relation to psychiatric events, focusing on enduring changes that may expose patient to an unexpected risk. Animal behaviour was analysed 5 min after the injection (D20). For tissues collection, 40 animals (20 females, 20 males) were randomly picked 8 h after the first (D1), the second (D7), or the third (D13) AKB48/vehicle injection to assess baseline 5-HT_{2A} receptor and SERT expression levels and perform histological and immunohistochemical studies (Fig. 1).

2.4. Behavioural studies

The effects of 2C-I and 25I-NBOMe were evaluated using a series of behavioural tests (visual object and visual placing response test, and reaction time test) routinely used in our laboratories to describe the effects of hallucinogenic phenethylamines in mice (Tirri et al., 2022) and widely validated for the preclinical characterization of the pharmaco-toxicological effects of NPSs in rodents (Arfè et al., 2021; Bilel et al., 2023; 2022). Experiments were performed between 8:30 a.m. and 2:00 p.m. in a thermostatic (temperature: 20–22 °C, humidity: 45 %–55 %) and light (150 lx) controlled room with a background noise of 40 ± 4 dB, by trained observers working in blind and in pairs. To reduce the stress induced by manipulation, voluntary and involuntary sensorimotor responses were performed in a consecutive manner according to the following sequence: observation of visual placing and object (frontal + lateral view) responses, and evaluation of the reaction time. Animals' behaviour was videotaped and analysed offline by a different trained operator.

2.4.1. Evaluation of visual object response

The visual object response test was used to assess the mouse's ability to see an approaching object from three perspectives, that is, from the front, the right and the left, causing it to move to avoid it, turning its head or retreat. For the frontal visual response, a white horizontal bar was moved frontally to the mouse's head; the manoeuvre was repeated three times. For the lateral visual response, a small dentist's mirror was moved into the mouse's field of view in a horizontal arc until the stimulus was between the animal's eyes. The procedure (bilaterally) was repeated three times. The total value was calculated by adding the score of 1 if there was a reflection in the mouse movement or the score of 0 if not (overall max score: 9). Evaluation of the visual object response was measured at 0-5-30-60-120-180-240-300 min post-injection.

2.4.2. Evaluation of visual placing response

The visual placing response test was performed using a tail suspension-modified apparatus able to bring the animal towards the floor at a constant speed of 10 cm/s. The downward movement of the mouse was videotaped and the frame-by-frame video analysis to evaluate the beginning of the mouse reaction while it was near the floor. When the animal started the reaction, an electronic ruler evaluated the perpendicular distance in millimetres between its eyes and the floor. A naïve mouse typically perceives the floor and prepares for contact at a distance of 1.5 ± 0.25 cm. Evaluation of the visual placing response was measured at 0-10-35-70-125-185-245-305 min post-injection.

2.4.3. Evaluation of the reaction time

This test measures motor reactivity of the animal in an open field. Mice were allowed to inhabit the centre of a square arena (150 × 150 cm) for 5 min, then elevated 3 cm above the surface (lifting from the tail), and finally left to fall. When the animal touched the floor, the latency time for the first forelimb movement was recorded. Evaluation of the reaction time response was measured at 0-10-35-70-125-185-245-305 min post-injection.

2.4.4. Acoustic startle and prepulse inhibition (PPI) test

As previously reported (Bilel et al., 2020; Tirri et al., 2022; Corli et al., 2024a), mice were tested for acoustic startle reactivity and prepulse inhibition in startle chambers (Ugo Basile apparatus, Milan, Italy) consisting of a sound-attenuated, lighted, and ventilated enclosure holding a transparent non-restrictive Perspex® animal cage (90 × 45 × 50 mm). A loudspeaker mounted laterally within the animal holder produced all acoustic stimuli. The wave amplitude evoked by the

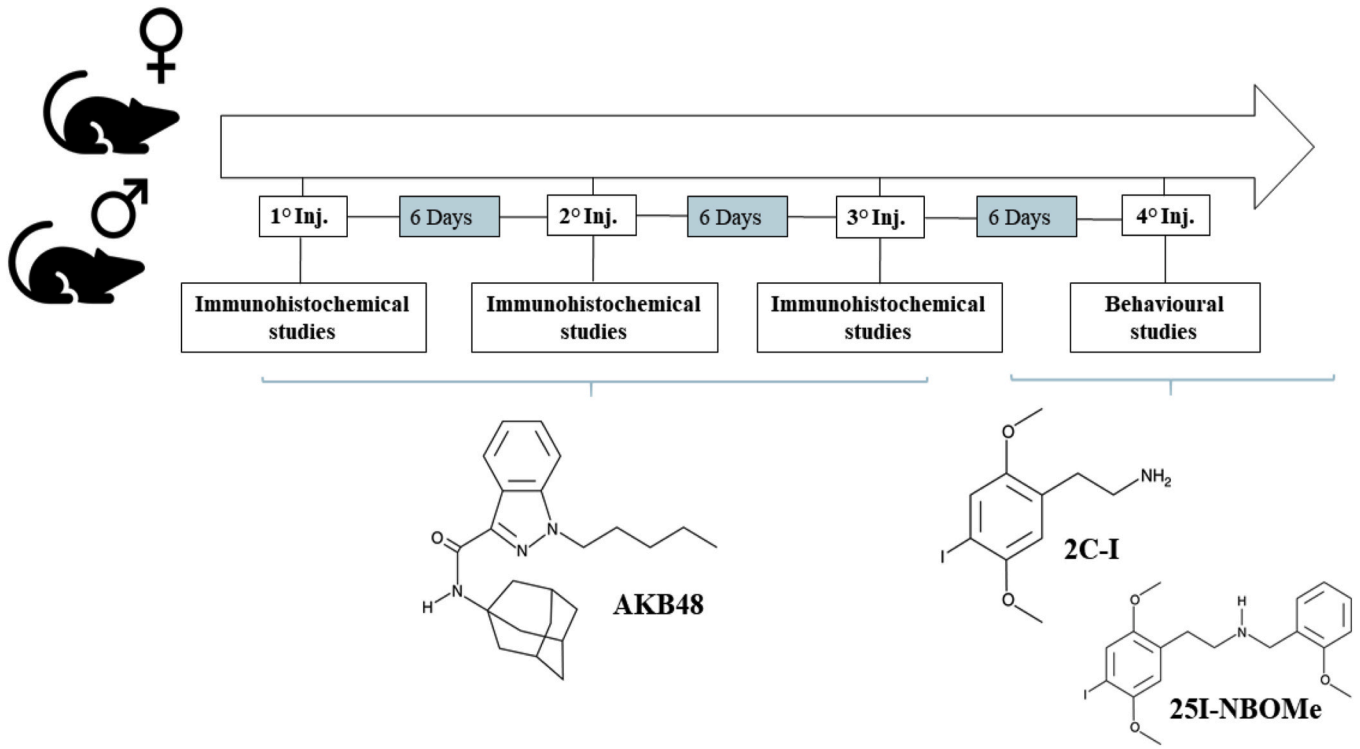

Fig. 1. Schematic representation of experimental design.

movement of the animals' startle response was detected by a load cell. At the onset of the startling stimulus, 300-ms readings were recorded and the wave amplitude was measured. Acoustic startle test sessions consisted of startle trials (pulse-alone) and prepulse trials (prepulse + pulse). The pulse-alone trial consisted of a 40-ms 120-dB pulse. Prepulse + pulse trials sequence consisted of a 20-ms acoustic prepulse, 80-ms delay, and then a 40-ms 120-dB startle pulse (100-ms onset–onset). There was an average of 15 s (randomly ranging from 9 to 21 s) 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 prepulse + pulse trials (with three different prepulses of 68-dB, 75-dB, and 85-dB) presented in a pseudo-randomized order. 2C-I and 25I-NBOMe were administered intraperitoneally, and animals were placed in the startle chambers 5 min after treatments. Mice were left in the chambers for 10 min (acclimation period) and the entire PPI test lasted 20 min. The amount of PPI is expressed as the percentage decrease in the amplitude of the startle reactivity caused by the presentation of the prepulse (% PPI).

2.5. Necropsy, tissue sampling and immunohistochemistry

2.5.1. Brain and cerebellum samples preparation

After mice were euthanized by cervical dislocation, brains and cerebella were immediately removed as previously described (De Luca et al., 2023, 2025; Corli et al., 2024a), washed in 0.9 % NaCl, fixed for 48 h at room temperature in 4 % paraformaldehyde in 0.1M phosphate buffer (pH 7.4), and postfixed in the same fixative solution at 4 °C for 1.5 h. Tissues were then immersed in absolute ethanol for 1 h, followed by acetone, and ultimately embedded in Paraplast X-TRA (Sigma Aldrich, Milan, Italy). Serial sectioning was achieved by manual rotary microtome. Eight-micrometre-thick sections of brain hemispheres and cerebellar vermis were sliced in coronal and sagittal planes, respectively and collected on silane-coated slides for histochemical and immunohistochemical studies. Firstly, to achieve the punctual neuroanatomical areas identification, a brightfield low-magnification analysis of haematoxylin & eosin (H&E)-stained samples was performed (Priori et al., 2023; Corli et al., 2024a; De Luca et al., 2023), allowing the choice of precise sections of dorsolateral prefrontal cortex (DLPC), and cerebellum, which were further treated for immunohistochemical analysis. Six slides (about 20 randomized sections) per animal were analysed.

2.5.2. Brightfield immunohistochemistry

Immunohistochemical reactions were carried out simultaneously on slides from different experimental conditions to avoid possible staining differences due to small differences in the procedures. Immunohistochemistry was performed using commercial antibodies on male and female mice cerebellar and cerebral specimens, to investigate the expression and distribution of the 5-HT_{2A} receptor and the serotonin transporter (SERT).

Paraffin-embedded sections were deparaffinized in xylene, rehydrated through a series of graded alcohol solutions and rinsed in phosphate-buffered saline (PBS, Sigma). Then, sections were incubated overnight at room temperature in a dark moist chamber with a specific primary antibody (Table 1)

Biotinylated secondary antibody (Table 1) was used to reveal the sites of antigen/antibody interaction, while the 3,3′-diaminobenzidine tetrahydrochloride peroxidase substrate (Sigma, St. Louis, MO, USA) was used as the chromogen. The nuclear counterstaining was achieved by employing Carazzi's haematoxylin. Then, sections were dehydrated in ethanol, cleared in xylene, and mounted in Eukitt (Kindler, Freiburg, Germany).

As negative controls, some sections were incubated with PBS in the absence of the primary antibodies: No immunoreactivity was observed in this condition. The Immuno-related procedures used comply with the recommendations made by the British Journal of Pharmacology (Alexander et al., 2018).

Table 1
Primary/secondary antibodies employed in immunohistochemical reactions.

	Antigen	Immunogen	Manufacturer, species, mono-polyclonal, cat./ lot	Dilution
Primary antibody	ST (H-8)	Purified antibody specific for an epitope mapping between amino acids 69–88 within an N-terminal cytoplasmic domain of ST of human origin.	Santa Cruz Biotechnology (Santa Cruz, CA, USA), Mouse monoclonal IgG, Cat# sc-518084	1:100
	5-HT_{2A} (A-4)	Purified antibody raised against amino acids 1–75 mapping within an N-terminal extracellular domain of SR-2A of human origin	Santa Cruz Biotechnology (Santa Cruz, CA, USA), Mouse monoclonal IgG, Cat# sc-166775	1:100
Secondary antibody	Biotinylated horse anti-mouse IgG	Gamma immunoglobulin	Vector Laboratories (Burlingame, CA, USA), Horse, Cat# PK-6102	1:200

2.5.3. Histochemical and immunohistochemical analyses

Samples were observed in brightfield microscopy using an Olympus BX51 optical microscope (model BX51TF), and images were acquired with an Olympus CAMEDIA C4040ZOOM camera (Olympus Italia S.r.l., Segrate, MI, Italy). For the assessed marker, six slides (about 20 randomized sections) per animal were examined, for a total of n = 5 animals per experimental condition. In all experimental groups, cerebellar and brain specimens with diverse immunolabelling extent were considered. Immunohistochemical labelling extent was evaluated on acquired digitized section images under exposure time avoiding any pixel saturation effect. The labelling intensity was measured utilizing densitometric analysis (Image-J 1.48i; NIH, Bethesda, MA, USA), as previously reported (Cizkova et al., 2021; Roda et al., 2020a, 2020b). First, the colour of images was inverted to obtain a positive signal lighter on a dark background (instead of the immunoperoxidase staining results), thus correlating the intensifying of immunopositivity with the increasing optical density (OD) values calculated by the software (expressed as mean of light intensity). The mask shape was adjusted depending on the spatial distribution, signal localization, layer, and cell types of the cerebellar or cerebral specimens under measurement (using the polygon selection tool to ensure the punctual evaluation of the positivity area only). The immunohistochemical intensity (expressed as OD) was evaluated in three randomized images/section (making at least 10 measurements per image) per six slides per animal for a total of n = 5 animals from each experimental group, with the operator blinded to the experimental condition. The labelling was measured as the mean intensity value over the area for all sections and reported as the mean of the measurements per slide for each animal. Additionally, immunoreactive cells density (number of immunopositive cells per area in mm^2) was assessed as previously described (Roda et al., 2023) and reported as the mean for each individual animal.

2.6. Data and statistical analysis

In sensorimotor response experiments, data was expressed in arbitrary units (visual object response) and percentage of baseline (visual placing response, reaction time). The amount of PPI was calculated as a percentage score for each prepulse + pulse trial type:

$\%PPI = \left\{\left[\frac{(startle\ response\ for\ prepulse+pulse\ trial)}{(startle\ response\ for\ pulse-alone\ trial)}\right] x100\right\}$. Startle magnitude was calculated as the average response to all the pulse-alone trials. All numerical data are expressed as mean ± SEM and analysed by repeated measures analysis of variance (ANOVA). The effects of treatments over time were analysed by two-way ANOVA followed by Bonferroni's *post hoc* test for multiple comparisons. Analysis of the total average effect induced by treatments was performed with one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons. Post-hoc tests were run only if F achieved $P < 0.05$ and there was no significant variance inhomogeneity.

Immunohistochemical results were expressed as mean ± SEM. For data that passed the normality test (D'Agostino & Pearson test), the analysis was conducted using one-way ANOVA followed by Bonferroni's post hoc test for multiple comparisons. Conversely, for non-normally distributed results, the analysis was performed employing the Kruskal–Wallis test followed by Dunn's test. The differences were considered statistically significant for $P < 0.05$. * control vs. each experimental condition; ° 1st AKB-48 vs. other treatments; # 2nd AKB-48 vs. any experimental groups. All statistical analyses were performed by using GraphPad Prism 8.0 (GraphPad Software Inc., CA, USA).

3. Results

3.1. Evaluation of the visual object response

The Visual object response does not change in vehicle-treated mice over the 300-min observation, while 2C-I (1 mg/kg, i.p.) and 25I-NBOMe (1 mg/kg, i.p.) reduce the visual object response both in male and female mice (Fig. 2a–d). However, both 2C-I (Fig. 2a; effect of treatment [$F_{(3,160)} = 206.2$, $P < 0.0001$], time [$F_{(7,160)} = 12.62$, $P < 0.0001$], time × treatment interaction [$F_{(21,160)} = 5.029$, $P < 0.0001$]) and 25I-NBOMe (Fig. 2c; effect of treatment [$F_{(3,160)} = 166.0$, $P < 0.0001$], time [$F_{(7,160)} = 12.21$, $P < 0.0001$], time × treatment interaction [$F_{(21,160)} = 4.159$, $P < 0.0001$]) induce a deeper decrease in visual object response at 30 min post-injection in male mice pretreated with AKB48 with respect to untreated mice. On the other hand, the effect induced in females by 2C-I (Fig. 2b; effect of treatment [$F_{(3,160)} = 220.4$, $P < 0.0001$], time [$F_{(7,160)} = 13.41$, $P < 0.0001$], time × treatment interaction [$F_{(21,160)} = 5.203$, $P < 0.0001$]) and 25I-NBOMe (Fig. 2d;

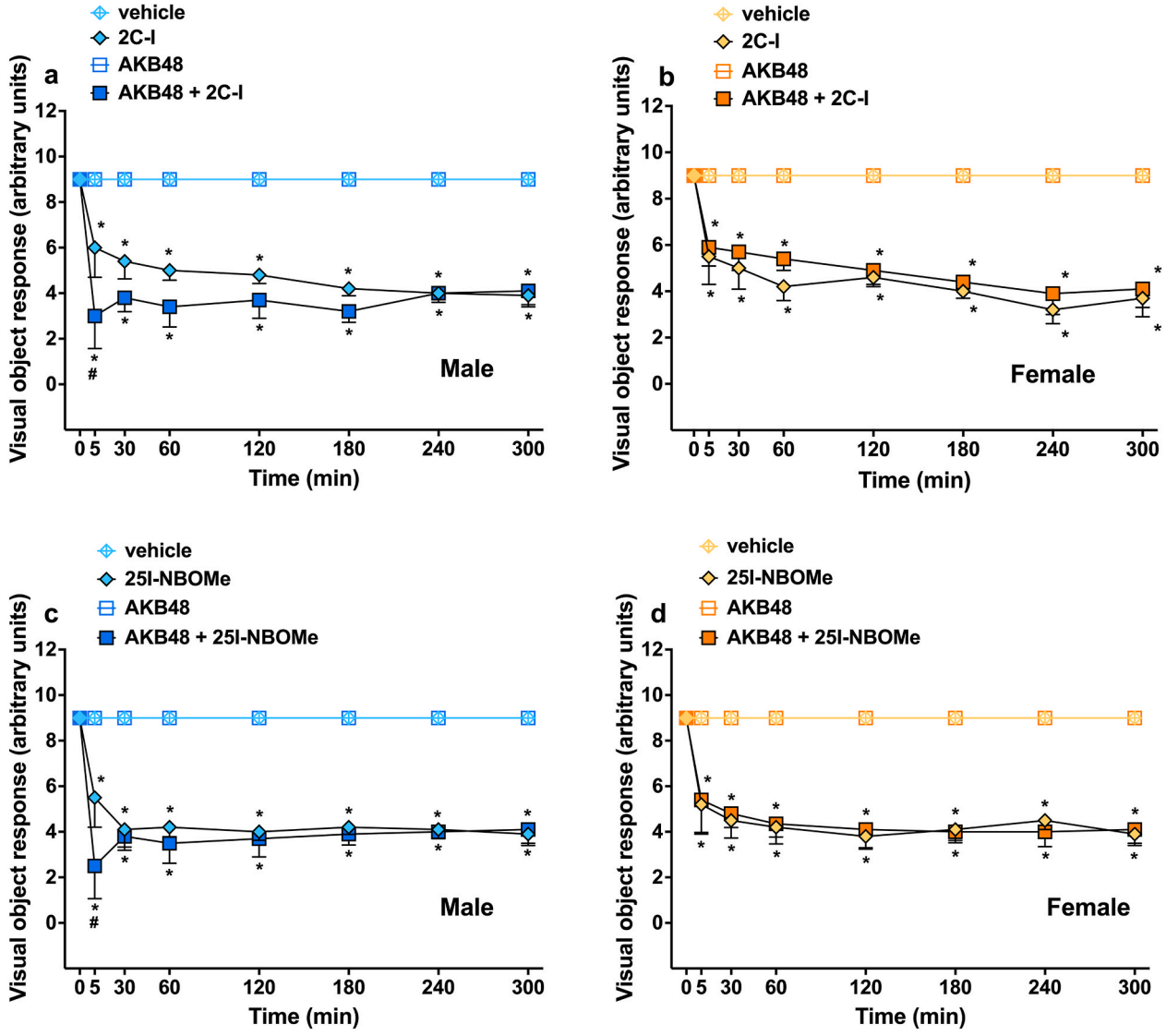

Fig. 2. Effect of 2C-I (1 mg/kg, i.p.; panels a–b) and 25I-NBOMe (1 mg/kg, i.p.; panels c–d) on the visual object in untreated and AKB48-treated male (left, in blue) and female (right, in orange) mice. Data are expressed as arbitrary units. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparisons for the treatment response curve at different times (a–d). *$P < 0.05$ versus vehicle; #$P < 0.05$ versus 2C-I or 25I-NBOMe; n = 8 per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

effect of treatment [$F_{(3,160)} = 170.7$, $P < 0.0001$], time [$F_{(7,160)} = 11.21$, $P < 0.0001$], time × treatment interaction [$F_{(21,160)} = 3.783$, $P < 0.0001$]) is not influenced by the pre-treatment with AKB48.

3.2. Evaluation of the visual placing response

The Visual placing response does not change in vehicle-treated mice over the 300-min observation, while 2C-I (1 mg/kg, i.p.) and 25I-NBOMe (1 mg/kg, i.p.) reduce the visual placing response both in male and female mice (Fig. 3, ad). In male mice pretreated with AKB48, 2C-I (Fig. 3a; effect of treatment [$F_{(3,176)} = 108.6$, $P = 0.0005$], time [$F_{(7,176)} = 6.762$, $P < 0.0001$], time × treatment interaction [$F_{(21,176)} = 2.535$, $P < 0.0001$]) and 25I-NBOMe (Fig. 3c; effect of treatment [$F_{(3,176)} = 79.74$, $P = 0.0060$], time [$F_{(7,176)} = 6.605$, $P < 0.0001$], time × treatment interaction [$F_{(21,176)} = 2.064$, $P < 0.0001$]) induce a deeper decrease in visual object response at 70–125 min and 15–70 min post-injection, respectively. Similarly, the effect induced in AKB48-treated female mice by 2C-I (Fig. 3b; effect of treatment [$F_{(3,176)} = 182.6$, $P < 0.0001$], time [$F_{(7,176)} = 11.00$, $P < 0.0001$], time × treatment interaction [$F_{(21,176)} = 4.091$, $P < 0.0001$]) is more pronounced at 15 min and that provoked by 25I-NBOMe (Fig. 3d; effect of treatment [$F_{(3,176)} = 186.3$, $P < 0.0001$], time [$F_{(7,176)} = 14.87$, $P < 0.0001$], time × treatment interaction [$F_{(21,176)} = 4.457$, $P < 0.0001$]) up to 35 min post-injection, with respect to the response evoked by the compounds in untreated female mice.

3.3. Evaluation of the reaction time

The latency time for the first forelimb movement (reaction time) does not change in vehicle-treated mice over the 300-min observation, while 2C-I (1 mg/kg, i.p.) and 25I-NBOMe (1 mg/kg, i.p.) increase the reaction time of both male and female mice (Fig. 4a–d). In male mice pretreated with AKB48, 2C-I (Fig. 4a; effect of treatment [$F_{(3,160)} = 108.6$, $P = 0.0005$], time [$F_{(7,160)} = 6.762$, $P < 0.0001$], time × treatment interaction [$F_{(21,160)} = 2.535$, $P < 0.0001$]) and 25I-NBOMe (Fig. 4c; effect of treatment [$F_{(3,160)} = 79.74$, $P = 0.0060$], time [$F_{(7,160)} = 6.605$, $P < 0.0001$], time × treatment interaction [$F_{(21,160)} = 2.064$, $P < 0.0001$]) induce a larger increase in the reaction time at 15, 70–185 min and 15–35 min post-injection, respectively. Similarly, the effect induced in AKB48-treated female mice by 2C-I (Fig. 4b; effect of treatment [$F_{(3,176)} = 182.6$, $P < 0.0001$], time [$F_{(7,176)} = 11.00$, $P < 0.0001$], time × treatment interaction [$F_{(21,176)} = 4.091$, $P < 0.0001$]) is

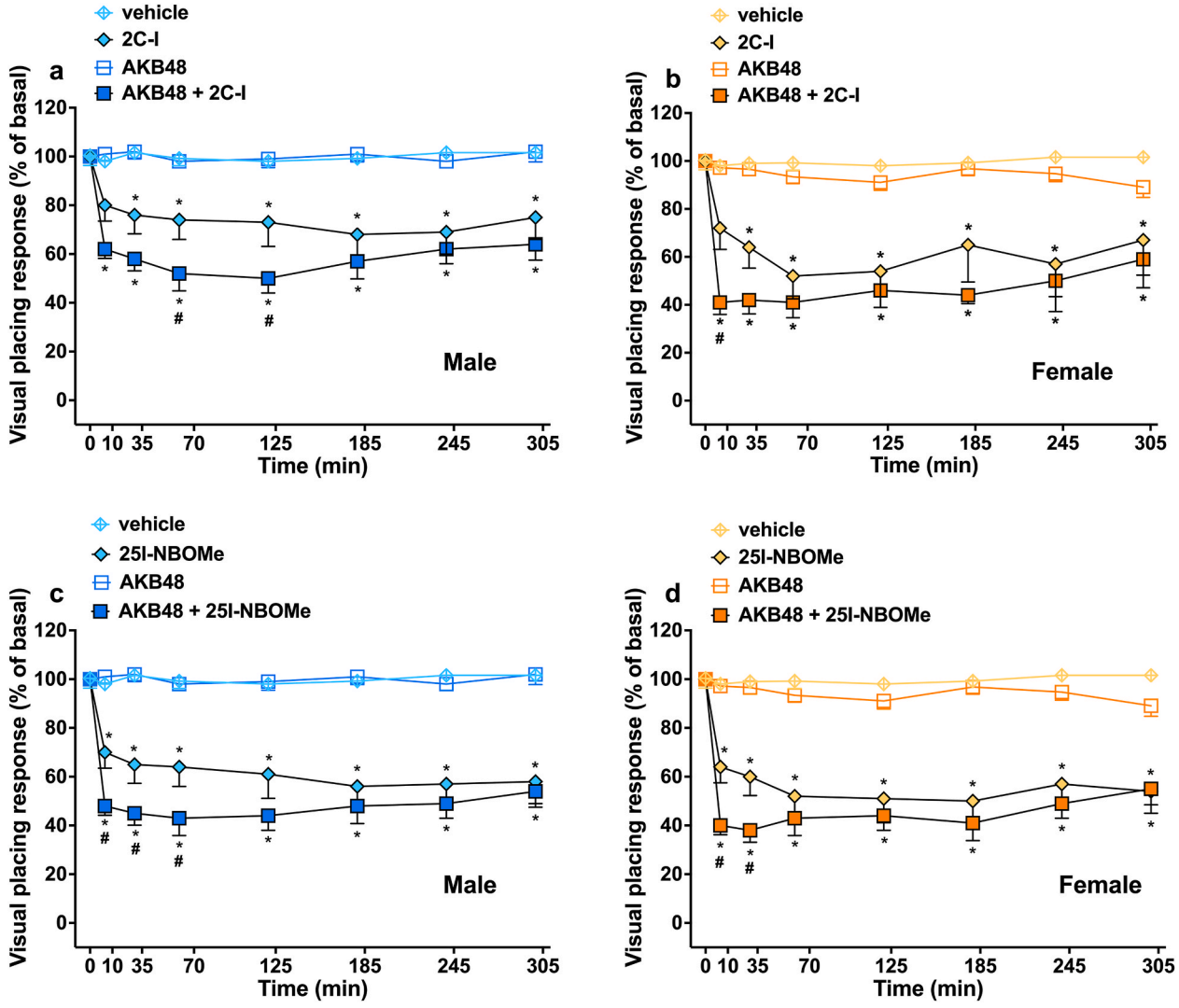

Fig. 3. Effect of 2C-I (1 mg/kg, i.p.; panels a–b) and 25I-NBOMe (1 mg/kg, i.p.; panels c–d) on the visual placing in untreated and AKB48-treated male (left, in blue) and female (right, in orange) mice. Data are expressed as % of basal values. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparisons for the treatment response curve at different times (a–d). *$P < 0.05$ versus vehicle; $^{\#}P < 0.05$ versus 2C-I or 25I-NBOMe; n = 8 per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

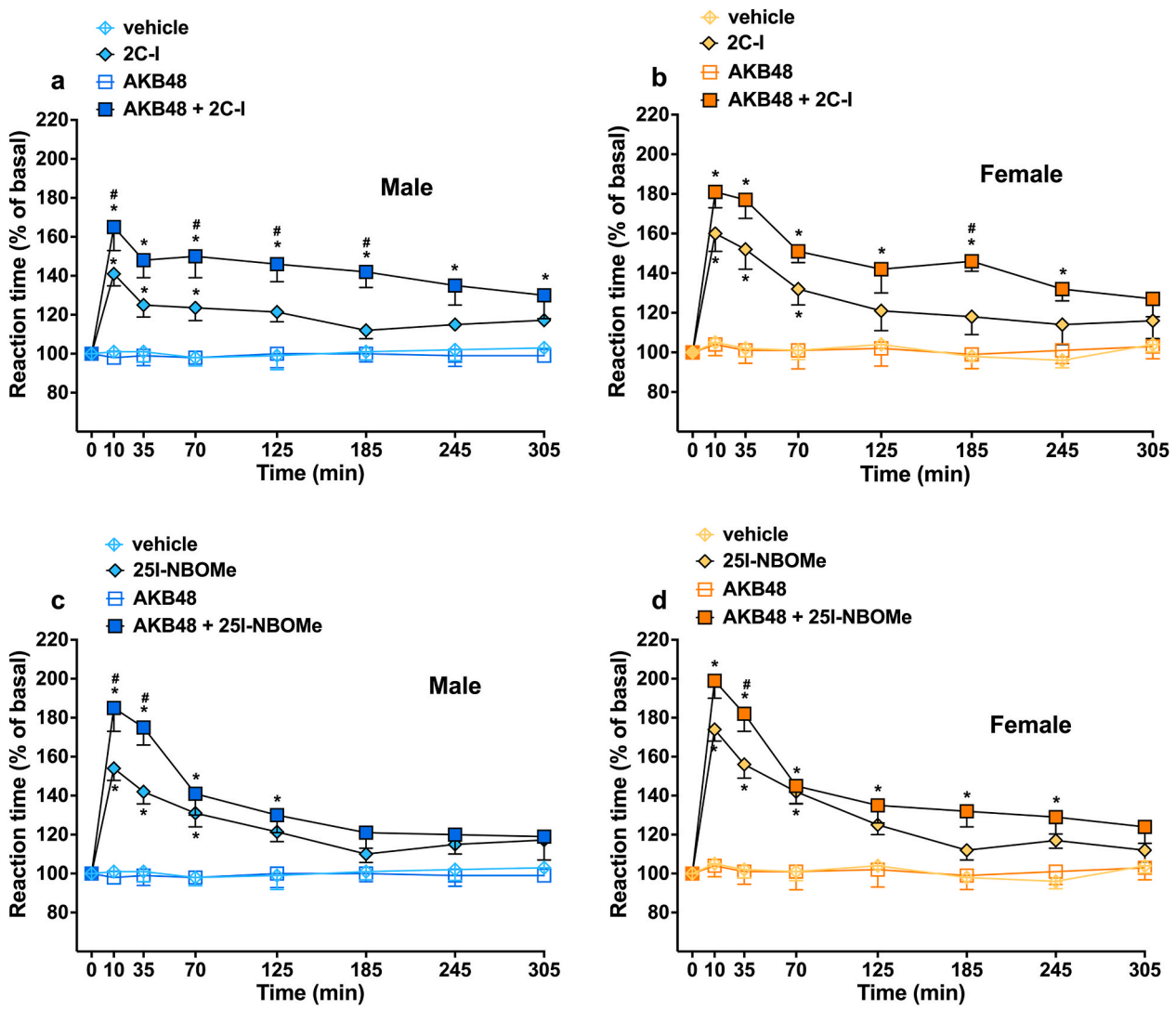

Fig. 4. Effect of 2C-I (1 mg/kg, i.p.; panels a–b) and 25I-NBOMe (1 mg/kg, i.p.; panels c–d) on the reaction time in untreated and AKB48-treated male (left, in blue) and female (right, in orange) mice. Data are expressed as % of basal values. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparisons for the treatment response curve at different times (a–d). *P < 0.05 versus vehicle; #P < 0.05 versus 2C-I or 25I-NBOMe; n = 8 per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

more pronounced at 185 min and that provoked by 25I-NBOMe (Fig. 4d; effect of treatment [$F_{(3,176)} = 186.3$, $P < 0.0001$], time [$F_{(7,176)} = 14.87$, $P < 0.0001$], time × treatment interaction [$F_{(21,176)} = 4.457$, $P < 0.0001$]) at 35 min post-injection, with respect to increase in the latency time for the first forelimb movement observed in untreated female mice.

3.4. Acoustic startle and prepulse inhibition (PPI) test

The acoustic startle and PPI response does not change in vehicle-treated mice (Figs. 5 and 6). Otherwise, systemic administration of 2C-I (1 mg/kg, i.p.) and 25I-NBOMe (1 mg/kg, i.p.), as well as repeated treatment with AKB48 (6 mg/kg; i.p.), provokes significant changes in startle amplitude in both male and female mice (Fig. 5a–d). In the 2C-I group of male mice, one-way ANOVA detects a significant effect (Fig. 5a; $F_{(3, 28)} = 6.241$, $P = 0.0022$) of AKB48 pretreatment alone, as well as the injection of 2C-I in AKB48-pretreated mice. Differently, ANOVA analysis only detects a significant effect of AKB48 pretreatment in female mice of 2C-I group (Fig. 5b; $F_{(3, 28)} = 4.318$, $P = 0.0127$). Moreover, one-way ANOVA detects a significant effect of 25I-NBOMe in untreated mice, AKB48 pretreatment, and injection of 25I-NBOMe in AKB48-treated mice, in both male (Fig. 5c; $F_{(3, 28)} = 15.32$, $P = 0.0022$) and female (Fig. 5d; $F_{(3, 28)} = 11.27$, $P = 0.0022$) 25I-NBOMe groups. Noteworthy, the reduction induced by the injection of 2C-I in male AKB48-treated mice is greater, with respect to that provoked by hallucinogen in untreated male mice. Even though one-way ANOVA does not detect this effect, the Unpaired T test revealed that the impairment observed after the injection of 25I-NBOMe in AKB48-treated male mice is significantly deeper with respect to that induced by the hallucinogen (Fig. 5c; $t = 2.472$, $df = 14$, $P = 0.0269$) and the pretreatment alone (Fig. 5c; $t = 2.589$, $df = 14$, $P = 0.0214$).

In addition, repeated treatment with AKB48 and acute administration of 2C-I and 25I-NBOMe inhibited the PPI in both male and female mice (Fig. 6a–d). One-way ANOVA detects a significant effect (Fig. 6a; $F_{(11, 84)} = 58.62$, $P < 0.0001$) of 2C-I administration in untreated male mice at prepulse intensities of 68 and 85 dB, AKB48 pretreatment at 75 and 85 dB, and injection of 2C-I in AKB48-pretreated male mice at 68, 75, 85 dB. Similarly, a significant impairment (Fig. 6b; $F_{(11, 84)} = 34.48$, $P < 0.0001$) is detected after 2C-I administration in untreated female mice at prepulse intensities of 68 and 85 dB, AKB48 pretreatment at 75 and 85 dB, and injection of 2C-I in AKB48-pretreated female mice at 68, 75, 85 dB. Again, ANOVA detects a significant effect (Fig. 6a; $F_{(11, 84)} = 53.69$, $P < 0.0001$) of 25I-NBOMe administration in untreated male

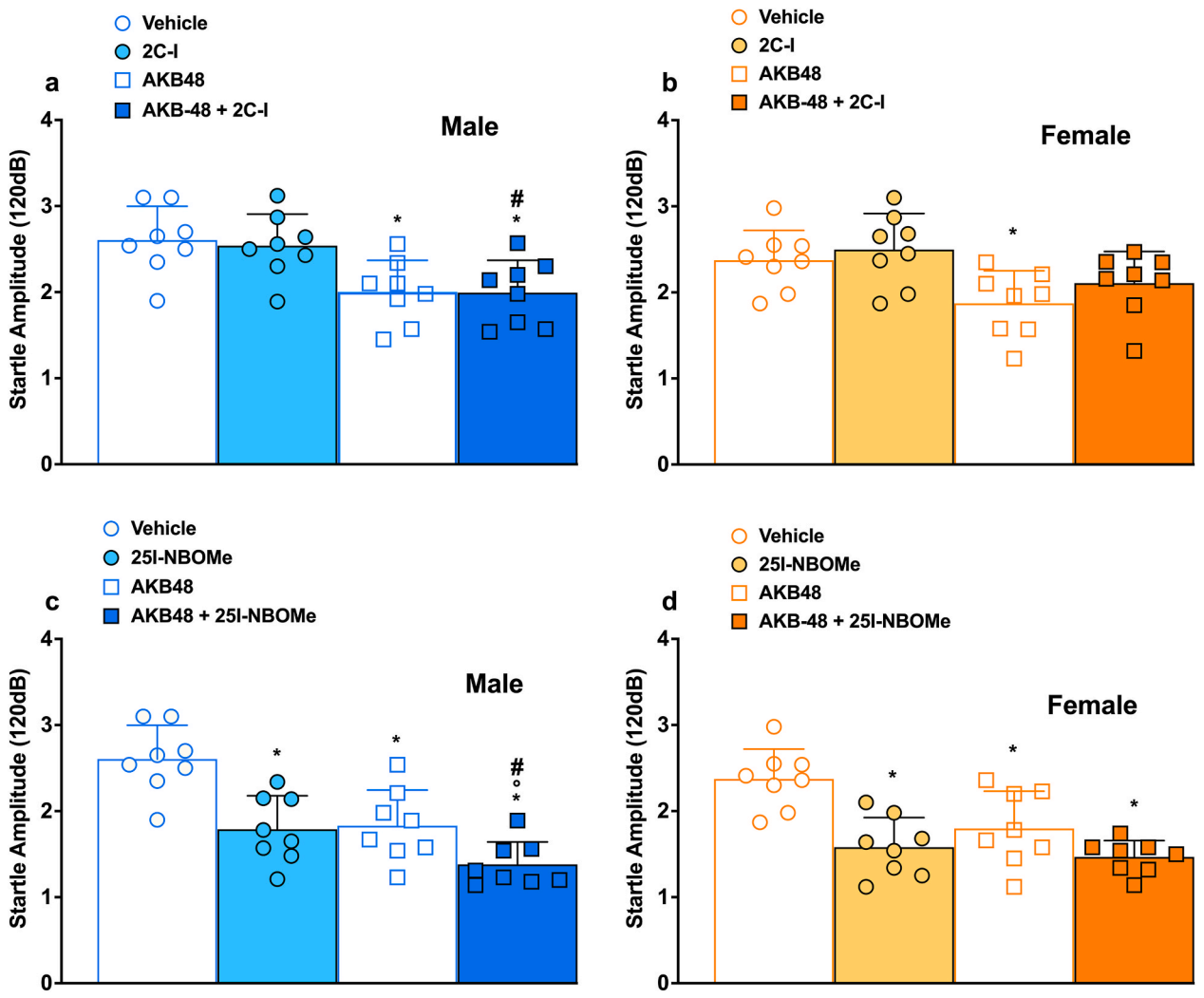

Fig. 5. Effect of 2C-I (1 mg/kg, i.p.; panels a–b) and 25I-NBOMe (1 mg/kg, i.p.; panels c–d) on the startle amplitude in untreated and AKB48-treated male (left, in blue) and female (right, in orange) mice. Data are expressed as the average response to all the pulse-alone trials. Statistical analysis was one-way ANOVA followed by Tukey's test for multiple comparisons and verified by Unpaired T test (a–d). *P < 0.05 versus vehicle; °P < 0.05 versus AKB48; #P < 0.01 versus 2C-I or 25I-NBOMe; n = 8 per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mice at prepulse intensities of 68, 75, 85 dB, AKB48 pretreatment at 75 and 85 dB, and injection of 25I-NBOMe in AKB48-treated male mice at 68, 75, 85 dB. Similarly, a significant impairment (Fig. 6b; $F_{(11, 84)} = 61.92$, $P < 0.0001$) is detected after 25I-NBOMe administration in untreated female mice, as well as the AKB48 pretreatment, and injection of 25I-NBOMe in AKB48-treated female mice at all the tested prepulse intensities. The PPI changes provoked by 2C-I and 25I-NBOMe in AKB48-treated male and female mice is more pronounced with respect to that induced by both the hallucinogen and the pretreatment alone.

3.5. *AKB48 effects on cerebellar and cortical serotonin pattern*

Immunohistochemical reactions were conducted on coronal and sagittal sections of the DLPFC and cerebellar vermis, respectively, of both male and female vehicle-treated (control) and AKB48-treated mice, i.e after the first, the second or the third drug-injection.

3.5.1. *5-HT$_{2A}$ receptor analisys*

As shown in Fig. 7, following immunohistochemical staining for the 5-HT$_{2A}$ receptor in the cerebellum of the different experimental groups, immunopositivity for this receptor was observed in the soma of Purkinje cells and in the mossy fiber rosettes of cerebellar cortex across all groups, although with varying intensity depending on the experimental condition. In particular, the analysis of 5-HT$_{2A}$ immunopositive OD revealed a highly significant immunopositivity reduction already after a single administration, in both males and females, compared with their respective control groups. The reduction remained significant when comparing the 2nd AKB group with the controls, in both sexes. A further decrease was evident after the third administration in both males and females (Kruskal Wallis test, K-W statistic = 91.3, $p < 0.001$ and Kruskal Wallis test, K-W statistic = 95.7, $p < 0.001$, for males and females, respectively).

The analysis of 5-HT$_{2A}$ immunolabeling in Purkinje cells, however, showed a different trend compared to that observed in mossy fibers. Specifically, after a single administration, a marked increase in 5-HT$_{2A}$ immunopositive OD was detected in Purkinje cells of both sexes. Nevertheless, in the 2nd AKB-48 groups a reduction of this immunopositivity was observed, which was more pronounced in females compared with males. A further decrease was then evident after the third injection in both sexes (Kruskal Wallis test, K-W statistic = 58.2, $p < 0.001$ and Kruskal Wallis test, K-W statistic = 75.6, $p < 0.001$, for males and females, respectively).

In line with the observations made in the cerebellar cortical mossy fibers, 5-HT$_{2A}$ immunopositive OD measured in pyramidal neurons (PyrNs) of the DLPFC (Fig. 8) was also found to be significantly increased after a single injection in both sexes, compared with their

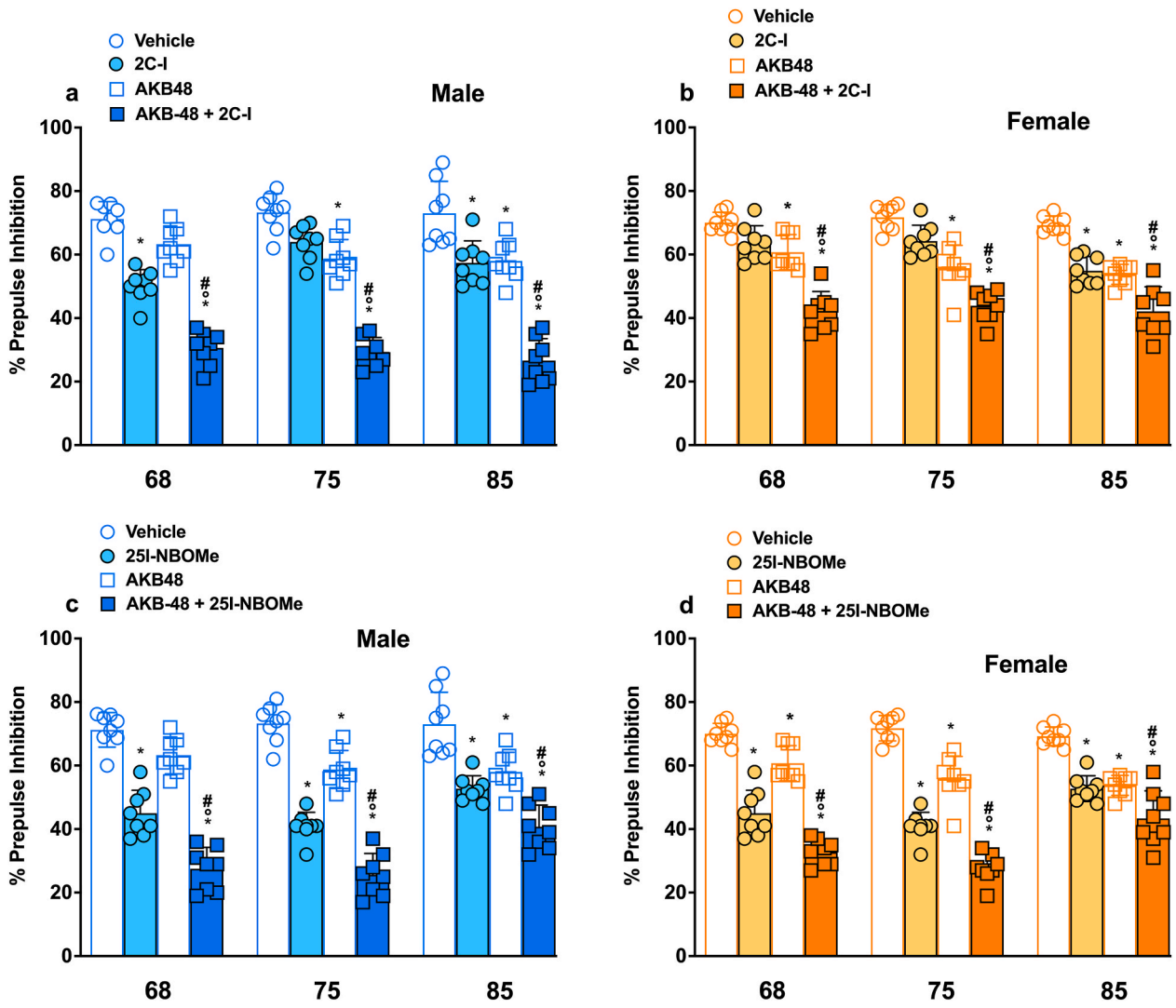

Fig. 6. Effect of 2C-I (1 mg/kg, i.p.; panels a–b) and 25I-NBOMe (1 mg/kg, i.p.; panels c–d) on the prepulse inhibition (PPI) in untreated and AKB48-treated male (left, in blue) and female (right, in orange) mice. Effects on PPI are shown for the three pre-pulse intensities (68, 75, and 85 dB) 15 min after treatment. Data are expressed as percentage of PPI. Statistical analysis was performed by one-way ANOVA followed by Tukey's test for multiple comparisons and verified by Unpaired T test (a–d). *P < 0.05 versus vehicle; °P < 0.05 versus AKB48; #P < 0.01 versus 2C-I or 25I-NBOMe; n = 8 per group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

respective control groups. The increase remained significant when comparing the 2nd AKB-48 groups with the controls in both sexes. However, following the third administration, a highly significant reduction in 5-HT_{2A} immunolabeling in these cortical neurons was detected compared with the previous experimental groups (one-way ANOVA, $F(3, 116) = 9.181$, $p < 0.001$ and one-way ANOVA, $F(3, 116) = 1.169$, $p < 0.001$, for males and females, respectively), in both sexes, with the decrease being much more pronounced in males.

Similarly, the analysis of 5-HT_{2A} immunopositive PyrNs cell density revealed an increase in the number of immunopositive cells after the first administration, compared with the corresponding sex-specific control groups, followed by a decreasing trend after the second and third administrations (Kruskal Wallis test, K-W statistic = 12.0, $p = 0.008$ and Kruskal Wallis test, K-W statistic = 12.3, $p < 0.006$, for males and females, respectively) in both sexes.

3.5.2. *SERT evaluations*

Fig. 9 shows the results of the immunohistochemical analyses for the SERT transporter in the cerebellum of the different experimental groups. The analysis of SERT immunopositive OD in the mossy fiber rosettes revealed a reduction in immunolabeling in the 1st AKB-48 group compared with the control group in both sexes, with a further decrease after the second and third administrations of the substance (Kruskal Wallis test, K-W statistic = 73.0, $p < 0.001$ and Kruskal Wallis test, K-W statistic = 101, $p < 0.001$, for males and females, respectively). This reduction was more pronounced in female mice.

After the first AKB-48 administration, a significant reduction in immunopositive OD was also observed in Basket cells. This reduction became even more pronounced after the second and third administrations (one-way ANOVA, $F(3, 112) = 7.33$, $p < 0.001$ and one-way ANOVA, $F(3, 116) = 17.5$, $p < 0.001$, for males and females, respectively).

A similar decreasing trend in SERT immunopositive OD was observed throughout the parenchyma of the internal granular layer (IGL) in treated animals. The reduction was already detectable after a single administration and became progressively greater in the 2nd AKB-48 and 3rd AKB-48 groups (Kruskal Wallis test, K-W statistic = 95.5, $p < 0.001$ and Kruskal Wallis test, K-W statistic = 102, $p < 0.001$, for males and females, respectively) in both sexes.

The analysis of SERT-immunopositive cell density in the IGL revealed only a slight reduction in the 1st AKB-48 male group compared with controls, whereas a very significant reduction was observed in the

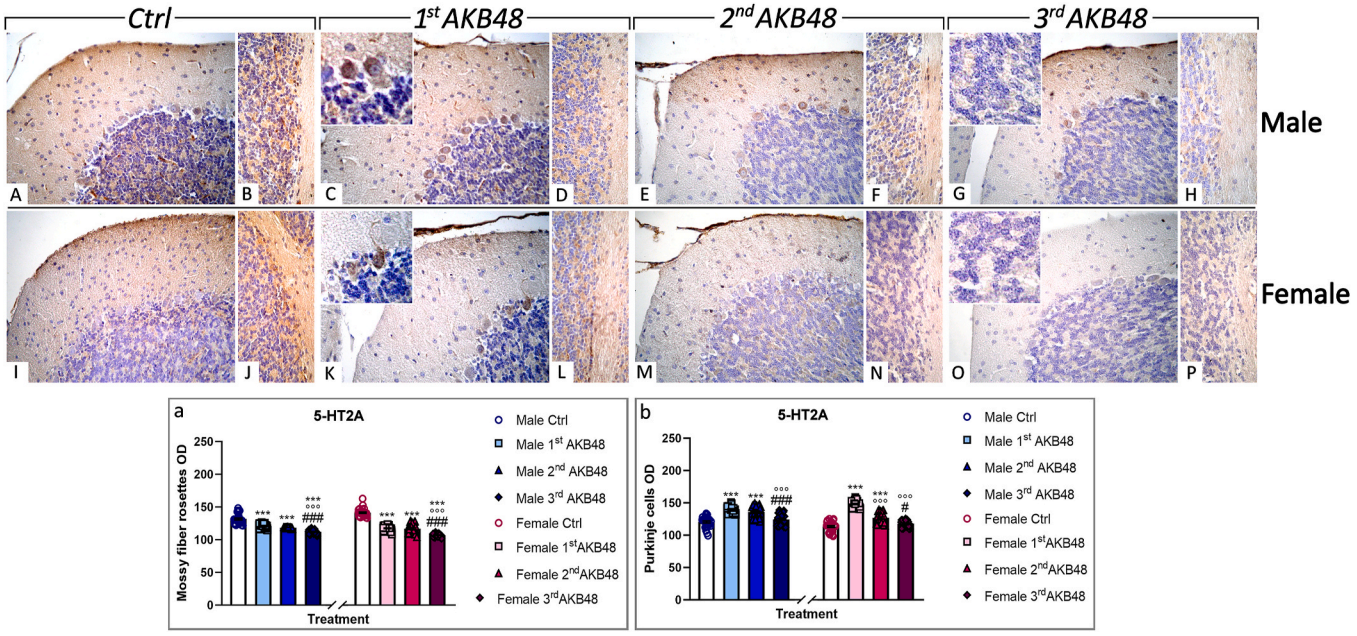

Fig. 7. Representative micrographs showing the effect of single and repeated systemic administration of AKB48 (6 mg·kg−1, i.p.) on cerebellar immunostaining patterns of SR2A receptor expression in male and female mice after vehicle administration (panels A–B and I-J, for male and female respectively), first AKB48 injection (panels C–D and K-L, for male and female respectively), second AKB48 injection (panels E–F and M-N, for male and female respectively), and third AKB48 injection (panels G–H and O-P, for male and female respectively). Light microscopy magnification: 400 × (A, C, E, G, I, K, M, O); 600 × (B, D, F, H, J, L, N, P); 1000 × (inserts). Histograms show the quantitative analysis of SR2A receptor-immunopositive OD in the mossy fiber rosettes and PCs in cerebellar cortex, of control and AKB48-treated mice (panels a–b).

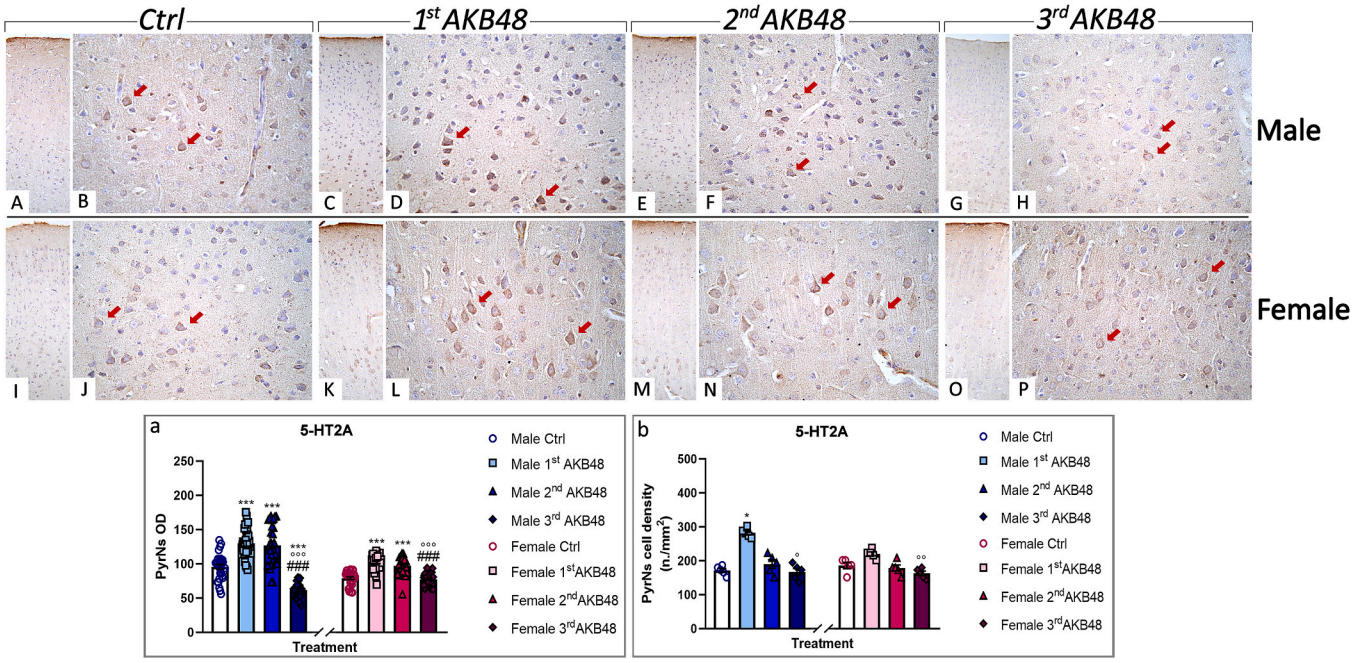

Fig. 8. Effect of single and repeated systemic administration of AKB48 (6 mg·kg−1, i.p.) on dorsolateral prefrontal cortex (DLPFC) immunostaining patterns of SR2A receptor expression in male and female mice after vehicle administration (panels A–B and I-J, for male and female respectively), first AKB48 injection (panels C–D and K-L, for male and female respectively), second AKB48 injection (panels E–F and M-N, for male and female respectively), and third AKB48 injection (panels G–H and O-P, for male and female respectively). Light microscopy magnification: 200 × (A, C, E, G, I, K, M, O); 400 × (B, D, F, H, J, L, N, P). The red arrows indicate PyrNs. Histograms show the quantitative analysis of SR2A receptor PyrNs immunopositive OD and cell density (n./mm^2) in DLPFC of control and AKB48-treated mice (panels a–b). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

corresponding female groups. After the second administration, a further decrease in the density of immunolabeled cells was detected in both sexes. However, while no additional significant reduction was observed in males after the third administration, female mice showed a further decrease in the density of SERT-immunopositive cells (Kruskal Wallis test, K-W statistic = 15, p = 0.002 and one-way ANOVA, F(3, 16) = 0.133, p < 0.001, for males and females, respectively).

In the DLPFC of AKB-48–treated animals, a highly significant

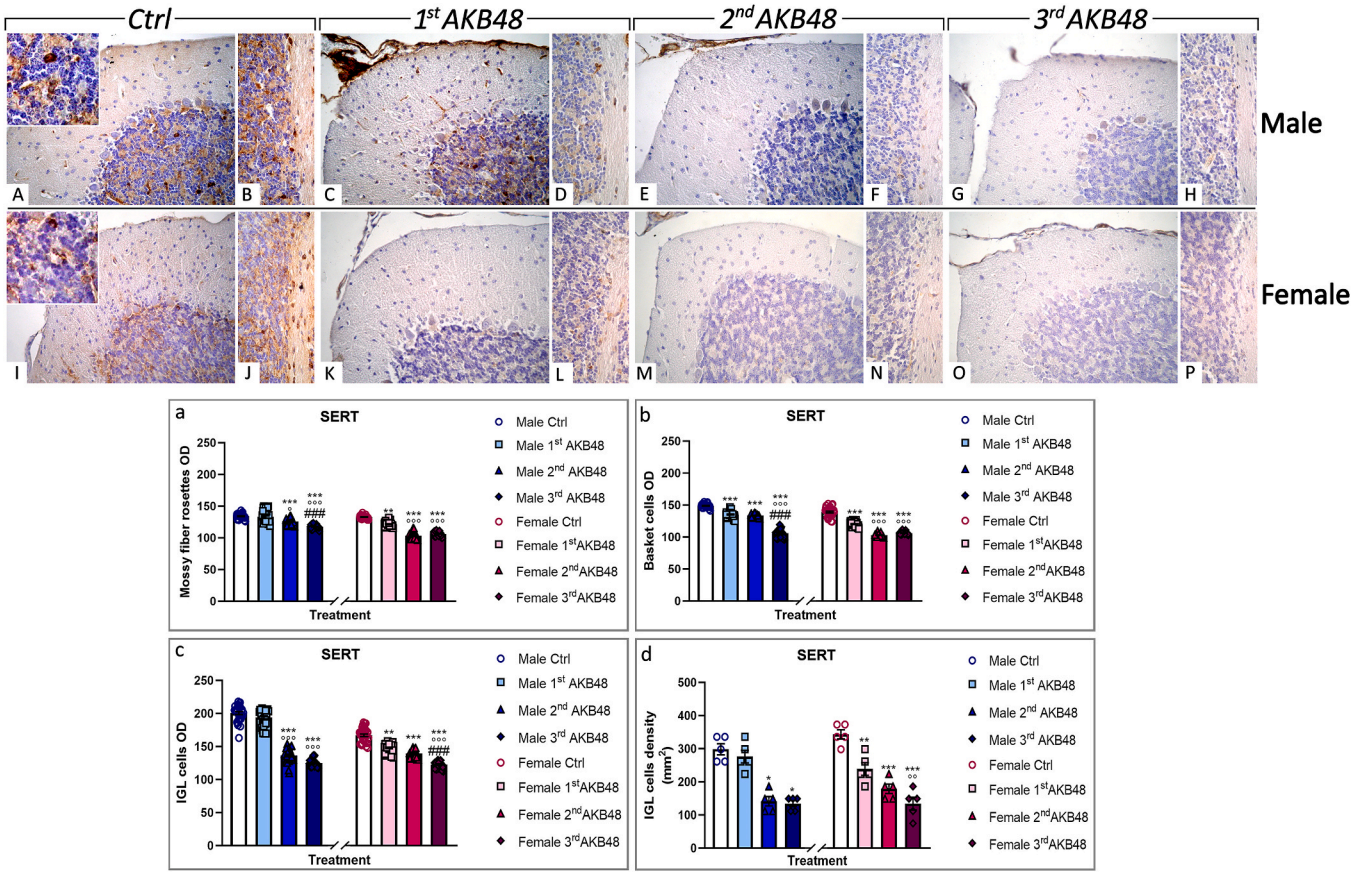

Fig. 9. Representative micrographs showing the effect of single and repeated systemic administration of AKB48 (6 mg·kg−1, i.p.) on cerebellar immunostaining patterns of ST trasporter expression in male and female mice after vehicle administration (panels A–B and I-J, for male and female respectively), first AKB48 injection (panels C–D and K-L, for male and female respectively), second AKB48 injection (panels E–F and M-N, for male and female respectively), and third AKB48 injection (panels G–H and O-P, for male and female respectively). Light microscopy magnification: 400 × (A, C, E, G, I, K, M, O); 600 × (B, D, F, H, J, L, N, P); 1000 × (inserts). Histograms show the quantitative analysis of ST transporter-immunopositive OD in the mossy fiber rosettes, basket cells and IGL, as well as ST transporter-immunopositive IGL cell density in cerebellar cortex, of control and AKB48-treated mice (panels a–d).

reduction in SERT PyrN immunopositive OD was already observed after a single administration, more pronounced in males than in females (Fig. 10). This reduction remained unchanged after the second administration in both sexes. However, while no further decrease was observed in male mice after the third administration, in female 3rd AKB-48 animals an additional reduction in pyramidal neuron immunopositive OD was detected (Kruskal Wallis test, K-W statistic = 68.20, $p < 0.001$ and Kruskal Wallis test, K-W statistic = 65.43, $p < 0.001$, for males and females, respectively).

Conversely, when analyzing the cell density of SERT-immunopositive pyramidal neurons, a marked increase was detected after a single AKB-48 administration in both sexes, with the increase being much more pronounced in males than in females. After the second administration, a decreasing trend in immunolabeling was observed. Although the density of immunopositive cells slightly decreased in males, this reduction was highly significant in females compared with their 1st AKB-48 groups, reaching values comparable to controls. In female 3rd AKB-48 animals, no further reduction was observed compared with the 2nd AKB-48 group, whereas in males the density of immunopositive cells decreased significantly, reaching values comparable to those of the respective control group (one-way ANOVA, $F(3, 16) = 1.640$ and one-way ANOVA, $F(3, 16) = 0.7684$, for males and females, respectively).

4. Discussion

The present study demonstrates for the first time that repeated treatment with Synthetic Cannabinoids (SCBs) can induce cerebellar and cortical neuroplasticity at serotoninergic 5-HT_{2A} receptors and serotonin transporter (SERT). This neuroadaptation results in increased sensorimotor (visual object and visual placing response), sensory gating (prepulse inhibition) and "trance-like" (reaction time) effects triggered by the synthetic hallucinogens 2C-I and 25I-NBOMe. Sex-dependent variations have been observed both in behavioural and immunohistochemical studies, in line with the influence of sex on in vivo and ex vivo alterations consequent the exposure to SCBs (Fattore et al., 2020; Corli et al., 2024a).

The injection of both 2C-I and 25I-NBOMe induces visual and acoustic sensorimotor alterations in male and female mice, with AKB48-pretreated mice being more sensitive to the effects induced by the compounds. Noteworthy, the decrease in visual object response observed in female AKB48-treated mice does not differ from that observed in untreated female mice. In line with this, male AKB48-treated mice appear to be more sensitive to the visual placing alteration provoked by both 2C-I and 25I-NBOMe with respect to female AKB48-treated mice. Indeed, the worsening effect is more prolonged in male than in female AKB48-treated mice. These findings are in line with previous research showing the visual impairment induced by new psychoactive substituted phenethylamines (i.e., 2C-I and 25I-NBOMe) (Tirri et al., 2022). NBOMe and 2C drugs have been shown to potently interact with serotoninergic 5-HT_{2A} receptors (Rickli et al., 2015), that play a key role in the response gain by which the impact of sensory stimuli is modulated in the visual cortex of mice (Barzan et al., 2024). Further studies have pointed out that exposure to a different hallucinogenic

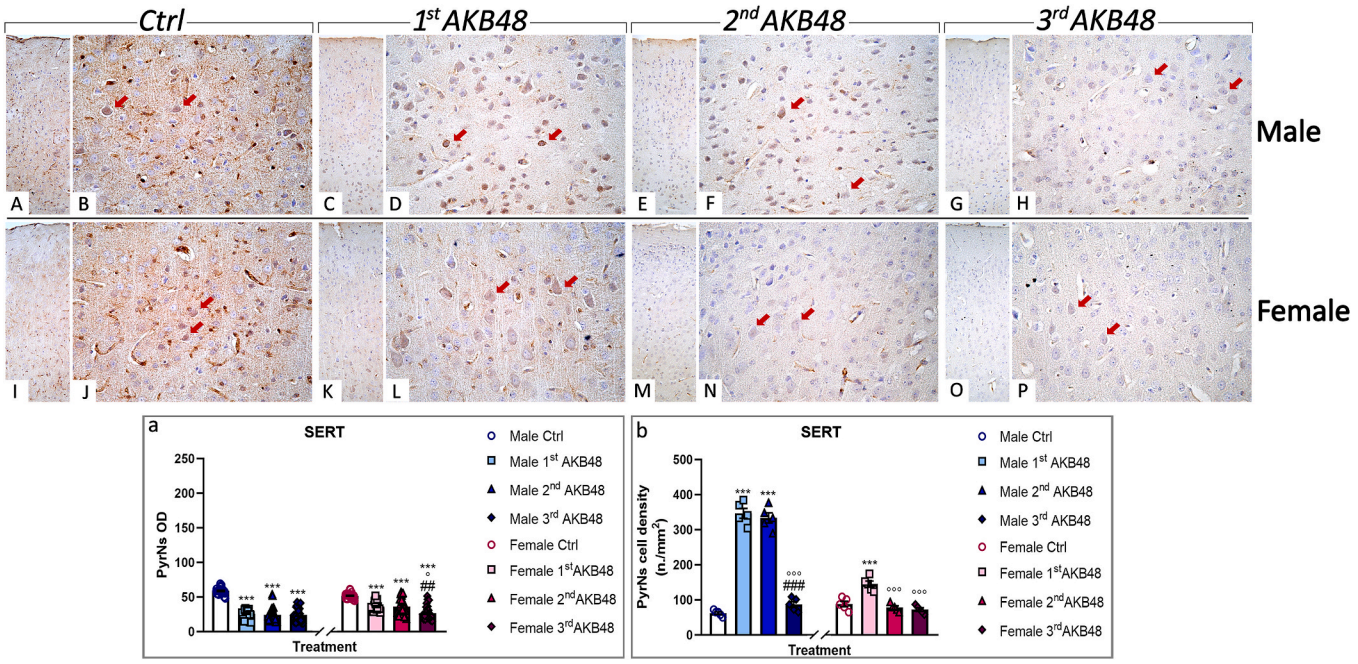

Fig. 10. Effect of single and repeated systemic administration of AKB48 (6 mg·kg−1, i.p.) on dorsolateral prefrontal cortex (DLPFC) immunostaining patterns of ST trasporter expression in male and female mice after vehicle administration (panels A–B and I-J, for male and female respectively), first AKB48 injection (panels C–D and K-L, for male and female respectively), second AKB48 injection (panels E–F and M-N, for male and female respectively), and third AKB48 injection (panels G–H and O-P, for male and female respectively). Light microscopy magnification: 200 × (A, C, E, G, I, K, M, O); 400 × (B, D, F, H, J, L, N, P). The red arrows indicate PyrNs Histograms show the quantitative analysis of ST transporter PyrNs immunopositive OD and cell density (n./mm2) in DLPF cortex of control and AKB48-treated mice (panels a–b). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

phenethylamine (e.g. 2,5-dimethoxy-4-iodoamphetamine, also known as DOI) causes reduction in the visual response gain and altered temporal dynamics in the primary visual cortex (Michaiel et al., 2019). Strengthening this hypothesis, scientific literature has stated that mechanisms underlying hallucinogenic effects (e.g., audio-visual synesthesia and significant alteration of visual perception) crucially involve serotoninergic 5-HT_{2A} receptors (Kometer et al., 2012, 2013). Noteworthy, Fay and Kubin (2000) has suggested that serotoninergic 5-HT_{2A} receptor may mediate post- and pre-synaptic effects in the motor and oculo-vestibular-precerebellar areas, based on its distribution. This possibly confirms that the tested compounds can induce altered sensory perceptions in mice, disrupting their ability to process visual stimuli in both static (visual object) and dynamic (visual placing) conditions. In fact, visual placing response requires the integration between sensory and vestibular pathways (Lambert et al., 2016). This might explain the worsening effect that affects the visual placing but not the visual object response in AKB48-treated female mice.

Given the well-known involvement of serotoninergic 5-HT_{2A} receptors in modulating sensory gating functions in rodents (Sipes and Geyer, 1997; Ou et al., 2023), the Prepulse-Inhibition (PPI) test have been carried out in both untreated and AKB48-treated mice injected with synthetic phenethylamines, as well as in mice only exposed to repeated treatment with AKB48. Our results show that 2C-I and 25I-NBOMe, as well as the repeated treatment with AKB48 alone, can significantly alter sensory gating in mice affecting both acoustic startle amplitude and PPI. As opposed to the inefficacy of 2C-I in altering startle reflex in untreated male mice, the injection of the compound in AKB48-treated male mice provokes a significant alteration of this parameter. However, the effect is similar to that induced by repeated injection of AKB48, thus suggesting that these changes may be solely due to the residual effect of repeated treatment with the synthetic cannabinoid. Differently, the inhibition evoked by the acute treatment with 25I-NBOMe in AKB48-treated male mice was more pronounced with respect to that induced by both the substituted phenethylamine in untreated mice and the repeated treatment with the synthetic cannabinoid alone. In line with this, a significant worsening effect on the PPI alteration induced by the hallucinogenic phenethylamines has been observed in both male and female AKB48-treated mice. In line with the impairment observed days after the last exposure to the treatment with AKB48 in male and female mice, a compelling study has revealed that the repeated treatment with JWH-018 lead to psychomotor agitation, accompanied by reduced social dominance, recognition memory and PPI in mice, suggesting that recurrent exposure to SCBs can result in the manifestation of psychotic-like symptoms (Bilel et al., 2023). As previously demonstrated by scientific literature, 25I-NBOMe induce a significant alteration at a dose of 1 mg/kg (Miliano et al., 2019; Tirri et al., 2022), while 2C-I appears to be ineffective in impairing the acoustic startle reflex in mice (Tirri et al., 2022). Together with the significant alteration in PPI, these findings confirm the pro-psychedelic effects of -NBOMe and 2C compounds already outlined by scientific literature (Halberstadt and Geyer, 2018; Miliano et al., 2019; Tirri et al., 2022; Syrová et al., 2023).

Together with the decrease in the visual sensorimotor responses and sensory gating, 2C-I and 25I-NBOMe induce an increase in the latency to the reaction provoked by the fall on a surface. AKB48-treated mice appear to be more sensitive to the compounds, with a longer worsening effect being observed in male with reference to female mice especially after the treatment with 2C-I. The present results agree with a previous study that has highlighted the close relationship between the sensory (visual and acoustic responses) inhibition and the reaction time alteration consequent the exposure to the potent hallucinogenic drugs belonging to the NBOMe and 2C series (Tirri et al., 2022). Previous studies by Halberstadt and Geyer (2014) have also shown the "head--twitches" effect induced by 2C-I and 25I-NBOMe representative for hallucinogenic effects in humans. Thus, the present findings may contribute to the behavioural prediction of a potential "trance-like" behaviour in humans, as it has been typically associated to the administration of these substances in preclinical models (Ponzoni et al., 2016).

To investigate the potential impact of the repeated exposure to SCBs on the serotoninergic neurotransmission system, we performed the immunohistochemical assessment of serotoninergic 5-HT_{2A} receptors and SERT in cortical and cerebellar samples taken from male and female mice after each AKB48 injection. Specifically, our attention focused on the dorsolateral prefrontal cortex, part of the associative corticostriatal circuit that plays a key role in the execution of motor skills, and on the cerebellum, a crucial CNS region involved in motor learning, coordination, posture, and several non-motor functions (Doyon and Benali, 2005; Hikosaka et al., 2002; Verwey et al., 2019; Prati et al., 2024; Nguyen et al., 2025). Here, we demonstrate that the worsening effect observed in behavioural disruption evoked by 2C-I and 25I-NBOMe is accompanied by a rapid cerebellar and cortical neuroplasticity at both serotoninergic 5-HT_{2A} receptors and SERT. Previous studies have shown that SCBs can induce an inhibitory effect on serotoninergic activity in mice brain, which is mediated by activation of presynaptic CB_1 cannabinoid receptors (Nakazi et al., 2000; Moranta et al., 2004) or CB_1 receptors expressed in a non-synaptic serotoninergic afferents subpopulation (Balázsa et al., 2008). SCBs has been linked to (i) inhibited serotonin release in the mouse brain cortex (Nakazi et al., 2000), (ii) decreased synthesis of 5-hydroxytryptophan/serotonin (5-HTP/5-HT) in brain regions enriched in 5-HT (i.e., cerebral cortex and hippocampus; Moranta et al., 2004), (iii) decreased 5-HT released in the hippocampus (Balázsa et al., 2008). These findings may possibly explain the neuroadaptation observed in the present study after the repeated exposure to the synthetic cannabinoid AKB48. A transient increase in serotoninergic 5-HT_{2A} receptors expression has been observed in both cerebellar and cortical areas, with the greater effect being observed in AKB48-pretreated female mice. This is in line with the upregulation and enhanced serotoninergic 5-HT_{2A} receptors activity displayed by non-selective cannabinoid receptors agonists via ERK1/2 signalling (Franklin and Carrasco, 2013). Further, our findings agree with the peculiar effect observed by Ibarra-Lecue and colleagues after the early chronic exposure to delta-9-tetrahydrocannabinol (THC) in mice. Authors have demonstrated that chronic administration of THC during adolescence results in altered expression, as well as in vivo and in vitro functions, of serotoninergic 5-HT_{2A} receptors receptor (Ibarra-Lecue et al., 2018). It is worthy to note that the upregulation observed in the present study tends to decrease with the number of injections, returning to basal levels after the third administration. Moreover, our results show a significant decrease in SERT expression lasting up to the third injection of AKB48. As opposed to male mice, this effect is observed after the first injection in AKB48-pretreated female mice. The key role played by CB_1 receptor in the modulation of serotonin release has been shown (Nakazi et al., 2000; Balázsa et al., 2008), also pointing out that the absence of its inhibitory action can result in increased synaptic 5-HT concentration (Burokas et al., 2014). Our previous study has revealed that long-term exposure to AKB48 lead to a rapid cortical and cerebellar down regulation of CB_1 receptors (Corli et al., 2024a), that occurs more rapidly in female mice and may possibly explain the shift in the effect induced by repeated injection of the cannabinoid on serotoninergic 5-HT_{2A} receptors expression levels in these central areas. The observed upregulation of 5-HT_{2A} may be a compensatory mechanism following CB_1 receptor downregulation. In fact, the tolerance mechanisms and neuroplasticity at CB_1 receptors evoked by repeated injection of AKB48 may result in increased 5-HT levels or serotonin signalling that could in turn influence serotoninergic receptors and transporters functions and expression (Koldzic-Zivanovic et al., 2006). Supporting this assumption, scientific literature has highlighted the serotonin- and PKC-mediated internalization and recycling of serotoninergic 5-HT_{2A} receptors (Bhattacharyya et al., 2002), as well as 5-HT-induced reduction of cell surface SERT expression (Jørgensen et al., 2014). Further, a more recent study has demonstrated that pharmacological modulation of CB_1 and 5-HT_{1A} receptors with a cannabinoid derivative normalized CB_1 receptor downregulation in the prefrontal cortex and improved behavioural outcomes in an MK-801 model of schizophrenia (Richardson et al., 2024). Previous studies have also shown that the chronic treatment with THC evokes a pro-hallucinogenic conformation of the receptor that has been linked to schizophrenia-like preclinical paradigm (Ibarra-Lecue et al., 2018). Hence, this data possibly explains the worsening effect observed in AKB48-pretreated mice after the exposure to hallucinogenic phenethylamines, despite the effects on serotoninergic 5-HT_{2A} receptors progressively decrease with the number of injection and its expression level is not significantly variated after the third injection. Yet, a compelling study by Viñals and colleagues has identified expressed and functionally active CB_1/5-HT_{2A} heterodimers in specific rodents' brain regions, which formation can influence the G-protein coupling of 5-HT_{2A} (Viñals et al., 2015). Specifically, this heteromers have been localized in dorsal striatum and somatomotor cortex (Viñals et al., 2015), involved in modulation of sensorimotor responses, sensory gating and reaction time in mice (Pozzi et al., 2010; Wang et al., 2023; Bilel et al., 2025). The same heterodimers have been localized in olfactory neuroepithelium cells of schizophrenic patients, in which their signalling is modulated by cannabis use (Guinart et al., 2020). Therefore, the possibility of these dimeric receptors contributing should be considered.

In line with similar basal expression of serotoninergic 5-HT_{2A} receptors between male and female observed in the present study, evidence of a sexual dimorphism in serotonin receptors and transporter levels in the brain has not been outlined by scientific literature. In fact, Ferrari et al. have stated that sex does not influence the density of serotoninergic 5-HT_{2A} receptors binding in hypothalamus or amygdala of neonatal rats (Ferrari et al., 1999). In addition, binding (Moses-Kolko et al., 2011) and mRNA expression for serotoninergic 5-HT_{2A} receptors (Zhou et al., 2002), as well as serotonin receptors and transporter density in the brain (Chavez et al., 2010) are not regulated by reproductive hormones (e.g., estrogenic). Thus, this possibly suggests that neuroadaptations observed at serotoninergic receptors and serotonin trasporter could not influence synaptic 5-HT tone differently between male and female. Despite this, sex-specific responses have been pointed out after the exposure to serotoninergic 5-HT_{2A} receptors hallucinogenic agonists, with reference to both their pharmacodynamics and pharmacokinetics (Brookshire and Jones, 2009; Jaster et al., 2022; Vohra et al., 2022). Notably, the study by Jaster and colleagues has shown that female mice are more sensitive to the head twitches response induced by the hallucinogenic phenethylamine 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), while the brain and plasma concentrations of the compound at 30 and 60 min after the injection were lower in female than male mice (Jaster et al., 2022). This may possibly explain the more prolonged worsening effect observed in male AKB48-pretreated mice with respect to females, that surprisingly does not reflect the immunohistochemical changes observed under repeated treatment regimen with AKB48. Indeed, the neuroadaptation at serotoninergic 5-HT_{2A} receptors appears more evident after the first injection, as well as the decrease in SERT levels occurs more rapidly in female AKB48-pretreated mice. The observed sex-dependent differences highlight the importance of considering sex as a biological variable in research on drug interactions and their effects. However, further research must be carried out to clarify this point, with reference to a potential sex-based difference in the pharmacokinetic profile of 2C-I and 25I-NBOMe.

5. Conclusions

The present study demonstrates that repeated exposure to SCBs can result in a worsening effect on the response to synthetic hallucinogens. Interestingly, the effect has been related to cerebellar and cortical neuroplasticity at both serotoninergic 5-HT_{2A} receptors and SERT. In the translational paradigm, our data highlight the interaction between SCBs and psychedelic drugs that may be relevant to their long-term effects and psychiatric sequelae potentially related to their consumption (Fantegrossi et al., 2018). These findings suggest that individuals who

use both SCBs and hallucinogens may be at increased risk for adverse psychological and cognitive effects, raising concern with reference to the decreased performance in driving and hazardous works consequent to the intake of these compounds (Tirri et al., 2022). Ultimately, the study may provide a foundation for identifying individuals at higher risk for adverse drug reactions, given the renewed interest in studying the potential therapeutic effects of hallucinogens (Rivera-García and Cruz, 2023).

CRediT authorship contribution statement

Giorgia Corli: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Conceptualization. **Fabrizio De Luca:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Conceptualization. **Sabrine Bilel:** Writing – review & editing, Writing – original draft, Visualization, Validation, Formal analysis. **Marta Bassi:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis. **Elisa Roda:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Formal analysis. **Rosa Maria Gaudio:** Writing – review & editing, Visualization. **Liana Fattore:** Writing – review & editing, Writing – original draft, Visualization, Validation, Conceptualization. **Carlo Alessandro Locatelli:** Writing – review & editing, Writing – original draft, Visualization. **Matteo Marti:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Ethical statement

All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All procedures performed in the studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. Project activated in collaboration with the Presidency of the Council of Ministers-DPA Anti-Drug Policies (Italy).

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Declaration of competing interest

Declarations of interest: none.

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