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RESEARCH ARTICLE

Sex-specific behavioural, metabolic, and immunohistochemical changes after repeated administration of the synthetic cannabinoid AKB48 in mice

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Abstract

Background and Purpose: AKB48 is a synthetic cannabinoid illegally sold for its psychoactive cannabis-like effects that have been associated with acute intoxication and whose effects are poorly known.

Experimental Approach: Using a behavioural, neurochemical, and immunohistochemical approach, we investigated the pharmaco-toxicological effects, pharmacokinetics, and neuroplasticity at cannabinoid CB_1 receptors in the cerebellum and cortex induced by repeated AKB48 administration in male and female mice.

Key Results: The effects of AKB48 varied significantly depending on sex and treatment duration. The first injection impaired sensorimotor responses and reduced body temperature, analgesia, and breath rate to a greater extent in females than in males; the second injection induced stronger effects in males while the third injection of AKB48 induced weaker responses in both sexes, suggesting emergence of tolerance. The CB_1 receptor antagonist NESS-0327 prevented the effects induced by repeated AKB48, confirming a CB_1 receptor-mediated action. Blood AKB48 levels were higher in females than in males and repeated administration caused a progressive rise of AKB48 levels in both sexes, suggesting an inhibitory effect on cytochrome activity. Finally, immunohistochemical analysis revealed higher expression of CB_1 receptors in the cerebellum and cortex of females, and a rapid CB_1 receptor down-regulation in cerebellar and cortical areas following repeated AKB48 injections, with neuroadaptation occurring generally more rapidly in females than in males.

Abbreviations: AKB48, N-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide; DLLME, dispersive liquid–liquid microextraction; DLPFC, dorsolateral prefrontal cortex; EMCDDA, European Monitoring Centre for Drugs and Drug Addiction; JWH-018, 1-naphthalenyl (1-pentyl-1H-indol-3-yl)-methanone; JWH-210-D9, (4-ethyl-1-naphthalenyl)(1-pentyl-1H-indol-3-yl)-methanone-D9; ML, molecular layer; MRM, multiple reaction monitoring; NAc, nucleus accumbens; NESS-0327, N-piperidinyl-[8-chloro-1-(2,4-dichlorophenyl)-1,4,5,6-tetrahydrobenzo-[6,7]cycloheptal [1,2-c]pyrazole-3-carboxamide]; NPS, new psychoactive substance; OD, optical density; PCs, Purkinje neurons; SCBs, synthetic cannabinoids; UHPLC-MS/MS, ultra-high performance liquid chromatography tandem mass spectrometry.

Giorgia Corli and Elisa Roda equally contributed to this work.

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Conclusion and Implications: We have shown for the first time that AKB48 effects significantly vary with prolonged use and that sex affects the pharmacodynamic/pharmacokinetic responses to repeated administration, suggesting a sex-tailored approach in managing AKB48-induced intoxication.

KEYWORDS
AKB48, APINACA, CB_1 receptor, NESS-0327, NPS, sex difference, synthetic cannabinoids

1 | INTRODUCTION

The steadily increasing introduction of new psychoactive substances (NPS) in the clandestine market has changed patterns of drug consumption over the last decade (European Monitoring Centre for Drugs and Drug Addiction [EMCDDA], 2021). The term NPS refers to a large number of compounds illegally marketed as "legal alternatives" to traditional drugs of abuse to avoid legal restrictions on controlled substances. NPS are typically classified based on their chemical structures or pharmacological effects into different classes (Luethi & Liechti, 2020), among which synthetic cannabinoids (SCBs) currently dominate drug seizures in Europe (EMCDDA, 2021). AKB48, also known as APINACA, is an adamantyl-indazole compound with an adamantyl moiety linked to the indazole group through a carboxamide bond, which was detected for the first time in 2012 in Japanese herbal smoking blends (Kim et al., 2019). It was then reported by the US Drug Enforcement Administration (DEA) as one of the most abundant SCBs seized (Odoardi et al., 2016; Uchiyama et al., 2012) and categorized in Schedule I of the Controlled Substances Act (DEA, 2016; NFLIS, 2013). Toxicological screening has reported its presence in biological samples from intoxicated patients (Karinen et al., 2015; Pinson et al., 2020; Vikingsson et al., 2015) and a simultaneous screening method for the detection of NPS has recently allowed its identification in other matrix samples (Kim et al., 2021).

As with other SCBs, AKB48 is taken to mimic the effect of the main psychoactive component of *Cannabis sativa* plant **Δ^9-THC** (Le Boisselier et al., 2017), but it induces more serious effects probably due to its greater affinity for the cannabinoid **CB_1 receptor**. Indeed, AKB48 retains nanomolar affinity for CB_1 and **CB_2** human (3.24 ± 0.28 nM and 1.68 ± 0.12 nM, respectively; Canazza et al., 2016; Uchiyama et al., 2013) and mouse (5.34 ± 0.44 nM and 1.93 ± 0.14 nM, respectively; Canazza et al., 2016) receptors and causes euphoria, a state of happiness and relaxation, easiness to laugh and talkativeness that occur rapidly and last up to 3 h (Santacroce et al., 2015). Nevertheless, users also reported several side effects such as stomach cramps, hypersalivation, excessive sedation, anxiety, increased heart rate, psychomotor impairment, paranoia, and irritability (Brewer & Collins, 2014; Santacroce et al., 2015; White, 2017). A recent in vitro study has shown that inflammation and oxidative stress play a key role in the toxicity profile of AKB48 (Oztas et al., 2019). In line with a number of intoxication cases reported in humans, preclinical studies reported severe behavioural (i.e., hypothermia, hypomotility, altered nociception, and catalepsy), neurological (i.e., seizures, myoclonia, and hyperreflexia), and neurochemical alterations (i.e., altered dopaminergic transmission in specific brain areas) after acute administration of AKB48 in rodents,

What is already known

- In vitro studies have related inflammation and oxidative stress to the AKB48 toxicity profile.
- In vivo acute administration of AKB48 in rodents provokes physiological, behavioural, and prepulse inhibition alterations.

What does this study add

- Sex and number of drug administrations significantly affect central and peripheral effects of AKB48.
- Sex affects the down-regulation of CB_1 receptors induced by repeated exposure to AKB48.

What is the clinical significance

- Pharmaco-toxicological effects induced by AKB48 may significantly vary with duration of use.
- Acute and repeated AKB48 use may be associated with sex-dependent health risks.

confirming its in vivo potential toxicity and suggesting that it might possess abuse potential (Bilel et al., 2019; Canazza et al., 2016; Ossato et al., 2017). In line with the acute analgesia, hypothermia, catalepsy, and hypolocomotion observed after AKB-48 and at a greater extent after its fluorinate analogue 5F-AKB48 injection (Canazza et al., 2016), a correlation between the typical "tetrad effect" and the high brain/blood concentrations ratio associated with SCBs has been suggested (Poklis, Amira, Wise, Wiebelhaus, Haggerty, Lichtman, & Poklis, 2012; Poklis, Amira, Wise, Wiebelhaus, Haggerty, & Poklis, 2012). Yet, information about its potential long-term effects is still scarce.

To fill this gap, the present study investigated the pharmaco-toxicological effects and the plasma AKB48 pharmacokinetic induced by repeated administration of AKB48 in mice. Because SCB agonists trigger signalling pathways and neuroadaptations that may occur differently in males and females (Fattore et al., 2007, 2010; Fratta & Fattore, 2013; Melis et al., 2013; Rosas et al., 2018), we hypothesised that the effects induced by their prolonged use might be influenced by the sex of the subject, which would emphasize the importance of a sex-tailored approach to intoxicated patients. Thus, we examined potential neuroadaptations induced by repeated exposure to AKB48 in both sexes by immunohistochemically assessing the expression levels of CB_1 receptors in cerebellum and prefrontal cortex, two brain areas densely populated by these receptors (Mackie, 2005; Tao et al., 2020), whose density and functionality are known to be significantly affected by sex and gonadal hormones in rats (Castelli et al., 2014) and mice (Liu et al., 2020).

2 | 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 a 12:12-h light–dark cycle (light on at 6:30 AM) 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.EXT.21) 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

N-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide (AKB48) was purchased from LGC Standards S.r.l (Milan, Italy), while the CB_1 receptor-preferring antagonist N-piperidinyl-[8-chloro-1-(2,4-dichlorophenyl)-1,4,5,6-tetrahydrobenzo-[6,7]cycloheptal[1,2-c]pyrazole-3-carboxamide] (NESS-0327) was purchased from Tocris (Bristol, UK), dissolved in 5% ethanol (BioUltra, for molecular biology, ≥99.8%; Sigma-Aldrich, Italy) + 2%TWEEN®80 (Sigma-Aldrich, Italy) + 96% saline (0.9% NaCl; Eurospital, Italy), and administered intraperitoneally (i.p.) at a dose of 6 $mg \cdot kg^{-1}$ and 1 $mg \cdot kg^{-1}$, respectively, in a volume of 4 $\mu l \cdot g^{-1}$. The same solution of ethanol, TWEEN®80, and saline was used as the vehicle. The doses of 6 $mg \cdot kg^{-1}$ (i.p.) for AKB48 and of 1 $mg \cdot kg^{-1}$ (i.p.) for NESS-0327 were selected on the basis of our previous studies (Bilel et al., 2019, 2023; Canazza et al., 2016; Ossato et al., 2017), as they were able to induce severe behavioural, neurological, and neurochemical impairments (Canazza et al., 2016) and to prevent the effects induced by the dose of 6 $mg \cdot kg^{-1}$ of the synthetic cannabinoid **JWH-018** in mice (Bilel et al., 2023), respectively.

A previous study carried out in our laboratories showed the acute in vitro and in vivo activity of AKB-48 at (0.01–6 $mg \cdot kg^{-1}$, i.p.) (Canazza et al., 2016). From the translational point of view, the dosage of 6 $mg \cdot kg^{-1}$ injected in mice corresponds to a dose of approximately 29.16 mg in humans (Human Equivalent Dose, or HED of 0.486 $mg \cdot kg^{-1}$ for a 60 kg male; Nair & Jacob, 2016). As stated by various scientific reports, SCBs contained in single packages of "Spice" products commonly are equivalent to 32.1 and 57 $mg \cdot g^{-1}$ (Ernst et al., 2019; Langer et al., 2016). Specifically, 76 $mg \cdot g^{-1}$ of a halogenated derivative of AKB-48 has been identified in herbal mixtures seized on European soil (Langer et al., 2016). Moreover, it has been shown that the rapid onset of potential tolerance mechanisms may lead the SCBs' consumers to increase dosages up to the intake of about 30 $mg \cdot day^{-1}$ (Cooper, 2016; Macfarlane & Christie, 2015; Nacca et al., 2013; Zimmermann et al., 2009). In line with this evidence, previous studies have revealed that repeated administration of JWH-018 and JWH-073 (3–10 $mg \cdot kg^{-1}$) results in the development of tolerance to pharmacological effects induced by the substance (Tai et al., 2015). As a confirmation of this, we have recently shown cognitive dysfunction and impaired neuroplasticity following repeated exposure to JWH-018 (Bilel et al., 2023), thus suggesting the relevance of investigating effects induced by repeated treatment with the same dosage of other SCBs both in male and female mice.

JWH-210-D9 was purchased from Cayman Chemicals (Michigan, USA). Water, chloroform, formic acid, and methanol were purchased from 3V-Chemicals (Rome, Italy). Ammonium formate was purchased from Agilent (Agilent Technologies, Santa Clara, CA, USA). All reagents and solvents were of LC/MS grade.

2.3 | Experimental design

Male and female mice were injected with AKB48 (6 $mg \cdot kg^{-1}$, i.p.) or its vehicle (0.004 $ml \cdot g^{-1}$, i.p.) once a week for three consecutive

weeks (13 days), for a total of three injections (D1, D7, and D13) with a 6-day period of washout between each consecutive injection, and were then assigned to behavioural experiments (n = 8 per group) or blood/tissue analyses (n = 24 per group). Animal behaviour was analysed 5 min after each injection (D1, D7, and D13). For blood/tissues collection, 48 animals (24 females, 24 males) were randomly picked after the first (D1), the second (D7), or the third (D13) AKB48/vehicle injection to assess baseline CB_1 receptor expression levels and perform histological and immunohistochemical studies (Figure 1). Separate groups of male and female animals were treated with the CB_1 receptor-preferring antagonist NESS-0327 (1 $mg \cdot kg^{-1}$, i.p.) 20 min before each administration of AKB48 to investigate the involvement of CB_1 receptors in AKB48-induced behavioural effects.

2.4 | Behavioural studies

The effects of AKB48 were evaluated using a series of behavioural tests (*visual object* and *visual placing response test*, *acoustic test*, *tactile test*, *tail pinch test*, *core temperature test*, *breath rate test*, and *mobility time test*) routinely used in our laboratories to describe the effects of synthetic cannabinoids in mice (Bilel et al., 2020; Canazza et al., 2016, 2017; Ossato et al., 2015, 2016; Vigolo et al., 2015) and widely validated for the preclinical characterization of the pharmacotoxicological effects of NPSs in rodents (Arfè et al., 2021; Bilel et al., 2021, 2022; De-Giorgio et al., 2020; Ossato et al., 2018; Tirri et al., 2022).

Experiments were performed between 8:30 AM and 2:00 PM 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, acoustic response, overall tactile (pinna, vibrissae, and corneal reflexes) response, determination of the mechanical (tail pinch), core temperature, breath rate, and motor activity (mobility time). Animals' behaviour was videotaped and analysed offline by a different trained operator.

2.4.1 | Evaluation of visual responses

Visual response was verified by means of two behavioural tests that evaluated the ability of the mouse to capture visual information when it is stationary (*visual object response*) or moving (*visual placing response*) as previously described (Ossato et al., 2015).

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 0-10-30-60-120-180-240-300 min post-injection.

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 $cm \cdot s^{-1}$. The downward movement of

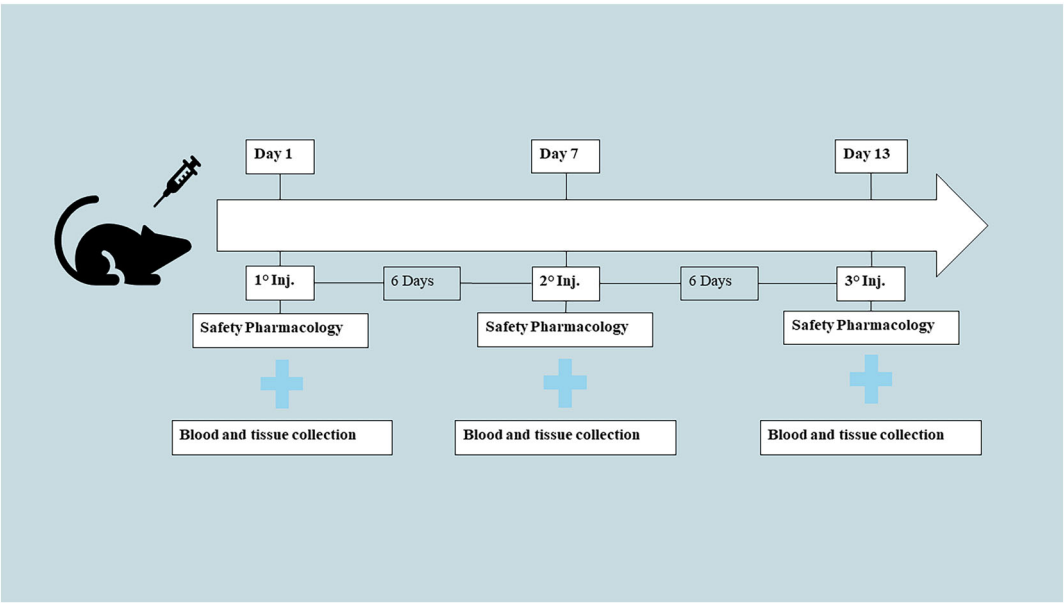

FIGURE 1 Experimental design and timeline.

the mouse was videotaped and the frame-by-frame video analysed 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 about 1.5 ± 0.25 cm. Evaluation of the visual placing response was measured at 0-15-40-70-130-190-250-310 min post-injection.

2.4.2 | Evaluation of acoustic responses

Acoustic response measures the reflex of the mouse in reply to a four different (for intensities and frequencies) *acoustic test* stimuli produced behind the animals (Marti et al., 2018). Each sound test was repeated three times, giving a value of 1 if there was a response and 0 if not present (for a total score of 3 for each sound and an overall score of 12). Evaluation of the acoustic response was measured at 0-10-30-60-120-180-240-300 min post-injection.

2.4.3 | Evaluation of tactile responses

Tactile tests were performed through *vibrissae*, *pinna*, and *corneal reflexes* (Bilel et al., 2020), and data were expressed as the sum of these parameters (overall score: 12). The *vibrissae* reflex was evaluated by touching the vibrissae (right and left) with a thin hypodermic needle once per side, giving a value of 1 if there was a reflex (turning of the head to the side of touch or vibrissae movement) or 0 if not present (overall score: 2). The *pinna* reflex was assessed by touching the pavilions (left and right) with a thin hypodermic needle, the interior pavilions first. This test was repeated twice per side, giving a value of 1 if there was a reflex and 0 if not present (overall score: 4). The *corneal* reflex was assessed by gently touching the cornea with a thin hypodermic needle and evaluating the response, assigning a value of 1 if the mouse moved only its head, 2 if it only closed its eyelid, and 3 if it closed its lid and moved its head. The procedure was conducted bilaterally (overall score: 6). The overall tactile procedures were measured at 0-10-30-60-120-180-240-300 min post-injection.

2.4.4 | Evaluation of nociception

Acute mechanical nociception was evaluated using the *tail pinch* tests (Canazza et al., 2016). A special rigid probe connected to a digital dynamometer (ZP-50N, IMADA, Japan) was gently placed on the distal portion of the tail, and progressive pressure was applied. When the mouse flicked its tail, the pressure was stopped, and the digital instrument saved the maximum peak of weight supported (g force). A cut-off (500 g force) was set to avoid tissue damage. The test was repeated three times, and the final value was calculated as the average of three obtained scores. Acute mechanical nociception was measured at 0-20-45-75-135-195-255-315 min post-injection.

2.4.5 | Evaluation of core body temperature

The *core (rectal) temperature* was evaluated by a probe (1 mm diameter). The conscious mouse was restrained manually by a trained operator, who, after lubricating it with liquid Vaseline, gently inserted the probe into the rectum of the mouse (to about 1 cm) and left it in position until the stabilization of the temperature (about 10 s). The probe was connected to a digital thermometer, and the core body temperature was measured at 0-15-40-70-130-190-250-310 min post-injection.

2.4.6 | Evaluation of breath rate

During the *breath rate* test, the animal was placed above an observation cage, leaving it awake and freely moving. The experimental protocol for the detection of respiratory parameters was non-invasive and required minimal handling (Foti et al., 2019), consisting in monitoring respiration patterns of the mice while it was videotaped by a camera (Ugo Basile, Milan, Italy). A blind operator analysed frame by frame the off-line video and evaluated the number of breaths per minute (brpm). Breath rate were measured at 0-10-25-55-125-185-245-305 min post-injection.

2.4.7 | Evaluation of the spontaneous locomotor activity

Spontaneous locomotor activity was investigated by the *mobility time* test. The mouse was placed in a square plastic cage (30 × 30cm), and horizontal motor activity was monitored for 5 min at 0-5-25-55-115-175-235-295 min post-injection by using a camera (Ugo Basile, Italy). The recordings were analysed off-line frame by frame by a blind trained operator. To avoid mouse olfactory cues, cages were carefully cleaned with a dilute (5%) ethanol solution and washed with water between consecutive trials.

2.5 | AKB48 blood pharmacokinetic studies

Serial blood specimen samples were obtained to investigate possible pharmacokinetic differences of AKB48 between male and female mice after repeated administration, accordingly to the main instructions reported by Golde et al. (2005). Mice were lightly anaesthetised with isoflurane (2%), and the samples were taken from the vein located in the submandibular area of the jaw. A 25G needle was used to carry out the sampling near the cheek pocket, to which a small vascular bundle flows. A total of 48 mice (24 male, 24 female) was picked (after the first, second, and third injection) and divided into two subgroups corresponding to the different collection time considered. To reduce the number of animals, two blood samples were collected (at 30–240 min or 120–300 min) from each animal after AKB48/vehicle treatment, one from the right and one from the left submandibular

area (up to 150 µl per sample) and stored into 1-ml vials containing ethylendiaminetetracetic-acid (EDTA: 4 mg·ml^{-1} of blood) as preservative and anticoagulant. At the end of the blood collection, mice were rehydrated by a subcutaneous injection of glucose solution (5%) to maintain volume and osmotic homeostasis and killed 5 h later to collect the tissue for immunohistochemistry studies.

2.5.1 | Sample preparation

A dispersive liquid–liquid microextraction (DLLME) was performed for sample purification. Blood samples (150 µl) were spiked with 10 µl of the synthetic cannabinoid JWH-210-D9 as an internal standard, achieving the final concentration of 20 ng·ml^{-1}, and deproteinized with 500 µl of methanol. The sample was centrifuged at 10,000×*g* for 10 min, and 500-µl surnatant was transferred into a 15-ml conical tube containing 1 ml of water, 0.2 g of NaCl, and 100 µl of carbonate buffer, pH 9. To obtain the formation of the cloudy solution, 350 µl of a mixture, chloroform/methanol 1:2.5, respectively, the extractant and the disperser solvent, was rapidly added to obtain the formation of a turbid mixture. The sample was sonicated for 2 min and then centrifuged at 4000×*g* for 5 min to sediment the fine droplets of the extractant phase at the bottom of the tube. The sediment phase (about 50 ± 5 µl) was transferred into a vial, evaporated under a gentle nitrogen stream, reconstituted in 20 µl of methanol and 80 µl of water with 0.1% formic acid, and 10 µl were injected in the ultrahigh-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) system.

2.5.2 | Instrumental analysis

The analytical method (UHPLC-MS/MS) and its validation has been previously described in detail (Odoardi et al., 2016). Chromatography was performed using an Agilent 1290 Infinity system, equipped with a binary pump with integrated vacuum degasser, high-performance well-plate autosampler, and thermostated column compartment modules. The detection system was an Agilent 6460 triple-quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) with a Jet-Stream electrospray ionization source. The column was a superficially porous Kinetex C18 column (2.6 µm, 100 × 2.1 mm, Phenomenex, Bologna, Italy). The column temperature was set at 40°C and the mobile phases used were as follows: (A) 5-mM ammonium formate containing 0.1% formic acid and (B) methanol with 0.1% of formic acid. The mobile phase gradient was from 45% to 100% B within 12 min, plus 3 min of equilibration, for cannabinoids analysis, and from 0% to 90% B within 11 min, plus 3 min of equilibration for stimulants. The flow rate was set to 400 µl·min^{-1}. The eluate was introduced into the mass spectrometer by means of electrospray ionization (ESI) in the positive mode. The optimized MS parameters were as follows: capillary voltage set to 4000 V, the ion source heated up to 350°C, nitrogen used as nebulizing and collision gas at 12 L·min^{-1} and 40 psi, respectively, EM voltage set to +1000 V, and nozzle voltage set to 2000 V. The detector operated in multiple reaction monitoring (MRM) mode. Transitions selected for AKB48 were 384, 135, 107, and 93.

2.6 | Brain tissue sampling: histopathology and immunohistochemistry

2.6.1 | Specimens preparation

A total of 48 mice (n = 6 per group) were employed for the immunohistochemical study. Mice were killed by cervical dislocation; brains were immediately excised as recently described (De Luca et al., 2023; Roda et al., 2022), washed in 0.9% NaCl, fixed by immersion for 48 h at room temperature in 4% paraformaldehyde in 0.1-M phosphate buffer (pH 7.4), and postfixed in the same fixative medium at 4°C for 1.5 h. Tissues were then kept in absolute ethanol for 1 h, followed by acetone, and finally embedded in Paraplast X-TRA (Sigma Aldrich, Milan, Italy). Serial sectioning was carefully carried out using a manual rotary microtome. Sections 8-microns thick of both cerebellar vermis and brain hemispheres were cut in the sagittal and coronal plane, respectively, and collected on silane-coated slides. Hence, because the CNS has more complex specialized structures compared with other tissues, a general staining method, that is, haematoxylin & eosin (H&E), was used to achieve an overview of tissue structures and anatomical order and area-specific setting (De Luca et al., 2023; Jordan et al., 2011; Li et al., 2022; Roda et al., 2019, 2023; Xiong & Gendelman, 2014). Therefore, using the brightfield examination of H&E-stained samples at low magnification, and based on the specific target area to be evaluated, neuroanatomical site identification was achieved, allowing the selection of precise sections of cerebellum and dorsolateral prefrontal cortex (DLPFC), which were further processed for histology and immunocytochemical analysis to gauge expected neurotoxicity (Houle, 2011). Five slides (about 20 randomized sections) per animal were analysed, for a total of n = 6 animals per experimental condition.

2.6.2 | Light microscopy and immunohistochemistry

Immunohistochemical reactions were carried out simultaneously on slides from different experimental groups to avoid possible staining differences due to small changes 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 CB_1 receptor.

Paraffin-embedded sections were deparaffinized in xylene, rehydrated through a series of graded alcohol treatments and rinsed in phosphate-buffered saline (PBS, Sigma). Then, sections were incubated overnight at room temperature in a dark moist chamber with a primary mouse monoclonal antibody against the CB_1 receptor (C-11) (Immunogen: purified antibody raised against amino acids 1–150 of the CB_1 receptor of human origin; RRID:AB_2889069) diluted 1:100 (Santa Cruz Biotechnology, CA, USA). A biotinylated horse antimouse

secondary antibody (RRID:AB_2336821) diluted 1:200 and an avidin biotinylated horseradish peroxidase complex (Vector Laboratories, Burlingame, CA, USA) were 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 finally 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).

2.6.3 | Histochemical and immunohistochemical evaluation

Sections 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, five slides (about 20 randomized sections) per animal were examined, for a total of n = 6 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; De Luca et al., 2023; Roda et al., 2021, 2023). 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 five slides per animal for a total of n = 6 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. Additionally, immunoreactive cells density (number of immunopositive cells per area in mm^2) was assessed as previously described (Roda et al., 2021).

2.7 | Data and statistical analysis

In sensorimotor response experiments, data are expressed in arbitrary units (visual object response, acoustic response, overall tactile response) and percentage of baseline (visual placing response). Core temperature values are expressed as the difference between control temperature (before injection) and temperature following drug administration (Δ°C). Antinociception (tail pinch test) is calculated as per cent of maximal possible effect {EMax% = [(test-control latency)/(cut-off time-control)] × 100}. Data are expressed as absolute values, that is, time (s) spent in the open field arena. Changes in breath rates per minute (brpm) are expressed as percentage of basal values. Concentration of AKB48 in plasma samples are reported as micrograms per litre.

All numerical data are expressed as mean ± SEM and analysed by repeated measures analysis of variance (ANOVA). Results from treatments showing significant overall changes were subjected to Tukey's post hoc test with a significant level set at $P < 0.01$. The effects of treatments over time were analysed by two-way ANOVA followed by Bonferroni'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. The total average effect induced by treatments was analysed by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons.

Immunohistochemical data were expressed as mean ± SEM. For data that passed the normality test, the 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 $P < 0.01$. All statistical analyses were performed by using GraphPad Prism 8.0 (GraphPad Software Inc., CA, USA) and R software. Data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2022).

2.8 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in https://www.guidetopharmacology.org/ and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Abood et al., 2023; Alexander et al., 2021).

3 | RESULTS

No differences were observed in the weight of mice and the amount of food consumed daily between vehicle- and AKB-48-treated animals, suggesting that the repeated treatment did not affect body weight and food consumption in male and female mice.

3.1 | Behavioural studies

3.1.1 | Evaluation of the visual response

The *Visual object* response did not change in vehicle-treated mice over the 300-min observation, while AKB48 (6 $mg \cdot kg^{-1}$, i.p.) reduced the

visual object response in a sex-dependent manner (Figure 2a,b). In males, the second drug injection (second AKB48) induced an effect that was greater and lasted longer (up to 240 min) than the first (first AKB48, up to 120 min) and the third (third AKB48, up to 60 min) injection (Figure 2a; effect of treatment [$F_{(3,224)} = 93.20$, $P < 0.0001$], time [$F_{(7,224)} = 30.44$, $P < 0.0001$], time × treatment interaction [$F_{(21,224)} = 5.775$, $P < 0.0001$]). On the other hand, the effect of AKB48 in females was inversely proportional to the succession of injections (Figure 2b; effect of treatment [$F_{(3,224)} = 55.76$, $P < 0.0001$], time [$F_{(7,224)} = 32.02$, $P < 0.0001$], time × treatment interaction [$F_{(21,224)} = 7.103$, $P < 0.0001$]). Notably, the first AKB48 injection inhibited the visual object response more in females than in males, while the opposite occurred after the second AKB48 injection (Figure 2c; $F_{(5,42)} = 137.3$, $P < 0.0001$). The CB_1 receptor antagonist NESS-0327, at a dose (1 mg·kg^{-1}, i.p.) ineffective per se, prevented the effect of AKB48 on the visual object response in both males ($F_{(7,56)} = 382.4$, $P < 0.0001$) and females ($F_{(7,56)} = 307.7$, $P < 0.0001$) (Figure S1A,B, respectively).

The *Visual placing* response did not change in vehicle-treated mice over the 300-min observation, while AKB48 (6 mg·kg^{-1}, i.p.) reduced the visual placing response in both sexes (Figure 2d,e) in a manner similar to its effects on the visual object response. Indeed, in males, the second AKB48 injection induced a greater and longer lasting effect than the first and third AKB48 injection, with the inhibition of the visual placing response induced by the second AKB48 injection persisting up to 5 h, while that induced by the first and third AKB48 injections persisted up to 130 and 70 min, respectively (Figure 2d; effect of treatment [$F_{(3,224)} = 127.8$, $P < 0.0001$], time [$F_{(7,224)} = 35.66$, $P < 0.0001$], time × treatment interaction [$F_{(21,224)} = 7.593$, $P = 0.0058$]). In females, the effects of AKB48 were inversely proportional to the succession of injections (Figure 2e; effect of treatment [$F_{(3,224)} = 43.44$, $P < 0.0001$], time [$F_{(7,224)} = 14.70$, $P < 0.0001$], time × treatment interaction [$F_{(21,224)} = 2.729$, $P = 0.0001$]). Again, the first AKB48 injection inhibited the visual placing response more in females than in males, while the opposite occurred after the second AKB48 injection (Figure 2f; $F_{(5,42)} = 57.53$, $P < 0.0001$). NESS-0327 (1 mg·kg^{-1}, i.p.) prevented the AKB48-induced effect on the visual placing response in both males ($F_{(7,56)} = 63.94$, $P < 0.0001$) and females ($F_{(7,56)} = 102.7$, $P < 0.0001$) (Figure S1C,D, respectively).

3.1.2 | Evaluation of the acoustic and tactile response

The *Acoustic* response did not change in vehicle-treated mice over the 300-min observation, while AKB48 (6 mg·kg^{-1}, i.p.) reduced the acoustic response differently in both sexes (Figure 3a,b). In males, the effect of the second AKB48 injection was more evident compared to the first and the third AKB48 injections and lasted up to 180 min

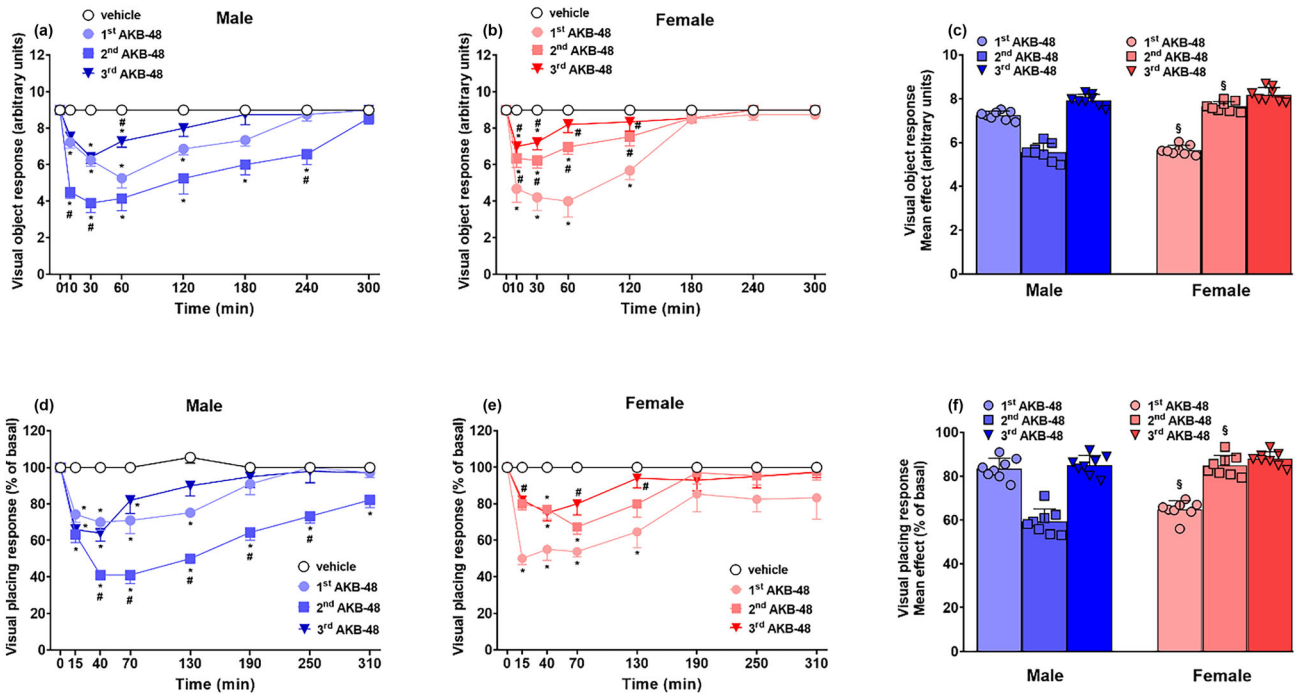

FIGURE 2 Effect of AKB48 (6 mg·kg^{-1}, i.p.) on the visual object (panels a–c) and the visual placing response (panels d–f) in male (*left*, in blue) and female (*middle*, in red) mice and comparison of mean effect of each treatment (*right*, in bars). Data are expressed as arbitrary units (a–c) or % of basal values (panels d–f). 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, b, d, e), and by one-way ANOVA followed by Tukey's test for multiple comparisons of each injection mean effect (c and f). *$P < 0.01$ versus vehicle; #$P < 0.01$ versus first AKB48 injection; §$P < 0.01$ versus corresponding male group; n = 8 per group.

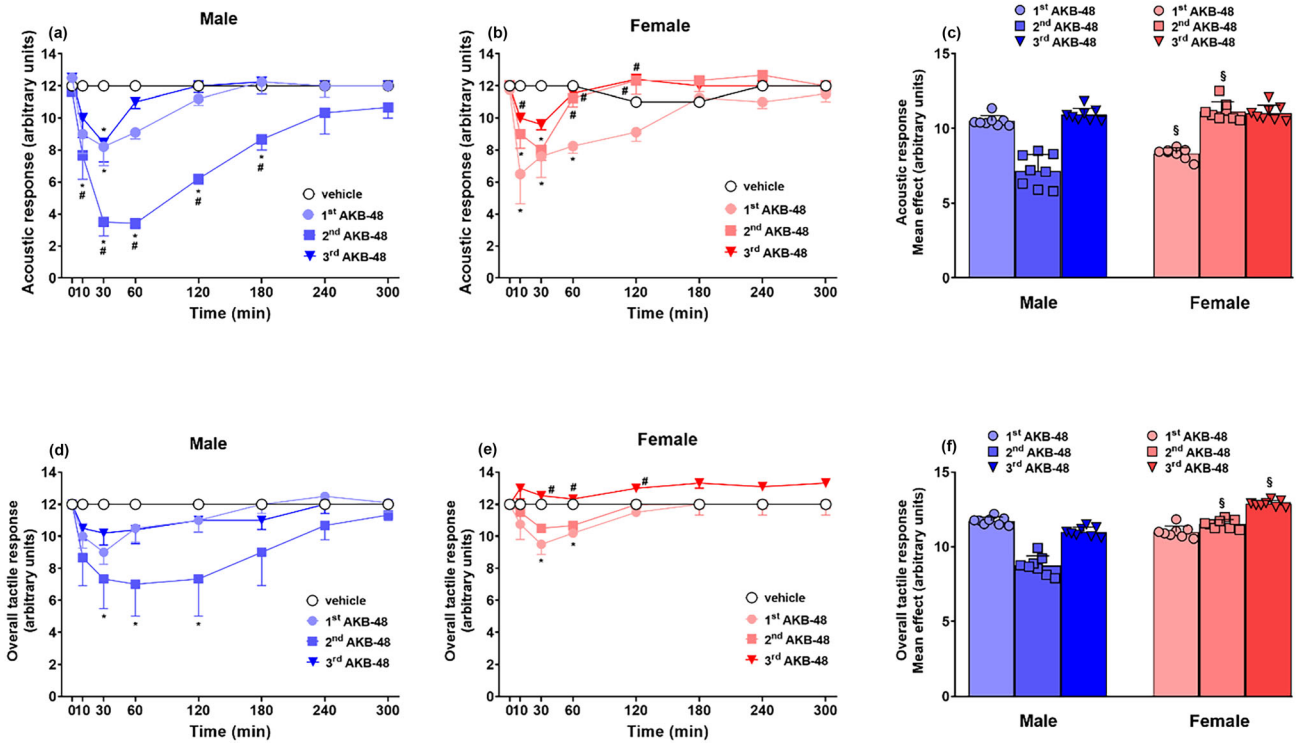

FIGURE 3 Effect of AKB48 (6 mg·kg^{-1}, i.p.) on the acoustic object (panels a–c) and the overall tactile response (panels d–f) in male (*left*, in blue) and female (*middle*, in red) mice and comparison of mean effect of each treatment (*right*, in bars). Data are expressed as arbitrary units (a–f). 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, b, d, e), and by one-way ANOVA followed by Tukey's test for multiple comparisons of mean effect of each injection (c and f). *P < 0.01 versus vehicle; #P < 0.01 versus first AKB48 injection; §P < 0.01 versus corresponding male group; n = 8 per group.

(Figure 3a; effect of treatment [$F_{(3,224)} = 59.98$, $P < 0.0001$], time [$F_{(7,224)} = 18.94$, $P < 0.0001$], time × treatment interaction [$F_{(21,224)} = 4.527$, $P < 0.0001$]). Differently from males, in females the stronger effect was observed after the first AKB48 injection, which was significantly different from the effect induced by the third AKB48 injection (Figure 3b; effect of treatment [$F_{(3,224)} = 22.87$, $P < 0.0001$], time [$F_{(7,224)} = 15.51$, $P < 0.0001$], time × treatment interaction [$F_{(21,224)} = 3.732$, $P < 0.0001$]). Notably, the first AKB48 injection inhibited the acoustic response more in females than in males, in contrast to the second AKB48 injection, whose effect was more evident in males than in females (Figure 3c; $F_{(5,42)} = 58.42$, $P < 0.0001$). NESS-0327 (1 mg·kg^{-1}, i.p.) prevented the effect induced by AKB48 on the acoustic response in both males ($F_{(7,56)} = 98.03$, $P < 0.0001$) and females ($F_{(7,56)} = 68.86$, $P < 0.0001$) (Figure S2A,B, respectively).

The *overall tactile* response did not change in vehicle-treated mice over the 300-min observation, while AKB48 (6 mg·kg^{-1}, i.p.) differently reduced the tactile responses in male and female mice (Figure 3d,e). In males, the second AKB48 injection decreased (~30%) overall tactile response from 30 up to 120 min (Figure 3d; effect of treatment [$F_{(3,224)} = 13.14$, $P < 0.0001$], time [$F_{(7,224)} = 3.957$, $P = 0.0004$], time × treatment interaction [$F_{(21,224)} = 0.9254$, $P = 0.5585$]), while in females a slight effect (~10%) was evident only from 30 up to 60 min after the first AKB48 injection (Figure 3e; effect of treatment [$F_{(3,224)} = 28.46$, $P < 0.0001$], time [$F_{(7,224)} = 6.612$, $P < 0.0001$], time × treatment interaction [$F_{(21,224)} = 1.863$, $P = 0.0146$]). The overall tactile response was more inhibited in males than in females after both the second and third AKB48 injections (Figure 3f; $F_{(5,42)} = 105.0$, $P < 0.0001$). NESS-0327 (1 mg·kg^{-1}, i.p.) prevented the effect induced by AKB48 on the overall tactile response in both males [$F_{(7,56)} = 124.9$, $P < 0.0001$] and females [$F_{(7,56)} = 58.45$, $P < 0.0001$] (Figure S2C,D, respectively).

3.1.3 | Evaluation of mechanical pain, core temperature and breath rate

The threshold to acute mechanical pain stimulus in the *tail pinch* test did not change in vehicle-treated mice over the 315-min observation, while AKB48 (6 mg·kg^{-1}, i.p.) significantly increased it with a maximum reached at 75 min in both sexes (Figure 4a,b). In particular, the most evident effects were observed after the second AKB48 injection in males (Figure 4a; effect of treatment [$F_{(3,196)} = 28.63$, $P < 0.0001$], time [$F_{(6,196)} = 7.588$, $P < 0.0001$], time × treatment interaction [$F_{(18,196)} = 2.881$, $P = 0.0002$]), while in females a significant antinociceptive effect was observed only after the first AKB48 injection (Figure 4b; effect of treatment [$F_{(3,196)} = 26.25$, $P < 0.0001$], time [$F_{(6,196)} = 2.801$, $P = 0.0123$], time × treatment interaction [$F_{(18,196)} = 1.530$, $P = 0.0828$]). Notably, the second AKB48 injection

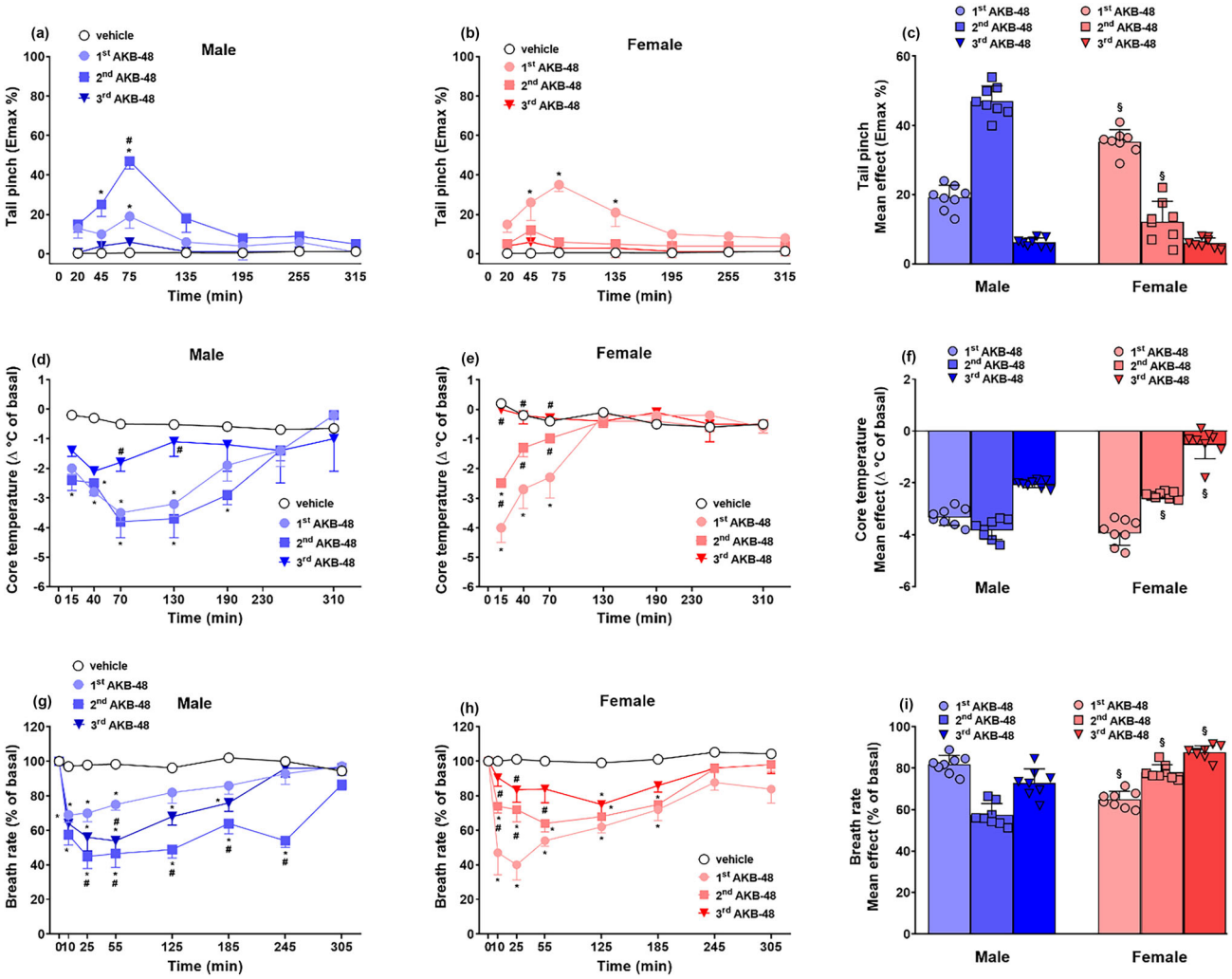

FIGURE 4 Effect of AKB48 (6 mg·kg^{-1}, i.p.) on the tail pinch response (panels a–c), core temperature (panels d–f) and breath rate (panels g–i) in male (*left*, in blue) and female (*middle*, in red) mice and comparison of mean effect of each treatment (*right*, in bars). Data are expressed as percentage of maximum effect (Emax %; a–c), difference between control temperature (before injection) and temperature following drug administration (Δ°C of basal; d–f) or % of basal (g–i). Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparisons for the treatment response curve at different times (*left* and *middle* panels), and by one-way ANOVA followed by Tukey's test for multiple comparisons of mean effect of each injection (*right* panels). $^{*}P < 0.01$ versus vehicle; $^{\#}P < 0.01$ versus first AKB48 injection; $^{\S}P < 0.01$ versus corresponding male group; n = 8 per group.

increased the threshold to the acute mechanical pain stimulus more in males than in females, as opposed to the first AKB48 injection whose effect was stronger in females than in males (Figure 4c; $F_{(5,42)} = 166.6$, $P < 0.0001$). Again, NESS-0327 (1 mg·kg^{-1}, i.p.) prevented the effect induced by AKB48 on the tail pinch response in both males ($F_{(7,56)} = 16.94$, $P < 0.0001$) and females ($F_{(7,56)} = 31.76$, $P < 0.0001$) (Figure S3A,B, respectively).

Body temperature did not change in vehicle-treated mice over the 300-min observation, while AKB48 (6 mg·kg^{-1}, i.p.) reduced the core body temperature differently in the two sexes (Figure 4d,e). Indeed, in males the first AKB48 second AKB48 injections induced a deep and long-lasting hypothermic effect, up to 130 and 190 min, respectively (Figure 4d; effect of treatment [$F_{(3,196)} = 23.38$, $P < 0.0001$], time [$F_{(6,196)} = 7.133$, $P < 0.0001$] time × treatment interaction [$F_{(18,196)} = 2.324$, $P = 0.0025$]). In females, the effect of AKB48 was greater and longer (up to 70 min) after the first AKB48 injection than after the second injection(Figure 4e; effect of treatment [$F_{(3,196)} = 27.31$, $P < 0.0001$], time [$F_{(6,196)} = 11.52$, $P < 0.0001$], time × treatment interaction [$F_{(18,196)} = 8.598$, $P < 0.0001$]). The third AKB48 injection did not induce significant effects in male and female mice. The decrease in core temperature was more marked in males than in females after both the second and third AKB48 injections (Figure 4f; $F_{(5,42)} = 91.3$, $P < 0.0001$). NESS-0327 (1 mg·kg^{-1}, i.p.) prevented the effect of AKB48 on the core temperature in both males ($F_{(7,56)} = 111.1$, $P < 0.0001$) and females ($F_{(7,56)} = 125.6$, $P < 0.0001$) (Figure S3C,D, respectively).

Breath rate did not change in vehicle-treated mice over the 300-min observation, while AKB48 (6 mg·kg^{-1}, i.p.) reduced differently the breath rate in male and female mice (Figure 4g,h). In males, the second AKB48 injection induced an effect that was greater and

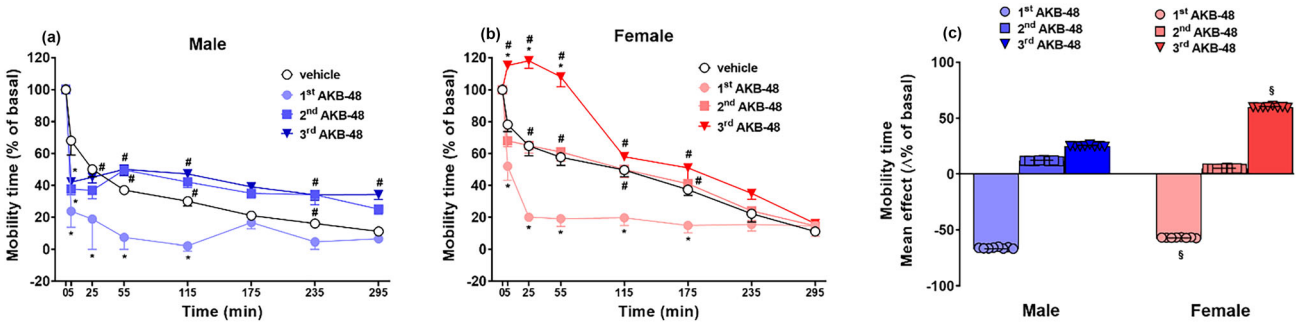

FIGURE 5 Effect of AKB48 (6 mg·kg^{-1}, i.p.) on mobility time in male (a) and female (b) mice and comparison of mean effect of each treatment (c). All data are expressed as % of basal values. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparisons for the treatment response curve at different times (a and b) and by one-way ANOVA followed by Tukey's test for multiple comparisons of each injection mean effect (c). *P < 0.01 versus vehicle; #P < 0.01 versus first AKB48 injection; §P < 0.01 versus corresponding male group; n = 8 per group.

lasted longer (up to 245 min) than the first (up to 55 min) and the third (up to 185 min) injections(Figure 4g; effect of treatment [$F_{(3,224)} = 83.96$, $P < 0.0001$], time [$F_{(7,224)} = 27.94$, $P < 0.0001$], time × treatment interaction [$F_{(21,224)} = 5.199$, $P < 0.0001$]). In females, the effect of AKB48 was inversely proportional to the succession of injections, with the first AKB48 inducing a deeper and longer effect than the subsequent administrations (Figure 4h; effect of treatment [$F_{(3,224)} = 57.10$, $P < 0.0001$], time [$F_{(7,224)} = 17.24$, $P < 0.0001$], time × treatment interaction [$F_{(21,224)} = 3.398$, $P < 0.0001$]). Interestingly, after the first AKB48 injection, the breath rate was more inhibited in females than in males, as opposed to the effect of the second injection, which was stronger in males than in females (Figure 4i; $F_{(5,42)} = 44.59$, $P < 0.0001$). NESS-0327 (1 mg·kg^{-1}, i.p.) prevented the effect induced by AKB48 on the breath rate in both males [$F_{(7,56)} = 80.18$, $P < 0.0001$] and females [$F_{(7,56)} = 79.23$, $P < 0.0001$] (Figure S3E,F, respectively).

3.1.4 | Evaluation of spontaneous locomotor activity

Spontaneous locomotor activity gradually declined in vehicle-treated mice over the 300-min observation (Figure 5a,b). Differently from the other tests, the effect of AKB48 (6 mg·kg^{-1}, i.p.) administration on *mobility time* was inversely proportional to the succession of injections in both sexes. In particular, in males the first AKB48 injection induced an immediate hypomotility that persisted up to 115 min (Figure 5a; effect of treatment [$F_{(3,224)} = 40.38$, $P < 0.0001$], time [$F_{(7,224)} = 96.30$, $P < 0.0001$], time × treatment interaction [$F_{(21,224)} = 3.753$, $P < 0.0001$]). In females, the first AKB48 injection induced an immediate hypomotility while the third injection significantly increased animals' motor activity (Figure 5b; effect of treatment [$F_{(3,224)} = 114.6$, $P < 0.0001$], time [$F_{(7,224)} = 153.3$, $P < 0.0001$], time × treatment interaction [$F_{(21,224)} = 11.48$, $P < 0.0001$]). After the first AKB48 injection, the locomotor response was more inhibited in males than in females, while after the third injection it was enhanced only in females (Figure 5c; $F_{(5,42)} = 76{,}637$, $P < 0.0001$). NESS-0327 (1 mg·kg^{-1}, i.p.) prevented AKB48-induced effects on the motor activity in both males [$F_{(7,56)} = 42.25$, $P < 0.0001$] and females [$F_{(7,56)} = 144.9$, $P < 0.0001$] (Figure S4A,B, respectively).

3.2 | Pharmacokinetic studies

Following systemic administrations of AKB48 (6 mg·kg^{-1}, i.p.), the maximum peak plasma concentration was reached after 30 min in both sexes (Figure 6a,b), although with mean values that differed between males (0.40–17.97 ng·ml^{-1}) and females (0.75–38.97 ng·ml^{-1}). Plasma time-concentration profiles for AKB48 were significantly affected by drug dose in both males (Figure 6a, dose [$F_{(3,140)} = 21.30$, $P < 0.0001$], time [$F_{(4,140)} = 29.66$, $P < 0.0001$], time × treatment interaction [$F_{(12,140)} = 7.748$, $P = 0.0162$]) and females (Figure 6b, dose [$F_{(3,140)} = 34.29$, $P < 0.0001$], time [$F_{(4,140)} = 75.22$, $P < 0.0001$], time × treatment interaction [$F_{(12,140)} = 26.65$, $P = 0.0162$]), with blood concentrations rising linearly with each drug administration. Sex comparison of the blood levels at 30 min after each drug injection revealed that after the third AKB48 injection, the plasma drug concentration was higher in females than in males (Figure 6c; $F_{(5,138)} = 426.7$, $P < 0.0001$).

3.3 | AKB48 affects cerebellar and cortical CB_1 receptor expression pattern

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

3.3.1 | CB_1 receptor evaluation in the cerebellum

As shown in Figure 7, in the cerebellum the CB_1 receptor distribution was widespread in the width of the molecular layer (ML) in both sexes,

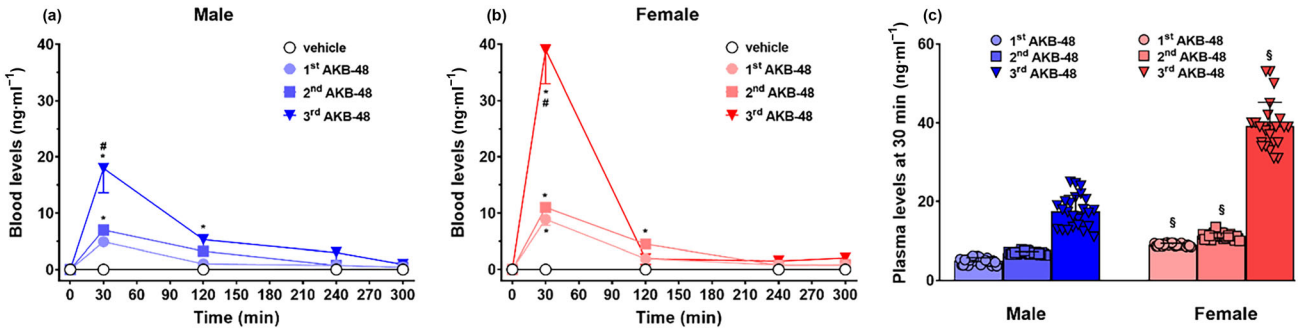

FIGURE 6 Time-concentration profiles for AKB48 in male (a) and female (b) mice and comparison of mean effect of each treatment (c) with data expressed as absolute values (ng·ml^{-1}). Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test for multiple comparisons for the treatment response curve at different times (a and b) and by one-way ANOVA followed by Tukey's test for multiple comparisons of plasma AKB48 level 30 min after injection (c). *P < 0.01 versus vehicle; #P < 0.01 versus first AKB48 injection; §P < 0.01 versus corresponding male group; n = 8 per group.

likely due to its presence on chief cerebellar cortex fibres, which gradually decreased after repeated AKB48 administration. In control animals, CB_1 receptor immunopositivity in the ML was manifestly stronger in female (E) than in male (A) mice. A marked immunopositivity appeared evidently localized in the large soma of Purkinje neurons (PCs) in both males (panels A–D) and females (panels E–H). The strongest immunopositivity appeared after the first AKB48 injection (panels b and f for males and females, respectively), and decreased after the second and at a greater extent after the third injection, with the greater reducing effects observed in females (panels G and H) compared with males (panels C and D).

Regarding ML immunoreactivity, repeated AKB48 significantly decreased CB_1 receptor expression levels in both sexes. In males (Figure 7a), a slight (not significant) reduction of CB_1 receptor expression levels was found after the first AKB48 injection as compared with control animals, a decrease that became significant after the second and even more after the third AKB48 injection (Kruskal–Wallis test, K-W statistic = 124, P < 0.001). Females (Figure 7b) displayed a significant reduction of CB_1 receptor expression levels starting from the first AKB48 injection, which became more evident after the second and at greater extent after the third AKB48 injection (Kruskal–Wallis test: K-W statistic = 139, P < 0.001).

Regarding PC immunoreactivity, a single AKB48 administration significantly increased CB_1 receptor expression levels (assessed as CB_1 receptor-immunopositive PC density) in both sexes. In males (Figure 7c), the first AKB48 administration greatly enhanced CB_1 receptor expression levels compared with controls, an enhancement that did not significantly differ after the second and the third AKB48 injections (one-way ANOVA, $F_{(3,44)} = 0.815$, P < 0.001). Compared with controls, CB_1R expression levels was significantly enhanced in males after the first AKB48 administration (Figure 7e), an enhancement that did not differ after the second but that was significantly reduced after the third AKB48 injection (Kruskal–Wallis test, K-W statistic = 135, P < 0.001). In females, after the first AKB48 injection, we observed significantly enhanced CB_1 receptor expression levels that did not differ after the second and the third AKB48 injection as compared with controls (one-way ANOVA, $F_{(3,44)} = 0.214$, P < 0.001). Compared with controls, CB_1 receptor expression levels were significantly increased in females after the first AKB48 administration (Figure 7f), which decreased after the second and returned to a level comparable to that measured in controls after the third AKB48 injection (Kruskal–Wallis test, K-W statistic = 132, P < 0.001).

3.3.2 | CB_1 receptor evaluation in the DLPFC

As shown in Figure 8, CB_1 receptor immunolabelling in male mice was characterized by a widespread distribution in the different DLPFC layers, with the strongest CB_1 receptor-immunopositivity observed in the soma of PyrNs localized in the internal pyramidal layer (layer V) (panels A–L). Notably, the CB_1 receptor-immunoreactivity decreased in a dose dependent manner, reaching the lower expression in mice after the third AKB48 injection, showing scarce, weakly immunopositive PyrNs (J–L).

In males, the first AKB48 injection slightly (not significantly) reduced CB_1 receptor expression levels, assessed as CB_1 receptor-immunopositive pyramidal neurons (PyrNs) density, compared with controls, while a significant decrease of CB_1 receptor expression levels was detected after the second AKB48 injection compared with both controls and the first AKB48 injection (Figure 8a). After the third AKB48 injection, CB_1 receptor expression levels increased to a level comparable to that observed after the first injection (one-way ANOVA, $F_{(3,44)} = 0.360$, P < 0.001). Compared with controls, CB_1 receptor expression levels assessed as CB_1 receptor-immunopositive PyrNs (layer V) OD was already significantly reduced after the first AKB48 injection, a reduction that gradually became more evident after the second and even more after the third AKB48 injection (Figure 8b; Kruskal–Wallis test, K-W statistic = 109, P < 0.001). Among the different DLPFC layers, significant differences in CB_1 receptor expression levels in males was observed both in the external granular layer (EGL, layer II) and the external pyramidal layer (EPL, layer III), where the third AKB48 injection caused an extremely significant decrease with respect to controls, and the first and the second

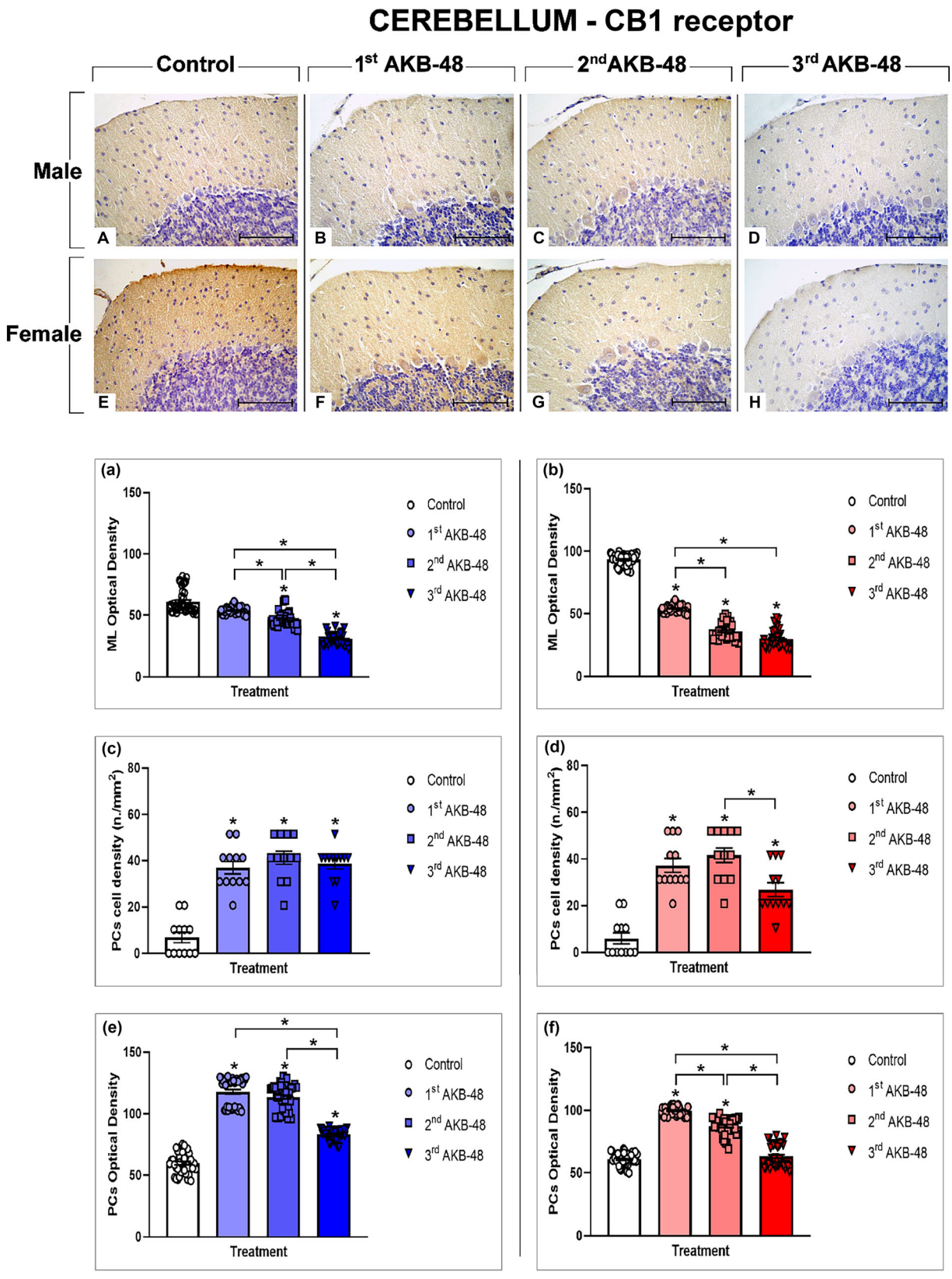

FIGURE 7 Effect of single and repeated systemic administration of AKB48 (6 mg·kg^{-1}, i.p.) on cerebellar immunostaining patterns of CB_1 receptor expression in male (panels A–D) and female (panels E–H) mice. Light microscopy magnification: 400× (A–H); scale bar: 100 μm (A–H). Histograms show the quantitative analysis of CB_1 receptor-immunopositive cells/fibres density and OD in cerebellum of males (panels a, c, e) and females (panels b, d, f), as determined in the molecular layer (ML) and Purkinje neurons (PCs) of control (*white* bars) and AKB48-treated mice. One-way ANOVA followed by Bonferroni's post hoc test (normally distributed data) or Kruskal–Wallis test followed by Dunn's test (non-normally distributed data): *$P < 0.01$.

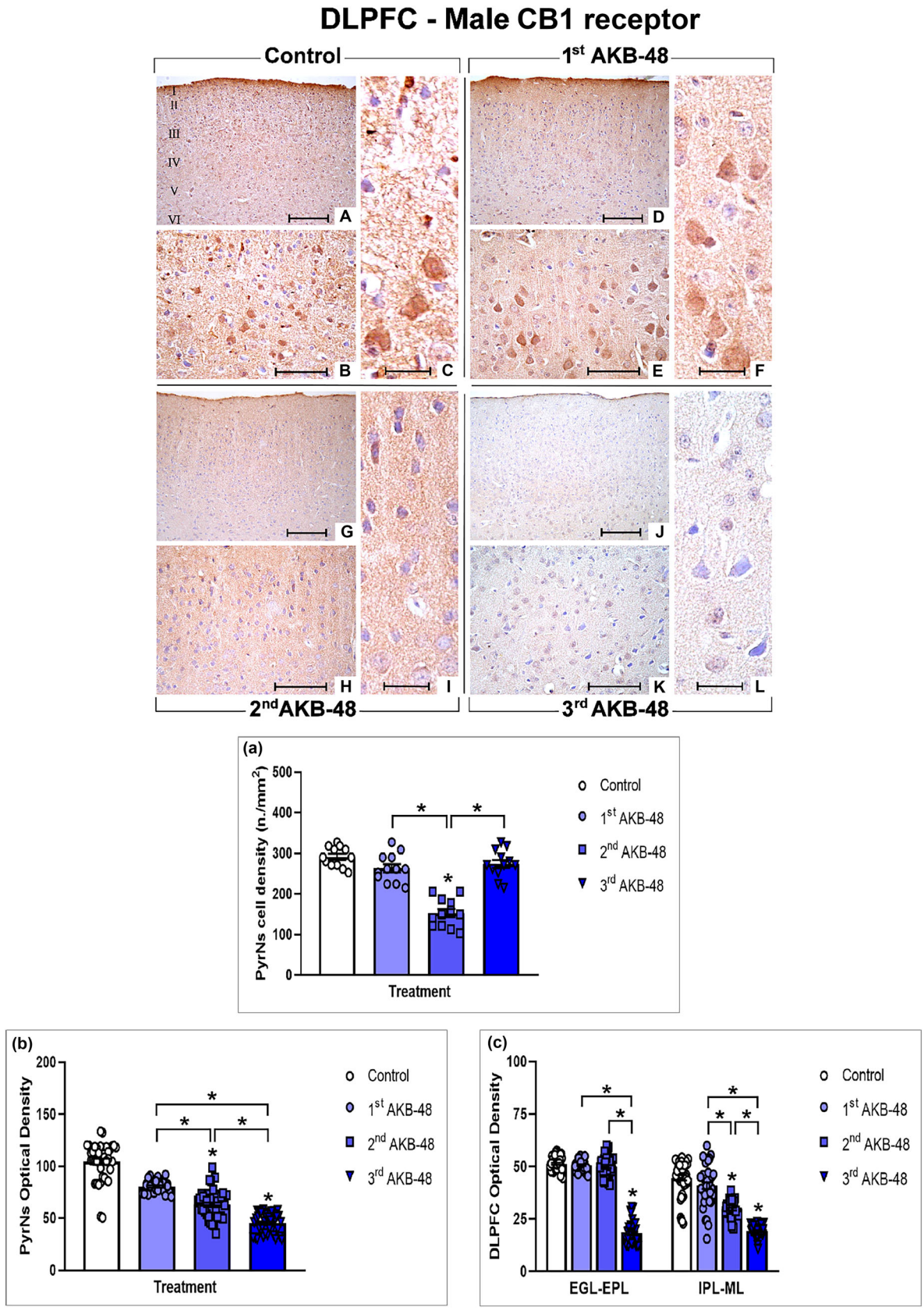

FIGURE 8 Legend on next page.

AKB48 injections (Figure 8c; Kruskal–Wallis test, K-W statistic = 89.9, $P < 0.001$). An OD reduction was also found in the internal pyramidal layer (IPL) and ML, with the third AKB48 injection inducing the most marked reduction in OD but where a significant reduction was detectable from the first AKB48 injection (Figure 8c; Kruskal–Wallis test, K-W statistic = 112, $P < 0.001$).

In females, the first AKB-48 injection slightly diminished CB_1 receptor expression levels, evaluated in terms of CB_1 receptor-immunopositive PyrNs density, as compared with controls (Figure 9a). Yet, a significant decrease of CB_1 receptor expression levels was detected after the second and third AKB48 injection with respect to both controls and the first AKB48 injection (one-way ANOVA, $F_{(3,44)} = 2.14$, $P < 0.001$). Compared with control females, CB_1 receptor-immunopositive PyrNs OD decreased after the first AKB48 injection, a reduction that gradually became more evident after the second and the third injections (Figure 9b; Kruskal–Wallis test, K-W statistic = 132, $P < 0.001$). Interestingly, the assessment of CB_1 receptor expression levels focused on the different DLPFC layers in females highlighted a slight OD reduction in the EGL (layer II) and EPL (layer III) after the first AKB48 injection, which became more evident after the second and third injections (Kruskal–Wallis test, K-W statistic = 118, $P < 0.001$), while no significant effect of AKB48 was observed in the IPL (layer V) and ML (layer VI) (Figure 9c).

Comparison between the two sexes revealed that AKB48 systemic administration (6 $mg \cdot kg^{-1}$, i.p.) dose-dependently induced an evident decrease of CB_1 receptor expression levels in DLPFC in both sexes, with females exhibiting the most marked outcome, evident from the first AKB48 injection (Figures 8 and 9, for males and females, respectively). Remarkably, CB_1 receptor expression levels were higher in females compared with male mice even in physiological conditions, that is, in vehicle-treated control animals. The CB_1 receptor expression pattern was characterized by a widespread distribution in the different DLPFC layers, both in males and females, with the strongest CB_1 receptor-immunopositivity observed in the soma of pyramidal neurons localized in the internal pyramidal layer (layer V).

4 | DISCUSSION

This is the first study showing the effects caused by repeated administration of the third-generation synthetic cannabinoid AKB48 on (i) sensorimotor and motor responses, (ii) nociception, breath rate and core temperature, (iii) plasma AKB48 pharmacokinetics, and (iv) cerebellar and cortical CB_1 receptor neuroplasticity in mice. Importantly, use of male and female animals allowed the discovery of significant sex-dependent differences in both the in vivo and ex vivo studies, confirming the existence of significant differences in the effects of NPSs in the two sexes (Fattore et al., 2020).

In line with previous studies (Bilel et al., 2019; Canazza et al., 2016), we confirmed that acute AKB48 impairs sensorimotor and motor responses, induces hypothermia and analgesia, and reduces the breath rate in mice. AKB48-induced inhibition of visual, acoustic and tactile reflexes is likely due to its action on CB_1 receptors located in circuitries designated for sensorimotor responsiveness (Gómez-Nieto et al., 2014; Hemelt & Keller, 2008; Tzounopoulos et al., 2007; Yoneda et al., 2013). Our findings of altered auditory and visual sensory perceptions are in line with previous animal studies showing that AKB48 affects prepulse inhibition of the acoustic startle reflex in rats (Bilel et al., 2019) and with clinical observations reporting visual and auditory hallucinations in users after consumption of other synthetic cannabinoid agonists (Holt et al., 2022). However, here we showed for the first time that such alterations occur differently in the two sexes after repeated exposure to AKB48, with females and males consistently showing the maximal effect after the first and the second administration, respectively.

Significant sex-specific differences were also found in AKB48-induced analgesia, with females and males showing the highest increase in the threshold to mechanical pain stimulus after the first and the second drug administration, respectively. Similarly, the AKB48-induced reduction of breath rate was more evident in females after the first administration and in males after the second administration. However, the reduction in body temperature was deeper and lasted longer after the first AKB48 administration in females, while in males both the first and the second drug administration induced the maximal effect, with no significant dose-dependent differences. A slightly different picture emerged from the analysis of the spontaneous locomotor activity following repeated AKB48 administrations, since both females and males exhibited hypolocomotion after the first drug administration, and hyperlocomotion after the third drug administration. Yet, hypomotility was more marked in males than in females, while hypermotility was much more evident in females than in males. Importantly, we confirmed that, similarly to the synthetic cannabinoid JWH-018 (Bilel et al., 2023), AKB48 induces its behavioural and physiological effects through a CB_1 receptor-mediated mechanisms. In fact, the effects are all prevented by the administration of the selective CB_1 receptor antagonist NESS-0327, which displayed strong affinity for CB_1 receptors (Ki = 350 ± 5 fM) versus CB_2 receptors (Ki = 21 ± 0.5 nM; Ruiu et al., 2003).

CB_1 receptors are highly expressed in the rodent brain, including those regions regulating locomotion, pain sensitivity, body

FIGURE 8 Representative micrographs showing the effect of single and repeated systemic administration of AKB48 (6 $mg \cdot kg^{-1}$, i.p.) on dorsolateral prefrontal cortex (DLPFC) immunostaining patterns of CB_1 receptor expression in male mice after vehicle administration (panels A–C), first AKB48 injection (panels D–F), second AKB48 injection (panels G–I), and third AKB48 injection (panels J–L). Light microscopy magnification: 200× (A, D, G, J); 400× (B, E, H, K); 600× (C, F, I, L); scale bars: 150 μm (A, D, G, J); 100 μm (B, E, H, K); 30 μm (C, F, I, L). Histograms show the quantitative analysis of CB_1 receptor-immunopositive cells density and OD in DLPFC, as determined in the EGL-EPL and IPL-ML layers and, in particular, in the layer V, the internal pyramidal cell layer, typically containing pyramidal neurons (PyrNs), of control and AKB48-treated mice (panels a–c). One-way ANOVA followed by Bonferroni's post hoc test (normally distributed data) or Kruskal–Wallis test followed by Dunn's test (non-normally distributed data), *$P < 0.01$.

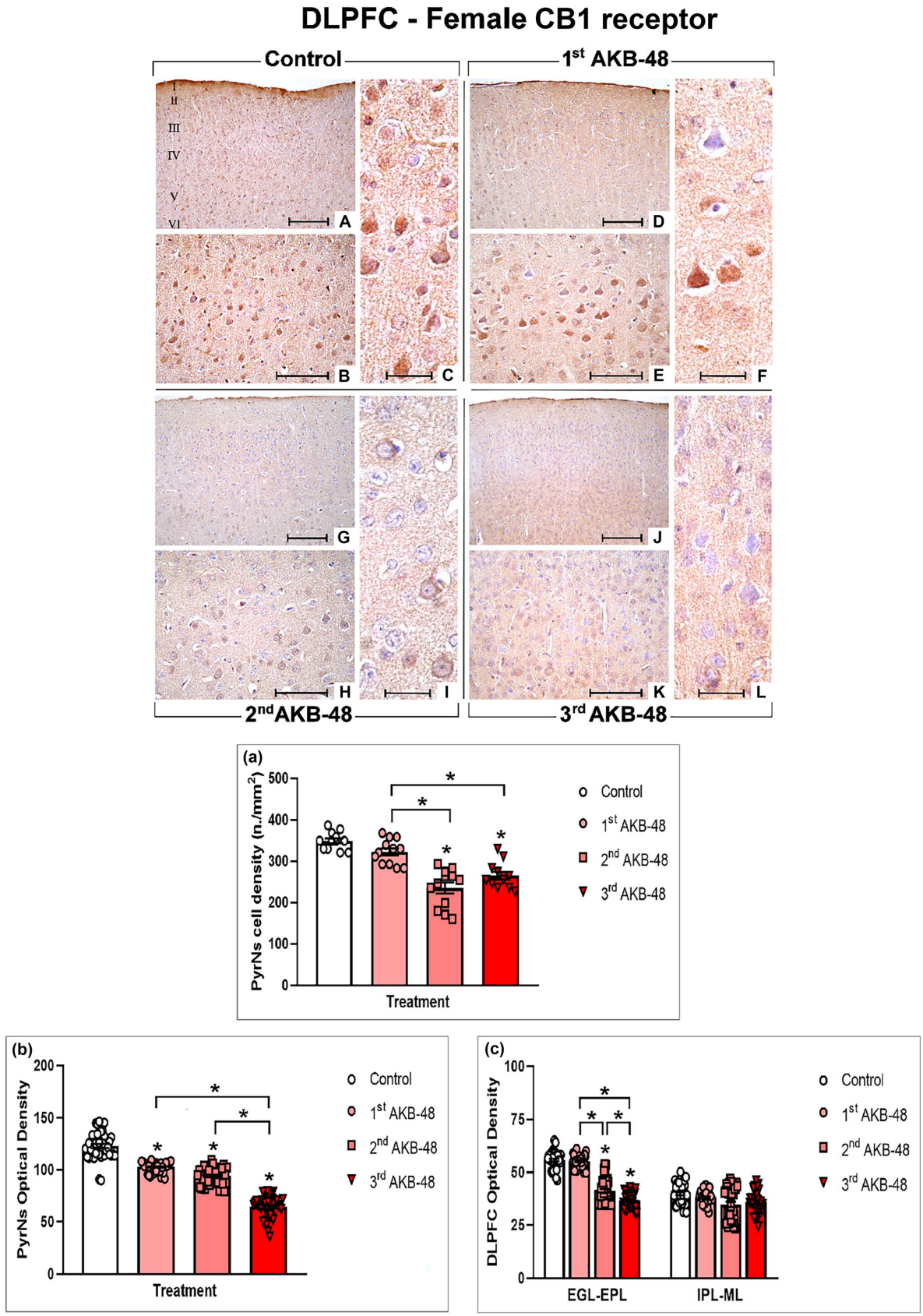

FIGURE 9 Legend on next page.

temperature, and cognitive functions (Busquets-Garcia et al., 2015; Egerton et al., 2006; Pertwee, 2001). Notably, their distribution and function are different in males and females, both in animals (Liu et al., 2020) and humans (Laurikainen et al., 2019), and are influenced by the circulating levels of sexual hormones (Antinori & Fattore, 2017; Castelli et al., 2014). It is therefore reasonable to assume that, as other synthetic cannabinoids of the third generation, AKB48 may exert its sex-specific action by stimulating cannabinoid signalling especially in the cortex, cerebellum, and basal ganglia (Bilel et al., 2019; Canazza et al., 2016; Ossato et al., 2017), thus affecting dopaminergic motor circuits and/or central glutamate neurotransmission involved in cognitive and motor functions (Funada et al., 2020; Morera-Herreras et al., 2012). The sexual dimorphic distribution of CB_1 receptor mRNA in mice (Liu et al., 2020) may also account, at least in part, for the sex-dependent effect of AKB48 on breath rate described in this study. Importantly, while the respiratory depressant effect of synthetic cannabinoid agonists has already been reported in rats (Pfitzer et al., 2004), cats (Doherty et al., 1983), and humans (Alon & Saint-Fleur, 2017), this is the first demonstration that such an effect occurs differently in males and females, not only in terms of the magnitude of the effect, which is greater in females than in males, but also in terms of timing of response, with females and males showing most marked respiratory depression after the first and second exposure, respectively.

It is worth noting that the first AKB48 injection induced sensorimotor impairment, hypothermia, analgesia, and breath rate reduction more in female than in male mice, while males typically show higher resilience to the first drug exposure, in line with previous evidence for a potentially higher susceptibility of females to the adverse effects of synthetic cannabinoids (Fattore et al., 2007, 2010; Wiley et al., 2017). The present study also strengthens the hypothesis that repeated administration of synthetic cannabinoids can lead to previously disclosed tolerance to their in vivo effects (Singh et al., 2011; Tai et al., 2015), which develops more rapidly in females than in males.

Females also showed consistently larger increases in AKB48 blood levels than males after each drug administration, with the third injection causing a rise that was approximately double in females with respect to males. These findings thus extend the sex specificity of the effects of AKB48 from behaviour and respiratory parameters to drug pharmacokinetics, in line with the sex-dependent differences previously described in Δ^9-THC metabolic pathways (Narimatsu et al., 1991; Tseng et al., 2004). Biochemical data also suggest a possible inhibitory effect of AKB48 on cytochrome activity, in line with previous in vitro studies disclosing the inhibitory action of synthetic cannabinoids on cytochrome P-450 enzymes (Ashino et al., 2014; Kim et al., 2020) that are among the major contributors to their metabolism (Holm et al., 2015; Stout & Cimino, 2014). In support to the higher susceptibility of females to the effects induced by the first injection of AKB48, immunohistochemical analysis revealed higher expression of CB_1 receptors in females with respect to male untreated mice, and a greater down-regulation of cerebellar CB_1 receptors in females following repeated AKB48 injections, which could be related to the development of tolerance to cannabinoid agonist effects (Romero et al., 1998; Rubino et al., 2000). The enhanced down-regulation of cortical and cerebellar CB_1 receptors in females is consistent with previous studies showing greater down-regulation or desensitization in the cerebellum, hippocampus, prefrontal cortex, and striatum of female rats compared with the respective male groups after repeated Δ^9-THC exposure (Farquhar et al., 2019), which could explain why women tend to progress to tolerance and dependence quicker than men after initiation of cannabis use.

5 | CONCLUSION

The present study investigated for the first time the behavioural, neurochemical, and immunohistochemical effects of repeated administration of AKB48 in male and female mice. Significant sex-dependent differences were found both in the magnitude of the effect and in the timing of repeated response, with males showing higher resilience and females showing higher responsiveness, respectively, to the first drug administration. Overall, the present data suggest that repeated administration of AKB48 may result in tolerance to behavioural and physiological impairment possibly related to variations in the drug blood concentration and brain CB_1 receptor expression. Clinical implications of this study include the indication of a higher health risk associated with the first use for female users and the possibility for male users to overcome the reduced effectiveness by escalating dose of drugs usually associated with severe toxicity.

AUTHOR CONTRIBUTIONS

Giorgia Corli: Conceptualization (equal); data curation (equal); investigation (equal); methodology (equal); supervision (equal); writing—original draft (equal); writing—review and editing (equal). **Elisa Roda:** Conceptualization (equal); data curation (equal); investigation (equal); supervision (equal); writing—original draft (equal); writing—review and editing (equal). **Micaela Tirri:** Data curation (equal); investigation (equal); writing—review and editing (equal). **Sabrine Bilel:** Data curation (equal); investigation (equal); writing—review and editing (equal). **Fabrizio De Luca:** Data curation (equal); formal analysis (equal);

FIGURE 9 Representative micrographs showing the effect of single and repeated systemic administration of AKB48 (6 mg·kg^{-1}, i.p.) on dorsolateral prefrontal cortex (DLPFC) immunostaining patterns of CB_1 receptor expression in female mice after vehicle administration (panels A–C), first AKB48 (panels D–F), second AKB48 (panels G–I), and third AKB48 injections (panels J–L). Light microscopy magnification: 200× (A, D, G, J); 400× (B, E, H, K): 600× (C, F, I, L); scale bars: 150 μm (A, D, G, J); 100 μm (B, E, H, K); 30 μm (C, F, I, L). Histograms showing the quantitative analysis of CB_1 receptor-immunopositive cells density and OD, in DLPFC of females, as determined in the EGL-EPL and IPL-ML layers and, in particular, in the layer V, the internal pyramidal cell layer, typically containing pyramidal neurons (PyrNs), of control and AKB48-treated mice (panels a–c). One-way ANOVA followed by Bonferroni's post hoc test (normally distributed) or Kruskal–Wallis test followed by Dunn's test (non-normally distributed data): *P < 0.01.

investigation (equal); writing—review and editing (equal). **Sabina Strano-Rossi:** Data curation (equal); formal analysis (equal); investigation (equal); writing—review and editing (equal). **Rosa Maria Gaudio:** Investigation (equal); methodology (equal); writing—review and editing (equal). **Fabio De-Giorgio:** Funding acquisition (equal); writing—review and editing (equal). **Liana Fattore:** Conceptualization (equal); data curation (equal); formal analysis (equal); funding acquisition (equal); supervision (equal); writing—original draft (equal); writing—review and editing (equal). **Carlo Alessandro Locatelli:** Conceptualization (equal); data curation (equal); funding acquisition (equal); visualization (equal); writing—review and editing (equal). **Matteo Marti:** Conceptualization (equal); funding acquisition (equal); project administration (equal); supervision (equal); writing—original draft (equal); writing—review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation and as recommended by funding agencies, publishers, and other organizations engaged with supporting research.

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