Progress in Neuropsychopharmacology & Biological Psychiatry 117 (2022) 110562

Contents lists available at ScienceDirect

Progress in Neuropsychopharmacology & Biological Psychiatry

journal homepage: www.elsevier.com/locate/pnp

Repeated administration of N-ethyl-pentedrone induces increased aggression and impairs social exploration after withdrawal in mice

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ARTICLE INFO

Keywords:
N-ethyl-pentedrone
Synthetic cathinones
Aggressive behavior
Monoamine levels
Addiction

ABSTRACT

N-ethyl-pentedrone (NEPD, 2-(ethylamino)-1-phenyl-1-pentanone) is one of the latest synthetic cathinone derivatives that emerged into the illicit drug market. This drug has psychostimulant properties and has been related with several intoxications and even fatalities. However, information about the consequences of its acute and repeated consumption is lacking. Thus, the aim of our study was to investigate the behavioral effects after both acute and repeated NEPD exposure as well as the neurochemical changes. Male OF1 mice were treated with an acute dose (1, 3 or 10 mg/kg, i.p.) or received repeated injections of these doses (twice/day, 5 days) of NEPD. Shortly after drug-exposure or during drug-withdrawal, anxiety-like behavior, aggressiveness, social interaction, depressive-like symptoms, body weight and temperature were assessed. Also, monoamine synthesis enzymes, levels of neurotransmitters and their precursors and main metabolites, as well as ΔFosB, were determined in striatum and prefrontal cortex from post-mortem tissue. Acute administration of NEPD induced anxiolytic effects and reduced social exploration whereas during withdrawal after repeated administration the anxiolytic effect had vanished, and the reduced social exploration was still present and accompanied with increased aggressive

Abbreviations: ^{13}C NMR, Carbon nuclear magnetic resonance; ^{1}H NMR, Proton nuclear magnetic resonance; 3-MT, 3-methoxytyramine; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hidroxytryptamine, a.k.a. serotonin; A + T, time spent attacking + threating; ACN, Acetonitrile; ANOVA, Analysis of variance; Arc, Activity-regulated cytoskeleton-associated protein; ARRIVE, Animal research: reporting in vivo experiments; BEH, Ethylene bridged hybrid; CA, Closed arms; CFSRE, Center for forensic science research and education; DA, Dopamine; DAT, Dopamine active transporter; DEA, Drug enforcement administration; DOPAC, 3,4-dihydroxyphenylacetic acid; EDTA, Ethylenediaminetetraacetic acid; ELISA, Enzyme-linked immunosorbent assay; EMCDDA, European monitoring centre for drugs and drug addiction; EPM, Elevated plus maze; ESI+, Electrospray ionization source in positive mode; FA, Formic acid; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; HCl, Hydrochloric acid; hDAT, Human dopamine active transporter; HLA, Horizontal locomotor activity; hSERT, Human serotonin transporter; HVA, Homovanillic acid; IC_{50}, half maximal inhibitory concentration; IEGs, Immediate early genes; IR, Infrared spectroscopy; ISM, Internal standard mixture; LC-MS/MS, Liquid chromatography-mass spectrometry/mass spectrometry; MDMA, 3,4-methylenedioxymethamphetamine, a.k.a. ecstasy; MDPV, 3,4-methylenedioxypyrovalerone; METH, Methamphetamine; MS, Mass spectroscopy; N_2, Nitrogen; NA, Noradrenaline (a.k.a. norepinephrine); NaCl, Sodium chloride; NEP, N-ethylpentylone (a.k.a. N-ethylnorpentylone, Ephylone); NEPD, N-ethylpentedrone (a.k.a. N-ethylnorpentedrone, α-ethylaminovalerophenone, α-ethylaminopentiophenone (α-EAPP)); NET, Noradrenaline transporter (a.k.a. Norepinephrine transporter); NH_4COOH, Ammonium formate; NPS, New psychoactive substances; NPY/CART, neuropeptide Y/ cocaine- and amphetamine-regulated transcript; OA, Open arms; OF1 mice, Oncins france 1 mice; PFC, Prefrontal cortex; PVDF, Polyvinylidene fluoride; SDS, Sodium dodecyl sulfate; SE, Social exploration; SEM, Standard error of the mean; SERT, Serotonin transporter; SI, Social interaction; SRM, Selected reaction monitoring; Str, Striatum; TH, Tyrosine hydroxylase; TLC, Thin layer chromatography; TPH, Tryptophan hydroxylase; Tris, Tris (hydroxymethyl)aminomethane; Tryp, Tryptophan; TST, Tail suspension test; Tyr, Tyrosine; UNODC, United nations office on drugs and crime; UPLC, Ultra performance liquid chromatography; α-PVP, α-Pyrrolidinovalerophenone.

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https://doi.org/10.1016/j.pnpbp.2022.110562
Received 9 January 2022; Received in revised form 22 April 2022; Accepted 26 April 2022
Available online 30 April 2022

behavior. Moreover, NEPD (10 mg/kg) induced slight hyperthermia and reduced weight gain during the repeated administration, whereas increased locomotor activity and lack of depressive symptoms were found during withdrawal. This was accompanied by increased plasma corticosterone and decrease in striatal dopamine. Finally, the long-lasting and robust increase in ΔFosB levels found in striatum after NEPD chronic exposure suggests a high risk of dependence. The increased aggressivity and locomotor activity, together with this potential of inducing dependence justify a warning about the risks of consumption of NEPD if translated to humans.

1. Introduction

New psychoactive substances (NPS) have experienced an increasing presence in the drug of abuse market since the beginning of this century. These include chemicals designed to mimic the effects of classical drugs of abuse such as cocaine, amphetamines, cannabis, or opioids, among others. As initially most of them are not controlled substances, they can be freely sold through online shops or through the *darknet* (European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), 2021; *United Nations Office on Drugs and Crime (UNODC)*, 2013) provided it is specified that they are not intended for human consumption. Thus, they were initially marketed as bath salts or research chemicals, as most popular presentations. However, they are really used as drugs of abuse and many cases of related intoxications and fatalities have been reported, which is a matter of concern (see Kraemer et al., 2019 and La Maida et al., 2021 as reviews). As sufficient evidence of abuse, risks for health and addiction potential are collected, some of these substances are progressively being classified and controlled. Consequently, new generations of nonscheduled chemically related substances break in to replace those that have been banned.

Cathinones (β-keto-amphetamines) are the second chemical group of NPS with more substances being surveilled by the EU early warning system (European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), 2021), following the synthetic cannabinoids. These substances exert psychostimulant effects and are purchased as replacement alternatives for cocaine or amphetamine derivatives such as MDMA or methamphetamine (METH). Cathinones exert their effects mainly by targeting the transporters for dopamine (DA; DAT), serotonin (5-HT; SERT) and noradrenaline (NA; NET), either as blockers and/or substrates (Baumann et al., 2018; Duart-Castells et al., 2021; Lopez-Arnau et al., 2012; Riley et al., 2020; Simmler et al., 2014) thus potentiating the effects of the monoamine/s involved.

"First generation" cathinones included mephedrone, methylone and 3,4-methylenedioxypyrovalerone (MDPV). These drugs became classified and controlled (Drug Enforcement Administration Department of Justice, 2011) and then, a "second-generation" emerged including α-pyrrolidinovalerophenone (α-PVP), pentylone and pentedrone (Drug Enforcement Administration, Department of Justice, 2014). Toxicity including fatalities and abuse potential have been demonstrated for several synthetic cathinones (Baumann et al., 2018; Luethi et al., 2017; Luethi and Liechti, 2020; World Health Organization (WHO), 2016; Zhou et al., 2019). This makes necessary to investigate the properties of the new substances to supply enough scientifically underpinned data to justify their control.

N-ethyl-pentedrone (NEPD, 2-(ethylamino)-1-phenyl-1-pentanone) is also known as alpha-ethylaminopentiophenone (α-EAPP), α-ethylaminovalerophenone or N-ethylnorpentedrone. It is a substituted cathinone that has been sold online as a designer drug since the mid-2010s.

NEPD shares a close structural relationship to its parent compound pentedrone, differing just by the presence of an ethyl group in the N-terminal position instead of a methyl group. In fact, it has been recently reported that this small structural change is able to increase the potency (from 2- to 3-fold) as DAT inhibitor (Nadal-Gratacós et al., 2021). Moreover, NEPD also resembles N-ethylpentylone, one of the most reported cathinones in the recent years (DEA, 2016, 2017, 2018), from which it differs in the absence of the methylenedioxy group in the aromatic ring.

To date, very little related scientific literature about NEPD is available and it is mostly preclinical (Eshleman et al., 2019; Duart-Castells et al., 2021; Nadal-Gratacós et al., 2021). However, in humans, several personal experiences with this drug can be found in websites such as Erowid (www.erowid.org) or The Drug Classroom (www.Thedrugclassroom.com). Also, the Center for Forensic Science Research and Education (CFSRE), in their project NPS Discovery (https://www.npsdiscovery.org/) reported the presence of NEPD in biological samples and seized drug materials during 2020 and 2021. Moreover, fatalities have been reported after poly-consumption of NEPD and mepirapim, a synthetic cannabinoid (Fujita et al., 2015) or mixed with several other psychoactive substances (Pieprzyca et al., 2021). In the Internet forums cited above, consumers describe stimulating, euphoric, and mildly entactogenic effects when administered, mainly insufflated. It is short-acting, with a single dose only producing its core effects for a couple hours. Because the total duration is short and euphoria declines even faster than stimulation, NEPD is frequently redosed or binged for one or more days. Although it can produce a euphoric rush, particularly with rapid-onset routes of administration, this rush is relatively weak. Aside from the rush, its effects include modest mood enhancement, stimulation, restlessness, talkativeness, and a tendency to be scatterbrained and unfocused. After a few doses or large amounts, NEPD can lose most of its mood enhancement and the somewhat positive effects of the "rush" shorten in duration.

Currently, NEPD is a controlled substance in several countries and is classified as a "'potentially harmful substance"', whereas in other states it is subjected to certain measures of control.

Our research group recently led a comparative study about the acute pharmacodynamic, psychostimulant and rewarding properties of some recently marketed cathinones, among which there was NEPD (Duart-Castells et al., 2021; Nadal-Gratacós et al., 2021). There, NEPD showed relatively higher potency at the human dopamine transporter (hDAT) than cocaine, with an IC_{50} for uptake inhibition which was around half of that of cocaine, and a binding affinity with a K_i 5-fold lower. Also, it showed poorer affinity for SERT, with a hDAT/hSERT activity ratio $>$ 1000 compared with that of cocaine (7.8). NEPD displayed a powerful psychostimulant effect by dose-dependently increasing locomotor activity at doses up to 10 mg/kg. It also induced place conditioning at a dose as low as 1 mg/kg, which indicates that it has powerful rewarding properties. Moreover, NEPD acutely induced increased expression of the immediate early genes (IEGs) Arc (activity-regulated cytoskeleton-associated protein) and c-fos in both dorsal and ventral striatum (Str), suggesting that it can induce neuroadaptation and neuroplasticity (Fumagalli et al., 2006; Gallo et al., 2018).

However, to date, additional information about the acute effects of NEPD and specially that concerning the behavioral and neurochemical effects of its repeated administration is lacking. Accordingly, the aim of this study was to investigate some of these effects after a single dose administration and after a 5-day treatment with two daily doses of NEPD followed by drug withdrawal. Three different doses were tested. Concretely, we assessed social interaction (SI) and the performance in the elevated plus maze (EPM) and in the tail suspension test (TST) after the acute dose and under withdrawal after the repeated administration of NEPD. Also, body weight and core temperature were measured during the repeated exposure. Two days after the repeated exposure, plasma corticosterone was determined, whereas monoamine neurotransmitters and their metabolites and precursors were analyzed after 3 and 21 days

of withdrawal. Locomotor activity was also assessed during withdrawal and, to estimate the addictive potential of NEPD, the levels of the transcription factor ΔFosB were assessed at short and long term after the repeated exposure.

Main results include decreased social exploration (SE) and increased aggressive behavior and basal locomotor activity on withdrawal, which was accompanied by increased plasma corticosterone and decrease in striatal dopamine. Also, a robust and persistent increase of ΔFosB in Str was found. This is a very particular feature for a drug of this family which deserves to be considered if translated to human consumption.

2. Materials and methods

2.1. Drugs and materials

The racemic form of hydrochloride salt of NEPD was synthesized according to the procedure described by Nadal-Gratacós et al. (2021). Chemical purity and identification of the obtained substance were evaluated by thin layer chromatography (TLC), infrared spectroscopy (IR), mass spectrometry (MS) and carbon and proton nuclear magnetic resonance (^{13}C NMR, ^{1}H NMR). Solutions for i.p. administrations of NEPD were freshly prepared in isotonic saline solution (0.9% NaCl, pH 7.4). Crystalline solid standards of Tryp, 5-HT, Tyr, DA, DOPAC, HVA were all purchased from Sigma-Aldrich (St. Louis, USA). NA was supplied by Tocris Bioscience (Ellisville, USA) and 3-MT was obtained from Merck (Darmstadt, Germany). 5-HIAA crystalline solid standard, as well as isotopically labeled standards of Tryp-1-^{13}C, 5-HTd^{4}, 5-HIAA-d^{5}, L-Tyr-$^{13}C_9$,^{15}N, DA-1,1,2,2-d^{4}, DOPAC-d^{5}, 3-MT-d^{4} and NA-d^{6} were supplied by Toronto Research Chemicals (TRC, Toronto, Canada).

2.2. Animals

The experimental protocols concerning the use of animals in this study were performed following the ARRIVE guidelines (du Sert et al., 2020), complied with the European Community Council guidelines (2010/63/EU), as amended by Regulation (EU) 2019/1010, and were approved by the animal Ethics Committees of the Universities of Barcelona and Valencia under supervision by the Autonomous Government of Catalonia (2018/9738).

All experiments were performed with male OF1 mice weighing 35–45 g and aged 61–65 days at the start of the procedure, which were purchased from Charles River (Lyon, France). Mice were divided into groups of four to six individuals and distributed in plastic cages (25 × 25 × 14.5 cm) with free access to standard laboratory diet and drinking water. Housing conditions included controlled temperature (22 ± 1 °C) and a 12 h light/dark cycle consisting in a lightness schedule from 8 AM to 8 PM for animals intended for biochemical, temperature and body weight gain assays and a darkness schedule from 8 AM to 8 PM (reversed light cycle) for the batch of mice used to perform the behavioral tests. To minimize suffering and discomfort of the experimental animals, they were supervised for symptoms as piloerection, immobility, self-mutilations, abnormal postures and vocalizations or extreme weight loss. If any of these signs was observed, the affected mouse was immediately euthanized by cervical dislocation.

2.3. Experimental design and treatment schedule

Behavioral studies and biochemical, temperature and body weight gain assays were performed with two independent batches of mice (n = 48 mice/batch). To randomly distribute the animals to the different experimental groups, mice were sorted on their body weight and the treatment to be administered (saline or NEPD 1, 3 or 10 mg/kg) was randomly assigned by a person different than the experimenter using the Random Allocation Software 1.0. No predetermined sample size calculation was performed, as the number of subjects for neurotransmitters, Western blot and behavioral experiments was based on previous studies of a similar nature (Duart-Castells et al., 2019; Buenrostro-Jáuregui et al., 2016; Daza-Losada et al., 2009; Ray et al., 2019). The detailed timeline and fate of the different mouse batches is depicted in Fig. 1.

Regarding behavioral studies, EPM and SI tests were performed after acute administration of saline or NEPD (1, 3 or 10 mg/kg, i.p.). Following a one-week washout period, the same mice were treated according to a schedule of a repeated administration of saline or NEPD (1, 3 or 10 mg/kg, i.p.) consisting in two daily injections (4 h apart) for 5 consecutive days. During the subsequent abstinence, EPM, horizontal locomotor activity (HLA), SI and tail suspension (TST) tests were performed 3, 4, 5 and 6 days after treatment, respectively (See Fig. 1). Behavioral experiments were conducted during the dark phase of the reversed light cycle.

Concerning biochemical, temperature and body weight gain assays, animals were treated according to the chronic administration schedule described above (saline or NEPD 1, 3 or 10 mg/kg, i.p.). During the treatment period, body temperature was assessed by means of a lubricated rectal probe connected to a digital thermometer 1 h after the second daily administration. Moreover, the mice were weighted daily and 72 h after treatment. Animals were euthanized by cervical dislocation 72 h after treatment, striatum (Str) and prefrontal cortex (PFC) brain areas were dissected out and monoaminergic neurotransmitters, precursors and metabolites were quantified. In addition, protein expression of neurotransmitters synthesis rate-limiting enzymes and the transcription factor ΔFosB were assessed in both brain areas of the mice treated with saline and 10 mg/kg NEPD. Furthermore, a set of mice treated with saline or NEPD 10 mg/kg was euthanized 21 days after treatment and monoamines, precursors, and metabolite levels, as well as those biochemical parameters altered 72 h after treatment, were also evaluated in both brain areas (see Fig. 1). Euthanasia of mice for biochemical determinations and temperature and body weight measurements were performed during the lights on period of the light cycle.

2.4. Monoamines, precursors, and metabolites quantification

To extract the target substances from Str and PFC, 300 μL of a cold extractant solvent solution (1% formic acid (FA) added to an Acetonitrile (ACN):H_2O, 90:10 solution) was added to the samples in Eppendorf tubes. Internal standard mixture (ISM) was used to spike all tested samples. The samples were homogenized in a bead mill (TissueLysser LT), at 50 osc/min for 90 s, using three stainless steel beads (3 mm diameter) per Eppendorf tube. Then, the homogenates were centrifuged at 13,000 rpm at 4 °C during 20 min, and the supernatants were filtered using 0.22 μm nylon filters (Scharlab, Barcelona, Spain) and stored in chromatographic vials at −80 °C.

LC-MS/MS analysis of the extracted solutions was based on the procedures previously described in (Gómez-Canela et al., 2018; Mayol-Cabré et al., 2020). Briefly, to accomplish neurochemicals separation, an Acquity UPLC BEH Amide column (150 mm × 2.1 mm ID, 1.7 μm particle size; purchased from Waters, Milford, MA, USA), provided with an Acquity UPLC BEH Amide pre-column (5 mm × 2.1 mm ID, 1.7 μm particle size; obtained from Waters, Milford, MA, USA), was used. To ensure elution, a binary mixture was used with a gradient program. The flow rate was fixed at 250 μL min^{-1} and 10 μL of each sample extract were injected for the analysis. First solvent (solvent A) contained 100 mM NH_4COOH (in ultra-pure water) with ACN (95:5) while the second solvent (solvent B) consisted in 30 mM NH_4COOH (in ultra-pure water) with ACN (15:85). Both solvents were acidified by adding FA to pH 3. Concerning the gradient program, the mobile phase was initially set at 100% solvent B and, after 4 min, decreased at 80% solvent B and held for 1 min. From minute 5 to 7, solvent B was set back to 100%. At the end, the beginning conditions were re-equilibrated in 3 min, resulting in a total run time of 10 min. The column worked at 30 °C and the chromatographic vials were kept in the autosampler at 10 °C. Analytes were assessed using electrospray ionization source in positive mode (ESI+). Nitrogen (N_2) was employed as desolvating agent (900 L h^{-1}) and cone

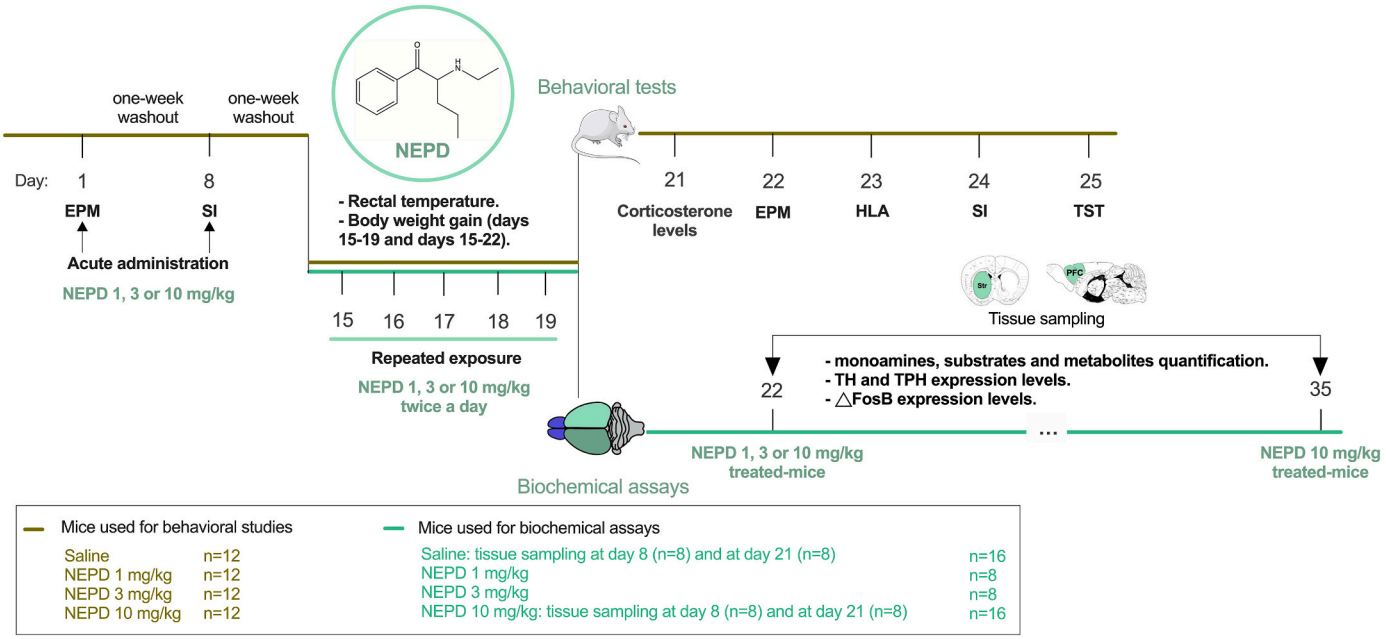

Fig. 1. Experimental design and NEPD administration schedule. NEPD chemical structure is shown above. Behavioral studies were performed both after acute and repeated exposure to NEPD. Biochemical parameters and corticosterone quantification as well as hyperthermia and body weight gain determinations were assessed during the withdrawal period after repeated administration of NEPD. Tissue sampling areas are indicated in the pictures of brain sections: Str (left, coronal plane) and PFC (right, sagittal plane).

gas (150 L h^{-1}). The desolvation temperature was set at 350 °C and the source was fixed at 100 °C. Capillary voltage was applied at 2.0 kV. The acquisition was performed in SMR mode, following the two most intense fragments of each precursor ion. First and second transitions were employed as the quantifier and the qualifier ions, respectively. Lastly, acquired data were processed using MassLynx® Software v.4.1. For quality assurance and parameters information, see supplementary material.

2.5. Western blotting

A general Western Blotting protocol was followed to analyze tyrosine hydroxylase (TH), tryptophan hydroxylase (TPH) and ΔFosB protein expression levels in Str and PFC areas. Briefly, 10 μg of the protein extracts were mixed with sample buffer (Tris-HCl 1 M, pH = 6.8; 10% glycerine, 10% (*w/v*) SDS, 5% (*v*/v) 2-β-mercaptoethanol, 0.05% bromophenol blue) and denatured boiling at 100 °C for 5 min. Denatured protein extracts were electrophoresed using a 10% acrylamide gel and then transferred to polyvinilidene fluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA, USA). Following the transfer phase, membranes were blocked for 1 h at room temperature with a solution of 5% defatted milk in Tris-buffer with 0.05% (v/v) Tween-20. Incubations with the primary antibodies (Anti-TH 1:10000, Anti-TPH2 1:10000, Anti-ΔFosB 1:5000) were performed overnight at 4 °C. After washing, membranes were incubated with the corresponding peroxidase-conjugated anti-IgG antibody (ECL™ Anti-Mouse IgG, 1:2500 and Anti-Rabbit IgG, 1:2000) at room temperature for 45 min. Immunoreactive protein was revealed using a chemoluminiscence-based detection kit (Immobilon Western, Millipore) and a BioRad ChemiDoc XRS system (BioRad, Inc., Madrid, Spain). Immunodetection of the proteins Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:5000), β-actin (1:2500) and β-tubulin (1:2500) was employed as loading controls to normalize the expression levels of the target protein. Target protein bands were analyzed using BioRad Image Lab Software and their densities were corrected with respect to those of their matching load controls. Finally, the results were normalized and expressed as the percentage of expression, with respect to the saline-treated group (100%).

2.6. Elevated plus maze

The EPM test was performed following the protocol described by (Daza-Losada et al., 2009). The apparatus consists of a central platform (5 × 5 cm) with two open arms (30 × 5 × 0.25 cm) and two enclosed arms (30 × 5 × 15 cm) elevated 45 cm above floor level. Open arms have a small edge (0.25) to provide the animals with additional grip. The floor of the apparatus was made of black Plexiglas and the walls of the enclosed arms were made of clear Plexiglas. To decrease experimental stress, mice were habituated to the room for 1 h prior to testing. Firstly, the mouse was placed on the central platform, facing an open arm, and was allowed to explore the maze for 5 min. The behavior displayed by the mice during the test was recorded with EthoVision XT 11. The apparatus was cleaned with a damp cloth between trials. The number of entries and the time spent by the animal during the test in each section of the maze (open arms, closed arms, and central platform) was tracked by an automated tracking system (EthoVision XT 11). An arm was considered to have been visited when the mouse placed all four paws on it. Time and percentage of time spent in open arms (OA) were measured to characterize the anxiolytic effects after acute administration of NEPD (1 (*n* = 12), 3 (n = 12) and 10 (n = 12) mg/kg) or saline (n = 12) and on the third day of withdrawal after repeated administration of NEPD (1 (n = 12), 3 (n = 12) and 10 (n = 12) mg/kg) or saline (n = 12) (Bourin et al., 2007; Blanco-Gandía et al., 2018).

2.7. Social interaction

The SI test consists of confronting an experimental mouse with a standard opponent in a neutral cage (61 × 30.5 × 36 cm) for 10 min following an adaptation period of 1 min. Standard opponents were rendered temporarily anosmic by intranasal lavage with a 4% zinc sulphate solution one day before testing (Smoothy et al., 1986). Since anosmic mice are not able to perceive a pheromone in the urine that functions as a cue for eliciting aggressive behavior in mice with a normal sense of smell, this kind of mouse induces an attack behavior in its

opponent but does not outwardly provoke or defense itself (Brain, 1981). The behavior was video recorded, and the recordings were analyzed using a custom-developed software that estimates the time spent performing different broad functional categories of behavior, each of which is characterized by a series of different elements and postures. In this study, SE and aggressive behaviors, considered as time spent both attacking and threating (A + T), were assessed after acute administration of NEPD (1 ($n = 12$), 3 (n = 12) and 10 (n = 12) mg/kg) or saline (n = 12) and on the fifth day of withdrawal after repeated administration of NEPD (1 (n = 12), 3 (n = 12) and 10 (n = 12) mg/kg) or saline (n = 12). More details of the behaviors evaluated can be found in Rodríguez-Arias et al. (1998).

2.8. Tail suspension test

The TST performance is based on the fact that rodents, after initial movements to escape, remained immobile when placed in an inescapable stressful situation. The stressful situation performed during the TST is being hung by the tail, so animals develop hemodynamic stress (Cryan et al., 2005). The time that each mouse remains immobile can be used as a measure of behavioral depressive-like symptoms since animals previously treated with common antidepressant drugs spend more time in escape-directed behavior than vehicle-treated mice (Pollak et al., 2010). In this study, TST was used to evaluate possible alterations in the time spent immobile induced by NEPD (1 ($n = 12$), 3 (n = 12) and 10 (n = 12) mg/kg) or saline (n = 12) repeated administration in the sixth day during subsequent withdrawal. The TST was performed following the procedure described by Vaugeois et al. (1997). Firstly, mice were suspended by the tail using adhesive tape, from a hook connected to a strain gauge that video-recorded their movements for 6 min. Then, an observer blinded to treatment analyzed the total time spent immobile using a computerized method.

2.9. Spontaneous horizontal locomotor activity

To evaluate possible affectations on spontaneous motor behavior of mice induced by repeated administration of NEPD (1 (n = 12), 3 (n = 12) and 10 ($n = 11$) mg/kg) or saline (n = 12) in the fourth day of withdrawal, an open-field apparatus made of black Plexiglas was used (32 × 30 × 32 cm). The lighting in the experimental room was of 150 lx at the center of the open field. The horizontal locomotor activity was tracked for 10 min and analyzed using EthoVision XT 11 software.

2.10. Determination of corticosterone levels

Forty-eight hours after the last chronic injection of NEPD (1 (n = 12), 3 (n = 12) and 10 (n = 12) mg/kg) or saline (n = 12), blood samples were taken under basal conditions. Blood sampling was carried out by tail-nick, which consisted of gently wrapping the animals with a cloth, making a 1–1.5 mm incision at the end of the tail and then massaging the tail vein while collecting 120 μL of blood into ice-cold EDTA capillary tubes (Sarsted, Granollers, Spain) within a 2 min period. All blood samples were taken at the same time (01:00 PM). Plasma levels were measured with an ELISA kit from Enzo® Life Sciences (Catalog No. ADI-900-097) for corticosterone, following the manufacturer's instructions. The sensitivity of the tests is 0.2, which is the antibody binding affinity for the antigen.

2.11. Data analysis

Statistical analyses were performed using GraphPad Prism 8.0 and IBM SPSS Statistics v.26 softwares. Kolmogorov-Smirnov normality test with Dallal-Wilkinson-Lilliefor *P* value was performed to find out if each set of data fitted a normal distribution. The α error probability was set at 0.05 ($p < 0.05$) and the p values for each statistical comparison were categorized and expressed as $p > 0.05$ (no statistically significant difference), $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). In addition, the presence of significant outlier values was tested applying Grubbs' test, using the QuickCalcs' calculator of GraphPad Software. Particularly, biochemical assays and body weight gain data were tested using one-way ANOVA and post-hoc Dunnett's test or with a two-tailed Student's *t*-test, depending on the number of experimental groups involved in each statistical comparison. Two-way ANOVA of repeated measures was performed to analyze possible increases of rectal temperature in NEPD-treated mice with respect the saline-treated group, followed by Tukey's multiple comparisons test to reveal statistical differences not only between treated and non-treated animals but also between NEPD consecutive administrations. The accumulated time that mice spent both attacking and treating (A + T) during the performance of the SI test was considered as aggressive behavior and was analyzed using the non-parametric Kruskall-Wallis test, followed by Dunn's multiple comparisons test. Data obtained from the performance of EPM, SE, spontaneous locomotor activity and TST behavioral tests were examined by one-way ANOVA and subsequent Dunnett's post-hoc test. All results are expressed as mean ± standard error of the mean (SEM).

3. Results

3.1. Behavioral effects induced by acute dose of NEPD

3.1.1. Elevated plus maze

EPM performance revealed that NEPD acute administration, at 3 and 10 mg/kg, induced anxiolytic-like effects in mice. One-way ANOVA of the results demonstrated that the variable Treatment had an effect in time ($F_{3,44} = 5.891$; $p < 0.01$) and percentage of time ($F_{3,44} = 6.422$; $p < 0.01$) spent in OA. Post-hoc test showed that mice treated with 3 and 10 mg/kg of NEPD spent more time and percentage of time in OA than saline (Fig. 2.A and Fig. 2.B, respectively). For further information on EPM parameters after acute administration of NEPD and statistical results, see Table S1 in the supplementary material.

3.1.2. Social interaction

A single acute dose of NEPD (1, 3, or 10 mg/kg) decreased the time that mice spent exploring other animals (social exploration, SE), with respect to saline-treated animals. One-way ANOVA of the results revealed an effect of the variable Treatment on the time spent in SE ($F_{3,44} = 14.27$; $p < 0.001$). Subsequent post-hoc test showed that all tested doses were able to induce this decrease in SE (Fig. 2.A). Acute administration of NEPD did not affect aggressive behavior, since no statistically significant effects on the time spent by animals in both attacking and threating (A + T) were revealed with a Kruskal-Wallis analysis of the data ($H_4 = 5.672$; $p > 0.05$) (Fig. 2.A.).

3.2. Effects induced by repeated administration of NEPD and during the following withdrawal period

3.2.1. Hyperthermia and body weight gain

The dose of 10 mg/kg NEPD induced slight but significant hyperthermia throughout the time of the treatment phase. This increase in rectal temperature measured 1 h after the second daily injection was similar each day of the repeated exposure period. Two-way ANOVA of temperature data demonstrated an effect of the variable Treatment ($F_{1,14} = 102.8$; $p < 0.001$), although no significant effects were found neither for the variable Time ($F_{4,56} = 0.6476$; $p > 0.05$) nor for the interaction Treatment x Time ($F_{4,56} = 0.6476$; $p > 0.05$) (Fig. 3.A.1). Due to the lack of significance for the variable time and the interaction Treatment x Time we analyzed the data grouping the values from all the animals in two groups (saline and NEPD) and performed a two-tailed Student's *t*-test which revealed that the increase in body temperature in NEPD-treated mice was statistically significant with respect to saline treated ($t_{78} = 12.72$; $p < 0.001$). (Fig. 3.A.2).

Concerning body weight gain, during the treatment phase NEPD-

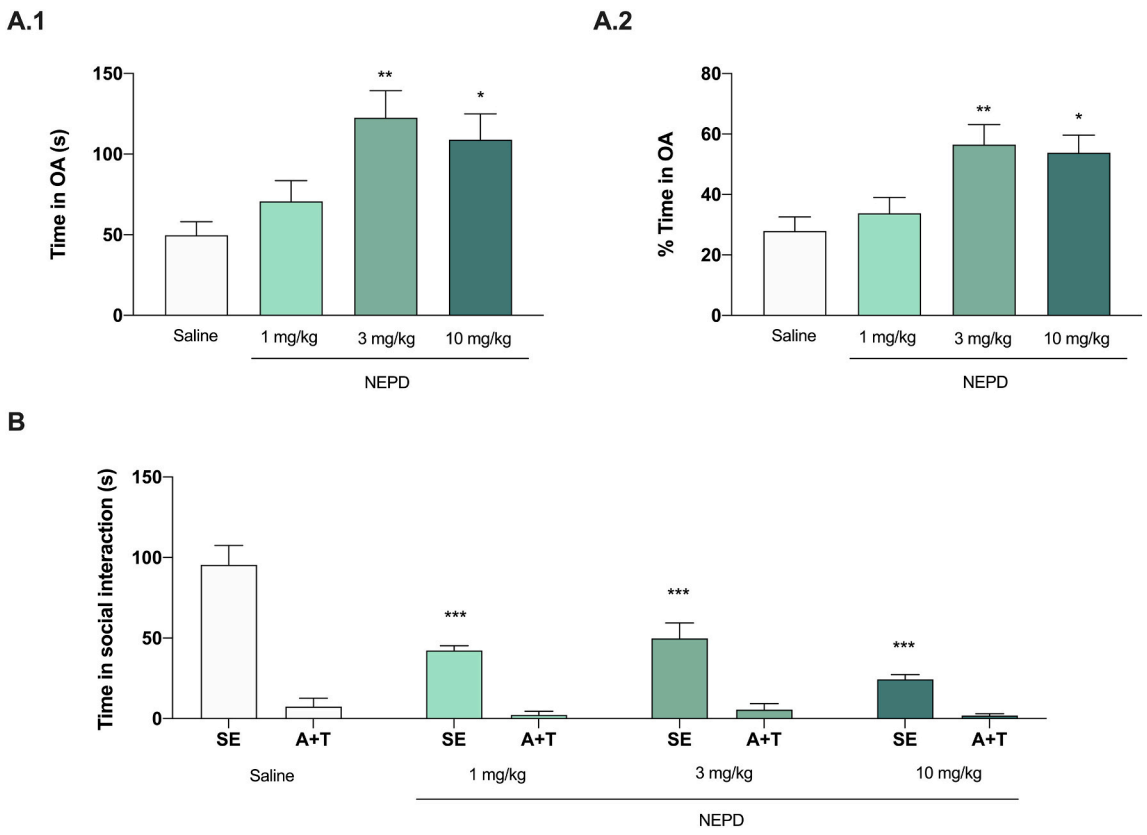

Fig. 2. Anxiolytic-like effects (panels A.1 and A.2) and alterations in SI (panel B) induced by acute administration of NEPD (1, 3, and 10 mg/kg) in mice. Anxiolytic-like effects are represented as mean ± SEM of time spent in OA (panel A) and percentage of time spent in OA (panel B) during the EPM test ($n = 12$ mice/group). SI data are expressed as mean ± SEM of accumulated time spent in SE or A + T (n = 12 mice/group). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. saline.

treated mice gained less weight than the saline group. One-way ANOVA of weight gain data revealed an effect of the variable Treatment ($F_{3,26} = 8.628$; $p < 0.001$) and post-hoc test showed that all tested doses of NEPD were able to decrease weight gain (Fig. 3.B). 72 h after treatment, body weight gain returned to saline levels in the mice treated with 3 and 10 mg/kg of NEPD. Unexpectedly, the animals treated with the lower dose of NEPD (1 mg/kg) still gained less weight than the saline-treated. The effect of the variable Treatment in body weight gain 72 h after treatment was demonstrated by one-way ANOVA ($F_{3,26} = 5.243$; $p < 0.01$). (Fig. 3. C).

3.2.2. Behavioral studies

In order to find out if repeated exposure to NEPD could induce depression or anxiety-like symptoms, as well as if it could modify SI and basal locomotor activity during the subsequent abstinence period, the following behavioral tests were performed.

3.2.2.1. Elevated plus maze. Repeated exposure to NEPD did not affect neither the time ($F_{3,44} = 0.4930$; $p > 0.05$) nor the percentage of time ($F_{3,44} = 0.9269$; $p > 0.05$) spent by mice 72 h after the treatment phase during EPM performance (Fig. 4.A.1 and Fig. 4.A.2, respectively). For further information on EPM parameters after administration of NEPD and statistical results, see Table S2 in the supplementary material.

3.2.2.2. Spontaneous horizontal locomotor activity. 4 days after treatment, the mice that received a repeated exposure to NEPD showed a significant increase in the distance travelled in comparison to the saline group. One-way ANOVA of the results revealed an effect of the variable Treatment on the distance travelled ($F_{3,43} = 11.57$; $p < 0.001$). Subsequent post-hoc test demonstrated that all the tested doses were able to increase the spontaneous horizontal locomotor activity of mice (Fig. 4. B).

3.2.2.3. Social interaction. Five days after the repeated administration phase, all NEPD-treated mice reduced SE time and increased their aggressive behaviors, although in those treated with 10 mg/kg NEPD the differences concerning aggressiveness did not reach statistical significance. Repeated exposure to NEPD significantly affected SE on the fifth day during withdrawal ($F_{3,44} = 17.15$; $p < 0.001$). Subsequent multiple comparisons test showed that all tested doses of NEPD reduced SE in comparison to saline. In addition, Kruskal-Wallis test revealed a significant effect of the variable Treatment on aggressive behaviors ($H_4 = 11.92$; $p < 0.01$), and subsequent post-hoc test demonstrated that repeated administration of 1 and 3 mg/kg of NEPD increased A + T with respect to the saline-treated group. Although a tendency to increase A + T was also observed in mice treated with the highest dose, no statistical significance was obtained, probably due to the higher variability in the individual values. (Fig. 4.A).

3.2.2.4. Tail suspension test. When the TST was performed on the sixth day during the abstinence period, no effect of NEPD on immobility time was found ($F_{3,44} = 1.562$; $p > 0.05$) for any tested dose (Fig. 5.A).

3.2.3. Costicosterone levels

On the second day during withdrawal, corticosterone levels on plasma were assessed. The effect of the variable Treatment on the corticosterone concentrations was demonstrated by one-way ANOVA analysis ($F_{3,44} = 2.821$; $p < 0.05$) and, although a tendency to increase corticosterone levels in plasma was observed in all NEPD-treated mice, subsequent multiple comparisons test only revealed statistically significant increases for those treated with 10 mg/kg with respect to the saline-treated group. (Fig. 6).

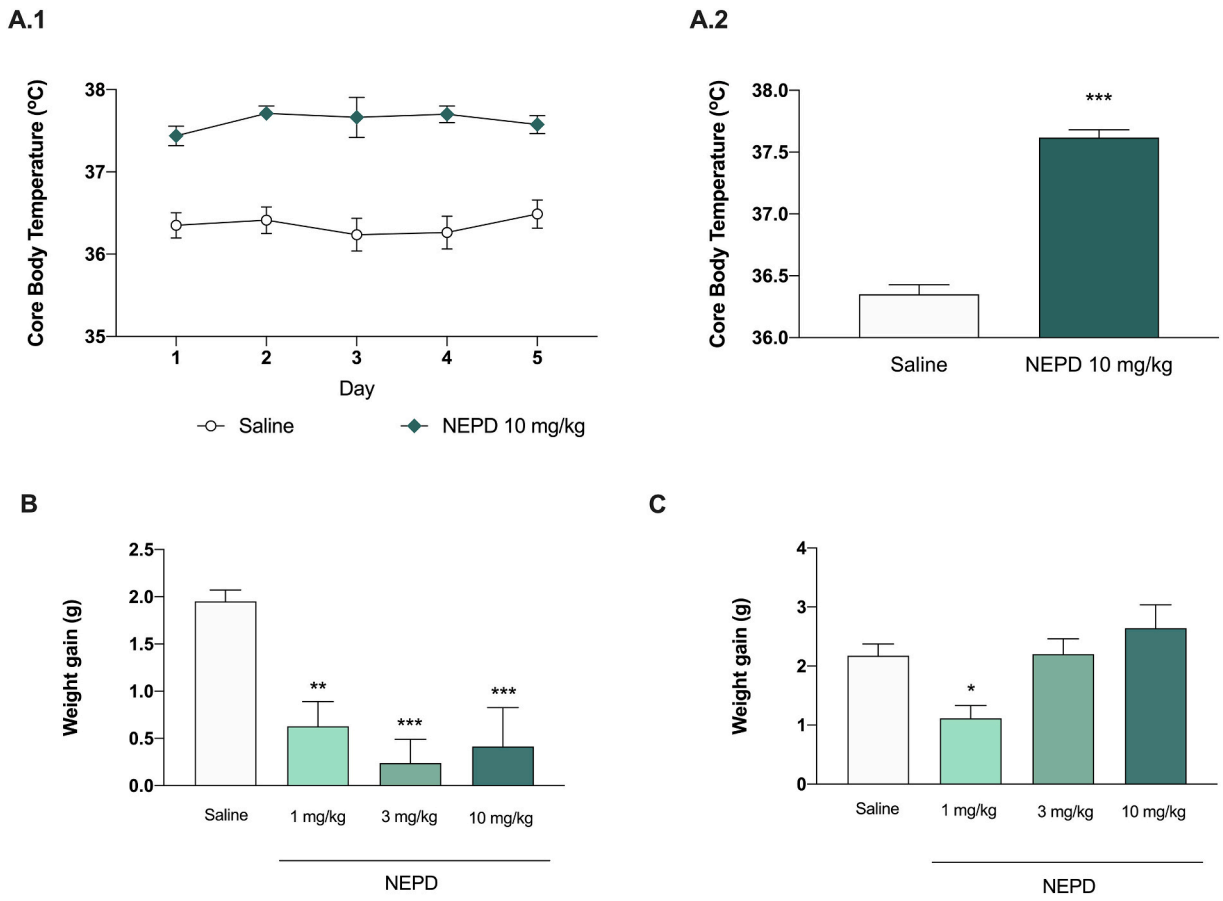

Fig. 3. Increases in body temperature and alterations in body weight gain caused by repeated administration of NEPD (1, 3, or 10 mg/kg; twice a day for 5 consecutive days) in mice. Panel A.1 depicts body temperature along the treatment days, whereas panel A.2 shows the means of the grouped temperatures as statistical analysis had shown no effect of the variable Time. Panels B and C show weight gain the last day of the treatment phase and 72 h after treatment, respectively. Hyperthermia is expressed as mean ± SEM of body temperature (n = 8 mice/group) and body weight gain data is represented as mean ± SEM of the increase in body weight (n = 7–8 mice/group). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. saline.

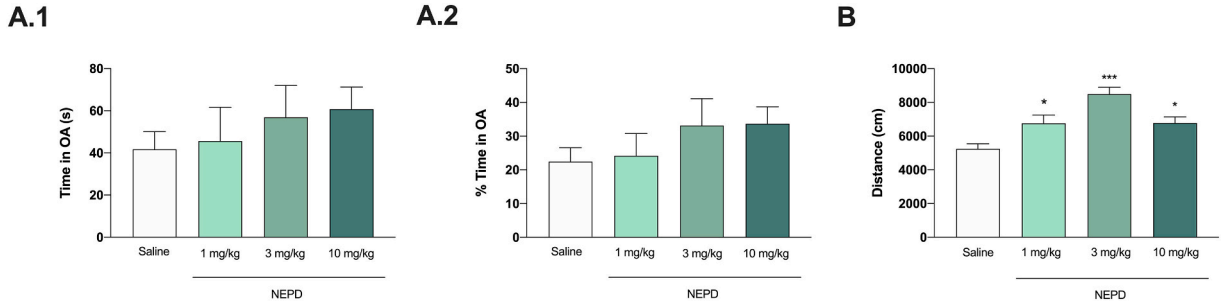

Fig. 4. Permanence in open arms of the EPM (panels A.1 and A.2) and basal horizontal locomotor activity (panel B), during withdrawal after repeated administration of NEPD (1, 3, and 10 mg/kg) in mice. Anxiolytic-like effects in the EPM are represented as mean ± SEM of time spent in OA (A.1) and percentage of time spent in OA (A.2) during the EPM test (n = 12 mice/group). Locomotor activity results are represented as mean ± SEM of travelled distance (n = 11–12 mice/group). * $p < 0.05$ and *** $p < 0.001$ vs. saline.

3.2.4. ΔFosB expression

Repeated exposure to 10 mg/kg NEPD induced a ΔFosB overexpression 72 h after the treatment phase in Str ($t_7 = 4.027$; $p < 0.01$) which remained increased 21 days after the last administration ($t_7 = 2.951$; $p < 0.05$) (Figs. 7.A and 7.B, respectively). On the other hand, this treatment did not induce alterations in ΔFosB protein expression in PFC ($t_8 = 0.5798$; $p > 0.05$) (Fig. 7.C).

3.2.5. Monoamines levels

With the aim of assessing possible alterations in monoaminergic levels induced by repeated exposure to NEPD (1, 3 and 10 mg/kg) and their relationship with the observed behavioral effects, DA, NA, and 5-HT concentrations were determined both in Str and PFC brain areas 72 h after the treatment phase. Moreover, some of their precursors and main metabolites were also analyzed. Since most notable alterations were initially expected in the 10 mg/kg-treated animals, protein expressions of its rate-limiting enzymes (TH and TPH) were determined in Str and PFC of mice exposed to this dose and saline. In addition, with the purpose to evaluate if the undergoing changes are reversible, the altered parameters were re-assessed 21 days after repeated administration with

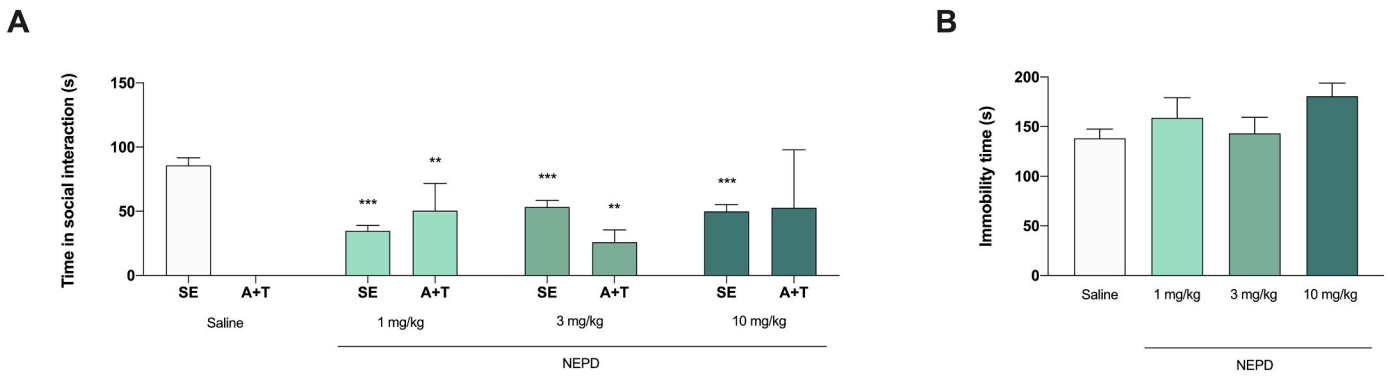

Fig. 5. Alterations in SI (A) and performance on the TST (B) during withdrawal after repeated administration of NEPD (1, 3, and 10 mg/kg; twice a day for 5 consecutive days). SI data is expressed as mean ± SEM of accumulated time spent in SE and A + T (n = 12 mice/group). TST results are expressed as the mean ± SEM of the immobility time (s) during TST performance (n = 12 mice/group), ** $p < 0.01$ and ***$p < 0.001$ vs. saline.

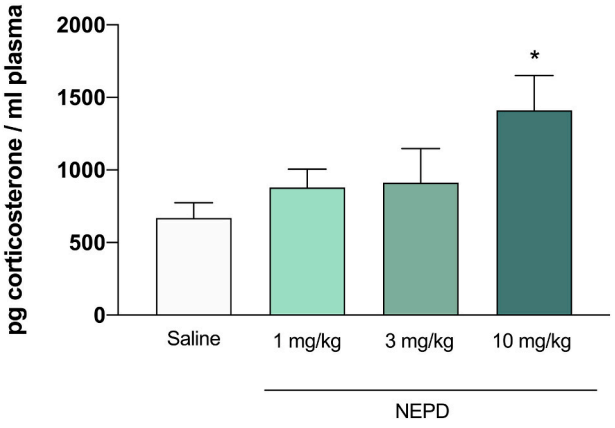

Fig. 6. Plasma corticosterone levels during withdrawal after repeated administration of NEPD (1, 3, and 10 mg/kg; twice a day for 5 consecutive days). Data are expressed as mean ± SEM of corticosterone concentrations in plasma (pg corticosterone/mL plasma) (n = 12 mice/group). * $p < 0.05$ vs. saline.

the 10 mg/kg dose.

3.2.5.1. Dopamine. Striatal DA levels were affected by repeated exposure to NEPD 72 h after treatment. Surprisingly, while no statistically significant alterations in DA content were observed after the 3 and 10 mg/kg repeated administration, a significant decrease was found in those animals previously exposed to 1 mg/kg. This decrease in DA returned to basal levels 21 days after treatment. Concerning PFC, repeated administration of NEPD did not induce a statistically significant effect on DA levels neither 72 h nor 21 days after treatment. (Table 1 and see Table S3 in supplementary material for further statistical information).

Repeated exposure to every tested dose of NEPD significantly increased both striatal and cortical Tyr concentrations with respect to saline 72 h after the last administration and had returned to basal levels 21 days after the treatment phase (Table 2 and see Table S4 in supplementary material for further statistical information). When evaluating TH protein expression 72 h after repeated administration of 10 mg/kg NEPD, no effect of the variable Treatment on TH expression was revealed neither in Str ($t_8 = 1.223$; $p > 0.05$) nor in PFC ($t_8 = 0.05944$; $p > 0.05$), so its levels were unaffected by treatment (Fig. S1 of supplemental material).

In addition, levels of some of the main metabolites of DA were assessed. Regarding Str, 1 and 3 mg/kg NEPD induced a decrease in DOPAC and 3-MT concentrations with respect to saline 72 h after the treatment phase, while 3 and 10 mg/kg NEPD increased HVA levels. Concerning PFC, only the 1 mg/kg dose was able to increase 3-MT levels while HVA concentrations were found increased in those animals previously treated with 3 and 10 mg/kg. All these alterations returned to basal concentrations 21 days after treatment. (Table 2 and see Table S4

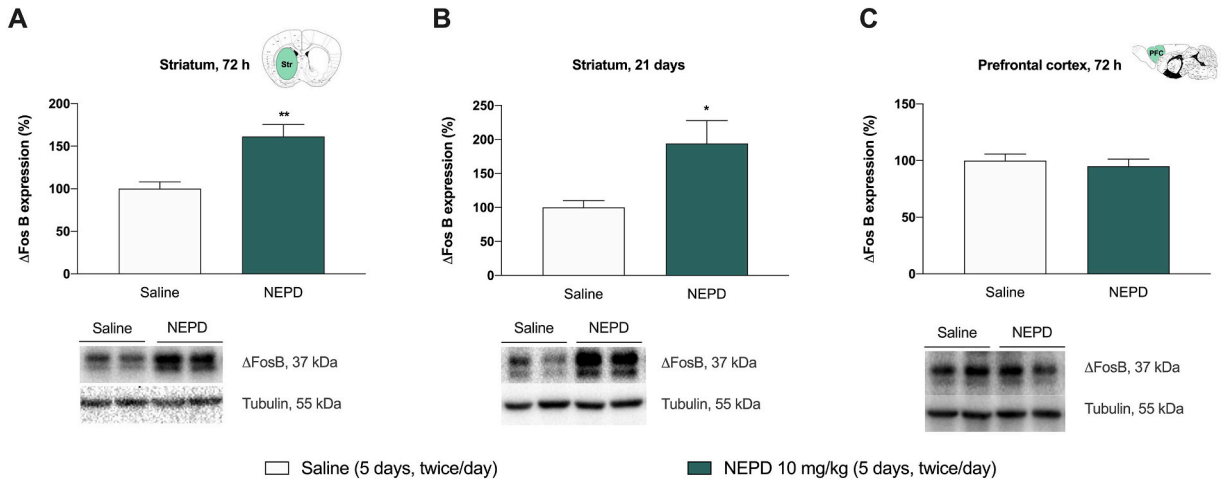

Fig. 7. Expression of ΔFosB after repeated administration of NEPD (10 mg/kg; twice a day during 5 consecutive days) in Str and PFC 72 h and 21 days after the repeated treatment phase. ΔFosB expression data is expressed as mean ± SEM of protein expression percentage vs saline ($n = 4$–5 mice/group). * $p < 0.05$ and ** $p < 0.01$ vs. corresponding saline.

Table 1
Monoamine neurotransmitters assessed 72 h or 21 days after a repeated administration with NEPD (1, 3 or 10 mg/kg; twice a day during 5 consecutive days) in Str and PFC. Data are expressed as means ± SEM of monoamine content (ng DA, NA or 5-HT/ mg tissue) and statistically analyzed by one-way ANOVA and Dunnett's post-hoc test (72 h) or Student's *t*-test (21 days). * $p < 0.05$, *** $p < 0.001$ vs. saline group ($n = 7$–8 mice/group).

	Striatum			Prefrontal cortex		
	DA	NA	5-HT	DA	NA	5-HT
	72 h					
Saline	23.79 ± 0.85	0.19 ± 0.01	0.76 ± 0.07	0.17 ± 0.08	0.67 ± 0.05	0.66 ± 0.05
1 mg/kg	↓10.11 ± 1.08***	↑0.29 ± 0.03*	0.75 ± 0.04	0.21 ± 0.05	0.66 ± 0.02	0.82 ± 0.08
3 mg/kg	19.89 ± 0.54	0.23 ± 0.02	0.76 ± 0.04	0.15 ± 0.03	0.63 ± 0.03	0.65 ± 0.04
10 mg/kg	22.49 ± 1.30	0.16 ± 0.02	0.77 ± 0.03	0.20 ± 0.03	0.56 ± 0.01	0.69 ± 0.04
	21 days					
Saline	16.91 ± 2.73	0.24 ± 0.04	0.51 ± 0.05	0.16 ± 0.05	0.75 ± 0.10	0.43 ± 0.04
10 mg/kg	16.51 ± 1.53	0.19 ± 0.03	0.50 ± 0.04	0.14 ± 0.05	0.66 ± 0.05	0.48 ± 0.04

in supplementary material for statistical information).

3.2.5.2. Noradrenaline. Repeated exposure to NEPD significantly affected NA concentrations in Str 72 h after the treatment phase. Remarkably, only those animals previously treated with the 1 mg/kg dose of NEPD showed an increase in their NA levels with respect to saline. Altered NA concentrations in Str returned to basal levels 21 days after treatment. Regarding PFC, no significant differences were evidenced in NA concentrations (Table 1 and see Table S3 in supplementary material for statistical information).

3.2.5.3. Serotonin. No statistically significant effects on 5-HT levels were found neither in Str nor in PFC 72 h and 21 days after repeated administration of NEPD. (Table 1 and see Table S3 in supplementary material for statistical information).

Accordingly, no alterations were found neither in its precursor Trp nor in its synthesis rate-limiting enzyme expression (TPH) (Fig. S1 in supplementary material. Str: $t_8 = 0.3610$, $p > 0.05$; PFC: $t_8 = 1.830$; $p > 0.05$). Moreover, Trp levels remained unaltered 21 days after the last injection in both brain areas. In addition, no changes in the main metabolite of 5-HT, 5-HIAA, were detected at any tested point in time or brain area. (Table 2 and see Table S4 in supplementary material for further statistical information of precursors and metabolites quantification).

4. Discussion

NEPD is a recently emerged cathinone derivative whose available information is, to date, very limited. Regardless, there are numerous reports about its human consumption and even related fatalities have occurred. Previous studies from our group reported powerful psychostimulant and rewarding effects of this drug in mice (Duart-Castells et al., 2021; Nadal-Gratacós et al., 2021), but its short- and long-term effects after repeated exposure are largely unknown. Thus, in this study we investigated the behavioral and neurochemical changes induced by acute and repeated administration of NEPD in mice.

Acute consumption of psychoactive drugs can produce pleasant or aversive effects, including either anxiolysis or increased anxiety, which might influence on their further abuse. In our experiments, NEPD increased the time in open arms in the EPM test at the doses of 3 and 10 mg/kg, suggesting an anxiolytic-like effect that subjectively may be interpreted as a positive and pleasant experience by the consumers. Similar effects were reported in rodents for other psychostimulants such as MDMA (Lin et al., 1999) and the cathinone derivatives mephedrone (Pail et al., 2015) and N-ethyl-pentylone (NEP) (Li et al., 2019). Although Lin et al. (1999) and Budzynska et al. (2015) reported an anxiogenic-like effect at lower doses of MDMA and mephedrone (10 mg/kg), respectively, this was not the case of the low dose of NEPD we tested (1 mg/kg), whose effects were not different to those observed in saline-treated mice. Moreover, we did not detect any anxiolytic-like effect of NEPD 72 h after the repeated administration, indicating that this effect only takes place when mice are under the effects of the drug and that there is no residual anxiety-related effect that may influence the behavior at this time point. This is a particular effect of NEPD, as it is very common that rodents under psychostimulant withdrawal show anxious behavior (Barr et al., 2010).

The consumption of synthetic cathinones has been related with several acute psychiatric disturbances including aggressive, violent, and bizarre behavior (James et al., 2011; Murray et al., 2012; Penders et al., 2012). For this reason, we assessed SI in mice after an acute administration of NEPD at three different doses and on withdrawal five days after the repeated administration. The acute effects of NEPD included a significant decrease in SE at the three doses tested. We recently reported similar effects for NEP (Espinosa-Velasco et al., 2021), although it impaired SE only at the highest dose tested (10 mg/kg). As mentioned before, NEP differs from NEPD by the presence of a methylenedioxy group in the aromatic ring. Such a difference indicates that certain modifications in the molecular structure of the cathinone may imply substantial changes in the behavioral effects, mostly driven by changes

Table 2
Precursors and metabolites of monoamine neurotransmitters assessed 72 h or 21 days after a repeated administration with NEPD (1, 3 or 10 mg/kg; twice a day during 5 consecutive days) in Str and PFC. Data are expressed as means ± SEM of monoamine concentrations (ng DA, NA, or 5-HT/ mg tissue) and statistically analyzed by one-way ANOVA and Dunnett's post-hoc test (72 h) or Student's *t*-test (21 days). * $p < 0.05$, *** $p < 0.001$ vs. saline group ($n = 7$–8 mice/group).

	Striatum						Prefrontal cortex					
	Tyr	DOPAC	3-MT	HVA	Trp	HIAA	Tyr	DOPAC	3-MT	HVA	Trp	HIAA
	72 h											
Saline	18.11 ± 0.60	2.71 ± 0.40	1.37 ± 0.06	16.33 ± 0.75	5.88 ± 0.33	0.50 ± 0.02	16.39 ± 0.54	0.62 ± 0.11	0.18 ± 0.03	14.29 ± 0.36	4.75 ± 0.27	0.40 ± 0.03
1 mg/kg	↑21.99 ± 0.88**	↓1.49 ± 0.11**	↓0.99 ± 0.11**	16.00 ± 2.62	5.79 ± 0.33	0.50 ± 0.09	↑20.95 ± 1.63**	0.82 ± 0.15	↑0.36 ± 0.02***	18.45 ± 1.72	5.13 ± 0.34	0.43 ± 0.04
3 mg/kg	↑20.99 ± 0.79*	↓1.81 ± 0.13*	↓1.09 ± 0.05*	↑27.76 ± 1.04***	5.58 ± 0.22	0.48 ± 0.03	↑19.88 ± 0.62*	0.44 ± 0.07	0.14 ± 0.01	↑23.56 ± 1.17***	4.72 ± 0.29	0.40 ± 0.02
10 mg/kg	↑24.05 ± 0.92***	3.37 ± 0.19	1.21 ± 0.06	↑43.38 ± 2.72***	5.55 ± 0.33	0.44 ± 0.01	↑21.91 ± 0.75**	0.72 ± 0.08	0.15 ± 0.01	↑29.15 ± 1.60***	4.90 ± 0.31	0.31 ± 0.03
	21 days											
Saline	23.85 ± 1.99	1.24 ± 0.13	1.21 ± 0.13	24.32 ± 1.29	7.77 ± 0.56	0.42 ± 0.04	24.32 ± 1.74	0.76 ± 0.08	0.38 ± 0.07	25.78 ± 1.68	7.97 ± 0.38	0.57 ± 0.03
10 mg/kg	24.13 ± 0.92	1.31 ± 0.15	1.28 ± 0.11	23.64 ± 1.04	7.28 ± 0.43	0.41 ± 0.02	25.41 ± 0.94	0.64 ± 0.06	0.34 ± 0.04	27.37 ± 1.13	6.98 ± 0.34	0.52 ± 0.04

in the hDAT/hSERT ratio.

SE in rodents has been related with serotonergic effects. For instance, MDMA, a drug with powerful serotonergic effects and low hDAT/hSERT ratio, increases SE in rats (Morley et al., 2005). Conversely, drugs with fewer serotonergic effects and high hDAT/hSERT ratio such as METH, acutely decrease SE in rats (Janetsian et al., 2015; Šlamberová et al., 2010). Our results with NEPD, which has a hDAT/hSERT ratio > 1000 (Nadal-Gratacós et al., 2021) agree with these previous findings.

Moreover, when SI was assessed on withdrawal 5 days after the repeated administration, SE was still reduced and an increase in aggressive behavior was detected as well. Long-lasting decreases in SE after repeated administration have also been described in cocaine-abstinent rodents (Wang et al., 2014) and recently we have reported the same effect for NEP (Espinosa-Velasco et al., 2021).

Concerning aggressive behavior induced by cathinones, our research group has reported that methylenedioxypyrovalerone (MDPV) (Duart-Castells et al., 2019) and NEP (Espinosa-Velasco et al., 2021) induce aggressive behavior in mice after acute administration. Conversely, NEPD did not acutely induce aggressive behavior, but increased aggression during withdrawal. This effect was also a very interesting and characteristic feature of NEPD, as it is not common in psychostimulants, but it is in other drugs such as opioids (Rodríguez-Arias et al., 1999; Piccin and Contarino, 2020). It also indicates that repeated administration of NEPD induces neurochemical changes that account for this increased aggressive behavior which, in turn, is not related with increased anxiety, as assessed from the EPM test results. In fact, it is accepted that aggressive behavior is the product of a complex multifactorial process, which involves the integration of the animal's environmental factors, motivational and physical states (Takahashi et al., 2012). Additionally, as SE decreases both in acute administration and after withdrawal from repeated NEPD exposure, it can be concluded that in this case the increase in aggressiveness does not run in parallel with the reduction in SE. Moreover, this aggression was clearly offensive, in the absence of any provocation by the opponent mouse. Both effects, the reduction in SE and the increase in aggressiveness during withdrawal, make it possible to foresee a deterioration of social behavior in humans who are habitual users of this substance.

Amphetamine derivatives can induce increases in core body temperature which, reaching a certain extent, can become a life-threatening side effect (Da Silva et al., 2014; Prosser and Nelson, 2012). As this effect has also been reported for cathinones, we monitored the changes in body temperature during NEPD chronic exposure at the dose of 10 mg/kg. NEPD induced significant increases in body temperature between 1 and 1.5 °C above the controls, which were constant along the days of repeated administrations. These increases did not apparently affect the welfare of the mice and cannot be considered a high hyperthermia, which might have induced lethality. However, a warning about the thermal dysregulation capability of this drug must be considered as it could be exacerbated by other factors as drug polyconsumption, high ambient temperature or overcrowding, as occurs with MDMA.

Cathinones, as well as amphetamines, produce anorectic effects and, in fact, some of them were synthesized as candidate drugs to treat obesity (Lafon, 1964; Wellman, 2012). The weight loss induced by some psychostimulants is largely due to reduced food intake (Samanin and Garattini, 1993) which is associated with the dopaminergic and serotoninergic mechanism involved in NPY/CART-mediated hypothalamic control of appetite (Chu et al., 2018). For this reason, we tested the effect of NEPD on weight gain during the repeated exposure and after three days of withdrawal. Accordingly, NEPD induced a significant decrease in weight gain during the chronic treatment at the three tested doses. These effects had reverted after three days of withdrawal, except, surprisingly, for the dose of 1 mg/kg, which showed a slower weight recovery.

Moreover, during withdrawal after the repeated exposure, the mice were also subjected to the TST, a behavioral paradigm useful for assessing depression-like symptoms. The results revealed that the abstinence after repeated administration of NEPD did not modify the immobility time, suggesting that this does not induce a depressive-like state in mice. In fact, depressive status has been related with decreases of 5-HT in certain brain areas (Rajkowska et al., 2017). Accordingly, MDMA and mephedrone, two psychostimulants which induce serotonergic deficits after repeated dosing, can induce depressive-like behaviors in rodents (McGregor et al., 2003; Martínez-Clemente et al., 2014). By contrast, in this study NEPD did not modify 5-HT levels at any of the doses and in any of the areas tested, which is consistent with its lack of depressive effects.

The mice treated with repeated doses of NEPD showed increased basal locomotor activity 4 days after treatment. Although neither anxiety nor depression were revealed by the EPM and the TST, this increased locomotion might be the manifestation of a sort of abstinence syndrome. The increased corticosterone levels, which occurs during withdrawal of several drugs and was statistically significant at the dose of 10 mg/kg is in line with this suggestion (Blanco-Gandía et al., 2018). Also, it has been suggested that locomotor enhancement may be related to aggressive behavior, which agrees with our findings (De-Giorgio et al., 2019). These two signs, together with the decreased SE, may suggest the occurrence of a psychosis-like behavior after repeated exposure to NEPD. In fact, studies with experimental rodent models of schizophrenia report increased basal locomotion and reduced social exploration and relate these behaviors with positive and negative symptoms, respectively (Karl et al., 2007; O'Tuathaigh et al., 2014; van den Buuse et al., 2009).

We determined the levels of monoamines and their main metabolites and precursors, aiming to relate their changes with the behavioral effects that we observed. After 72 h of withdrawal, very few of the differences resulted statistically significant in the brain areas we analyzed, although some tendencies were observed, making it difficult to draw robust conclusions from these results.

Nevertheless, in NEPD-treated animals, a significant increase in tyrosine (Tyr) was always present in Str and PFC. Tyr is the common substrate for catecholamines synthesis, and it might have been raised to increase DA and NA synthesis as a response to the effects of NEPD. However, these increases of Tyr were not dose-dependent and only NA content was significantly increased in Str at the dose of 1 mg/kg, accompanied by a decrease in DA. At the other two doses, a non-significant tendency to decrease in DA was observed in Str, without changes in NA, whereas in PFC a non-significant increase in DA was assessed. On the other hand, no changes in TH were observed in any case. These results, therefore, suggest that the increase in Tyr is a non-specific effect rather than a homeostatic response to the drug. In fact, NEPD may disrupt the blood–brain barrier such that the normally regulated transport of Tyr from the brain to the periphery is impaired as it was proposed to explain the increased Tyr after MDMA administration (Bankson et al., 2005). Another possibility might be the stimulation of peripheral β-adrenergic because of NEPD effects, and the subsequent increase in large neutral amino acids transport into the brain (Eriksson and Carlsson, 1988; Takao et al., 1992) which could increase brain Tyr. However, such effects remain to be explored.

Special attention must be paid to the results obtained from the mice treated with 1 mg/kg NEPD. This was the only group that showed statistically significant decrease in DA and increase in NA in Str, and it was also the only group that did not recover body weight after the repeated drug exposure. With the present results it is difficult to find an explanation to such differences and additional studies should be performed, but this suggests that the NEPD doses higher than 1 mg/kg may exert additional effects that to a certain extent tend to compensate these alterations. This might be related with increased probability to affect 5-HT at higher doses (Nadal-Gratacós et al., 2021) or even to the possibility to induce transporter up-regulation as described for other cathinones such as MDPV (Lopez-Arnau et al., 2019).

All these changes in monoamine markers had returned to normal levels after 21 days of withdrawal, which suggests that repeated administration of NEPD, at least after the schedule we used, does not

cause long-term effects on the monoaminergic system at the doses tested in this study, including neurotoxicity, which is in contrast with the long-term neurotoxic effects induced by amphetamine-like (monoamine releasers) compounds, such as MDMA and METH (for reviews see Cadet et al., 2007; Carvalho et al., 2012; Halpin et al., 2014; Moratalla et al., 2017; Rudin et al., 2021).

Nowadays it is well known that synthetic cathinones have rewarding and reinforcing properties as they activate the brain reward circuitry, as many other well-known drugs of abuse do. This suggests a potential for abuse and addiction in humans (see Riley et al., 2020 for a review). Moreover, there is growing evidence for a main role of ΔFosB in animal models of drug addiction (Nestler, 2008). ΔFosB is progressively accumulated after repeated drug exposure and has been linked to cocaine-induced reward and reinforcement (Colby et al., 2003; Kelz et al., 1999; McClung et al., 2004), suggesting an essential function in the neural mechanism involved in transitioning between recreational use and abuse. In this study we assessed a robust and very persistent increase of ΔFosB in the Str of mice that had received NEPD, suggesting that this cathinone can induce strong rewarding and reinforcing effects. In fact, this assumption is backed by the powerful rewarding effect our group had already described using the conditioned place preference paradigm (Duart-Castells et al., 2021). This, together with the long-term deficits in social behavior we observed in this study after the repeated administration of NEPD, may contribute to the establishment of an addictive-like behavior (Orben et al., 2020).

Finally, all these findings encourage to carry out further studies to fully elucidate the addictive potential, the neurochemical changes in the whole brain and other effects of this novel synthetic cathinone.

5. Conclusion

Acute administration of NEPD to mice induces anxiolytic-like effects and decreases SE. Repeated exposure to this drug induces slight hyperthermia and impairs weight gain during the treatment phase while produces decreased SE and increased aggressivity during withdrawal, as well as increased basal locomotion. In humans, these effects are compatible with impaired personal relationships or higher antisocial beliefs. Moreover, the high duration of the increased ΔFosB levels suggests a high addiction potential for this drug. Overall, these results justify a warning about the risks of consumption of NEPD.

Author contributions

Conceptualization, R.L.A., E.E. and M.R.A.; methodology, R.L.A, M. R.A. D.P. and C.G.C.; formal analysis, J.C., D.P. and M.R.A.; investigation, M.E.V., M.D.R., N.N.G., X.B. and M.B.; writing—original draft preparation, D.P., M.E.V., R.L.A.; writing—review and editing, R.L.A., E. E., M.R.A. and M.D.R; project administration, R.L.A., E.E. and M.R.A.; funding acquisition, R.L.A., E.E. and D.P. All authors critically reviewed the content and approved the final version for publication.

Disclosure statement

The authors have no conflicts of interest to disclose.

Ethical statement

We solemnly state that all authors have no conflicts of interest to disclose. This work has not been published previously, and it is not under consideration for publication elsewhere, and its publication is approved by all authors. If accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright-holder. The submission has been received explicitly from all co-authors. And authors whose names appear on the submission have contributed sufficiently to the scientific work and therefore share collective responsibility for the results.

The experimental protocols concerning the use of animals in this study were performed following the ARRIVE guidelines (du Sert et al., 2020), complied with the European Community Council guidelines (2010/63/EU), as amended by Regulation (EU) 2019/1010, and were approved by the animal Ethics Committees of the Universities of Barcelona and Valencia under supervision by the Autonomous Government of Catalonia (2018/9738). Efforts were made to reduce the number of animals used and suffering.

Role of the funding source

This study was supported by grants from Ministerio de Economía y Competitividad (grant number SAF2016-75347-R), Ministerio de Ciencia e Innovación (PID2019-109390RB-I00) and Plan Nacional sobre Drogas (2020I051). The funding sources did not have any other role in this study than financial support. DP, JC, RLA and EE belong to 2017SGR979 from Generalitat de Catalunya.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pnpbp.2022.110562.

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