Received: 23 September 2021 | Revised: 4 November 2021 | Accepted: 16 November 2021

DOI: 10.1111/jnc.15542

Journal of Neurochemistry JNC The Official Journal of the International Society for Neurochemistry WILEY

ORIGINAL ARTICLE

Behavioural and neurochemical effects after repeated administration of N-ethylpentylone (ephylone) in mice

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Funding information
Ministerio de Economía y Competitividad, Grant/Award Number: SAF2016-75347-R; Plan Nacional sobre Drogas, Grant/Award Number: 2020I051; Ministerio de Ciencia e Innovación, Grant/Award Number:

Abstract

N-ethyl-pentylone (NEP), also known as 'ephylone' and N-ethylnorpentylone, has been identified as one of the most recent novel psychostimulants to emerge into the illicit drug market and it has been associated with some intoxications and even fatalities. However, little is known about the consequences of its repeated consumption as well as the role of the monoaminergic system in such consequences. Thus, the aim of our study was to investigate the neurochemical profile and the behavioural effects after both acute and repeated NEP exposure. Male OF1 mice were acutely (1, 3, 10 mg/kg, i.p.) or repeatedly (1, 3, 10 mg/kg, i.p., 5 days, twice/day) exposed to NEP, and anxiety-like behaviour, aggressiveness, social interaction, depressive-like symptoms, body temperature, changes in monoaminergic enzymes and neurotransmitters levels as well as ΔFosB in striatum and prefrontal cortex (PFC) from post-mortem tissue were analysed short after drug-exposure or during drug-withdrawal. Acute administration of NEP induced anxiolytic effects but also an aggressive behaviour and social exploration deficits in mice, which persist during NEP-withdrawal. Moreover, NEP induced hyperthermia as well as depressive-like symptoms after repeated administrations that may be related to the decrease in serotonin and noradrenaline levels observed in striatum and PFC. Finally, the long-term increase in ΔFosB levels in striatum after NEP chronic exposure points to a high risk of dependence. Altogether indicates that NEP consumption induces different neurological and neuropsychiatric

Abbreviations: ^{1}H NMR, proton nuclear magnetic resonance; 3-MT, 3-methoxytyramine; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hidroxytryptamine, a.k.a. serotonin; 5-HT$_{2A}$, serotonin 2A receptors; ^{13}C NMR, carbon nuclear magnetic resonance; A+T, time spent attacking +threating; ACN, acetonitrile; ANOVA, analysis of variance; ARRIVE, animal research reporting in vivo experiments; BEH, ethylene bridged hybrid; CA, closed Arms; CNS, central nervous system; DA, dopamine; DAT, dopamine active transporter; DEA, drug enforcement administration; DOPAC, 3,4-dihydroxyphenylacetic acid; 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; HLA, horizontal locomotor activity; HVA, homovanillic acid; i.p., intraperitoneal; IQR, interquartile range; 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; MS, mass spectroscopy; N_2, nitrogen; NA, noradrenaline; NAcc, nucleus accumbens; NaCl, sodium chloride; NEP, N-ethyl-pentylone, a.k.a. N-ethylnorpentylone and ephylone; 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; Q1, first quartile; Q3, third quartile; RRID, research resource identifier; SDS, sodium dodecyl sulphate; SE, social exploration; SEM, standard error of the mean; 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; WB, western blotting.

PID2019-109390RB-I00; Generalitat de Catalunya, Grant/Award Number: 2017SGR979

disorders accompanied by changes in the monoaminergic system, posing a threat to public health.

KEYWORDS
aggressive behaviour, depressive-like symptoms, ephylone, monoamine levels, N-ethyl-pentylone, synthetic cathinones

1 | INTRODUCTION

From 2009 novel psychostimulants, especially synthetic cathinones, continue to emerge and account for most new psychoactive substances (NPS) reported to the United Nations Office on Drugs and Crime early warning advisory (United Nations Office on Drugs and Crime (UNODC) 2020) and European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) early warning system (European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) 2020). Among these compounds, the most consumed and abused are synthetic cathinones. These synthetic analogues mimic the psychostimulant effects of classical drugs of abuse, such as cocaine or amphetamine. They mainly act through the increase in dopamine (DA), serotonin (5-HT) and noradrenaline (NA) synaptic levels in the brain, and share subjective effects in humans such as euphoria, empathy, increased sociability, better ability to focus and learn, increased energy and alertness, while inducing some dangerous effects like hypertension, increased heart rate, decreased appetite, paranoia, hallucinations and aggressive behaviour (Tyrkko et al., 2016). Unfortunately, their use has been linked to several deaths and acute intoxications (Warrick et al., 2012; Zaami et al., 2018), for a review see (La Maida et al., 2021).

A synthetic cathinones subclass appears to be the beta-keto-methylenedioxyamphetamines (renowned with the suffix '-ylone'). Among them, we can find the 'second-generation' synthetic cathinone pentylone, which acts as a DA uptake blocker but also as a 5-HT releasing agent (Saha et al., 2019). However, when one NPS falls under legislative control, the drug market responds by producing different structurally related alternatives, through minor chemical modifications. Consequently, when pentylone was scheduled (DEA, 2014), new beta-keto-methylenedioxyamphetamines, such as N-ethyl-pentylone (NEP, also known as ephylone and N-ethylnorpentylone), broke into the illicit market. In fact, some fatalities and acute intoxications after NEP consumption have been recently reported (Blanco et al., 2021; Costa et al., 2019; Ikeji et al., 2018; Krotulski et al., 2018; Thirakul et al., 2017). In 2017 and 2018, NEP was the most commonly reported synthetic cathinone, accounting for approximately 50% of identifications (Drug Enforcement Administration (DEA) 2018). Altogether triggered the schedule of NEP by the Drug Enforcement Administration (DEA) in 2020 (Drug Enforcement Administration (DEA) 2021).

Regarding its mechanism of action, NEP is a DA, 5-HT and NA uptake inhibitor (tested using HEK-293 transfected cells and rat brain synaptosomes) and even more potent and with higher selectivity for DA transporter (DAT) than its structurally related analogue, pentylone (Costa et al., 2019; Eshleman et al., 2019; Nadal-Gratacós et al., 2021). Microdialysis studies in conscious rats also showed that acute NEP injection elicits a dose-dependent increase in DA and 5-HT levels in the nucleus accumbens (NAcc), being the effects on DA at least 10-fold greater than on 5-HT (Lin et al., 2019). Moreover, Gatch and colleagues also demonstrated that NEP is able to increase locomotor activity in rodents (ED_{50} = 0.73 ± 0.06 mg/kg, i.p.) similar to methamphetamine as well as to fully substitute for the discriminative stimulus effects of methamphetamine (ED_{50} = 1.7 ± 0.1 mg/kg, i.p.) and cocaine (ED_{50} = 2.0 ± 0.1 mg/kg, i.p.) (Gatch et al., 2019; Li et al., 2019). Regarding the pharmacokinetic and metabolism of NEP, Krotulski and collaborators identified the reduction of the beta-ketone group of NEP as the most abundant metabolite in microsomal preparations and in blood human samples from intoxication cases (Krotulski et al., 2018).

However, and although NEP use as a recreational drug and its main mechanism of action have been reported, the consequences of its repeated consumption are not, by far, fully studied and information about the consequences of NEP during withdrawal and its neurochemical long-term effects are rather limited. Thus, the aim of the present study was to investigate, in mice, the behavioural effects, such as anxiolytic-like effects, depressive-like symptoms and social interaction as well as the aggressive behaviour during withdrawal after repeated NEP exposure. The severity of the drug withdrawal is influenced by how long the exposure to the drug has been maintained. In our study, a short and intense exposure has been chosen, as an initial reference for more expanded exposure. Moreover, some of its acute behavioural effects were also studied for a more complete and better understanding.

Additionally, in order to determine the neurotoxicological consequences of the repeated exposure and the role of the monoaminergic system in the behavioural effects observed, DA, 5-HT, NA, their metabolites and precursors levels were analysed in mice striatum (Str) and prefrontal cortex (PFC) 3 and 21 days after NEP repeated exposure. In parallel, the expression of the enzymes responsible for DA and 5-HT biosynthesis, tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH), respectively, were also determined. Finally, and due to the risk of dependence that NEP may possess, the expression of ΔFosB, a transcription factor whose accumulation is related to compulsive behaviours (i.e. addiction)

(Nestler et al., 2001), was also analysed at different time points and mouse brain areas.

2 | MATERIALS AND METHODS

2.1 | Subjects

Experimental procedures and animal care were approved by the Animal Ethics Committees of the University of Valencia and Barcelona and supervised by the Autonomous Government of Catalonia (2018/9738) and complied with the Declaration of Helsinki and the European Community Council guidelines (2010/63/EU), as amended by Regulation (EU) 2019/1010. To minimize suffering and discomfort of the mice, they were regularly supervised for symptoms such as piloerection, immobility, abnormal postures and vocalizations, self-mutilation or extreme weight loss, whose limit to be considered as 'extreme' was set at >20% with respect to the initial body weight. If any of these signs was observed, the affected animal was immediately euthanized by cervical dislocation. All animal procedures were performed in accordance with the ARRIVE guidelines (McGrath & Lilley, 2015).

All experiments were performed with a total of 96 male OF1 mice (RRID:IMSR_CRL:612, Charles River), weighing 35–45 g (61–65 postnatal days) and randomly assigned to experimental groups. Animals were housed in groups of four to six in plastic cages (25 × 25 × 14.5 cm) with free access to drinking water and standard laboratory diet, in temperature-controlled conditions (22 ± 1°C) and under a 12 h light/dark–light cycle (reversed light schedule for behavioural experiments: darkness schedule from 8 am to 8 pm).

2.2 | Drugs and reagents

NEP was synthetized as racemic form of hydrochloride salt following the procedure reported by Nadal-Gratacós et al. (2021). Chemical purity and identification of the obtained compound were also assessed by thin layer chromatography (TLC), proton and carbon nuclear magnetic resonance (^{1}H NMR, ^{13}C NMR), infrared spectroscopy (IR) and mass spectrometry (MS). NEP solutions for intraperitoneal (i.p.) administration were freshly prepared in physiologic serum (saline, 0.9% NaCl, pH 7.4). Crystalline solid standards of DA hydrochloride (cat. no. H8502), DOPAC (cat. no. 11569), HVA (cat. no. 69673), 5-HT (cat. no. 14927), Tryp (cat. no. 1700501) and Tyr (cat. no. 1705006) were provided by Sigma Aldrich. Otherwise, 3-MT (cat. no. 75024) was obtained from Merck, NA (cat. no. 5169/50) was purchased from Tocris Bioscience and 5-HIAA (H953605) was supplied by Toronto Research Chemicals (TRC). Isotopically labelled standards, such as DA-1,1,2,2-d^{4} (cat. no. D533782), DOPAC-d^{5} (cat. no. D454253), 5-HIAA-d^{5} (cat. no. H953609), 5-HT-d^{4} (cat. no. S274982), 3-MT-d^{4} (cat. no. M262412), NA-d^{6} (cat. no. N674502), Trp-1-^{13}C (cat. no. T947513) and L-Tyr-$^{13}C_{9}$,^{15}N (cat. no. T899976) were all purchased from Toronto Research Chemicals (TRC).

2.3 | Treatment phases and experimental design

Behavioural tests and biochemical, temperature and body weight gain assays were performed with two independent batches of animals (n = 48 mice/batch).

Two primary endpoints were investigated. The first one is the effect induced by NEP on social interaction, anxiolysis, locomotion activity and depression in mice; and the second one is the appearance of displeasing effects after a repeated administration of NEP such as weight loss, hyperthermia and alterations in neurotransmitters levels and other addiction-related biochemical parameters. The present study was not pre-registered. Except statistical outliers determined by Grubb's test, no exclusionary criteria were utilized. Animals were randomly assigned to the different treatment groups by a different person than the experimenter. First, the subjects were sorted on their body weight and after, the treatment to be administered (saline, or the 3 doses tested in this study) was randomly assigned using the Random Allocation Software 2.0. No predetermined sample size calculation was performed, the number of subjects for behavioural, neurotransmitters and western blot experiments was based on previous studies of a similar nature (Buenrostro-Jáuregui et al., 2016; Daza-Losada et al., 2009; Duart-Castells et al., 2019; Ray et al., 2019).

Concerning behavioural studies, same mice were used for experiments after both acute and repeated administration of NEP, with one week of washout between each treatment (saline or NEP 1, 3 or 10 mg/kg, i.p.). First, elevated plus maze (EPM) and social interaction (SI) tests were performed after acute administration, one week apart. Following the washout period, mice were treated with saline or NEP (1, 3 or 10 mg/kg, i.p.), twice a day (4 h apart), for five consecutive days and, 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 (saline and NEP 1, 3 and 10 mg/Kg; n = 11–12 mice/group) (See Figure 1a). Behavioural experiments were performed during the dark phase of the light cycle.

Regarding biochemical, temperature and body weight gain assays, mice were treated following the chronic treatment schedule previously described (saline (n = 16) and NEP 1 (n = 8), 3 (n = 8) and 10 (n = 16) mg/kg). The body temperature of mice was assessed 1 h after the second daily injection using a lubricated rectal probe. In addition, animals were weighed daily and 72 h after treatment. Mice were euthanized by cervical dislocation 72 h after treatment, Str and PFC brain areas were dissected out (Paxinos & Franklin, 2004) and the monoaminergic neurotransmitters, substrates and metabolites levels were assessed. Furthermore, protein expression of neurotransmitters' synthesis rate-limiting enzymes, as well as the transcription factor ΔFosB, were assessed in both brain areas of saline and 10 mg/kg-treated mice. In addition, saline and 10 mg/

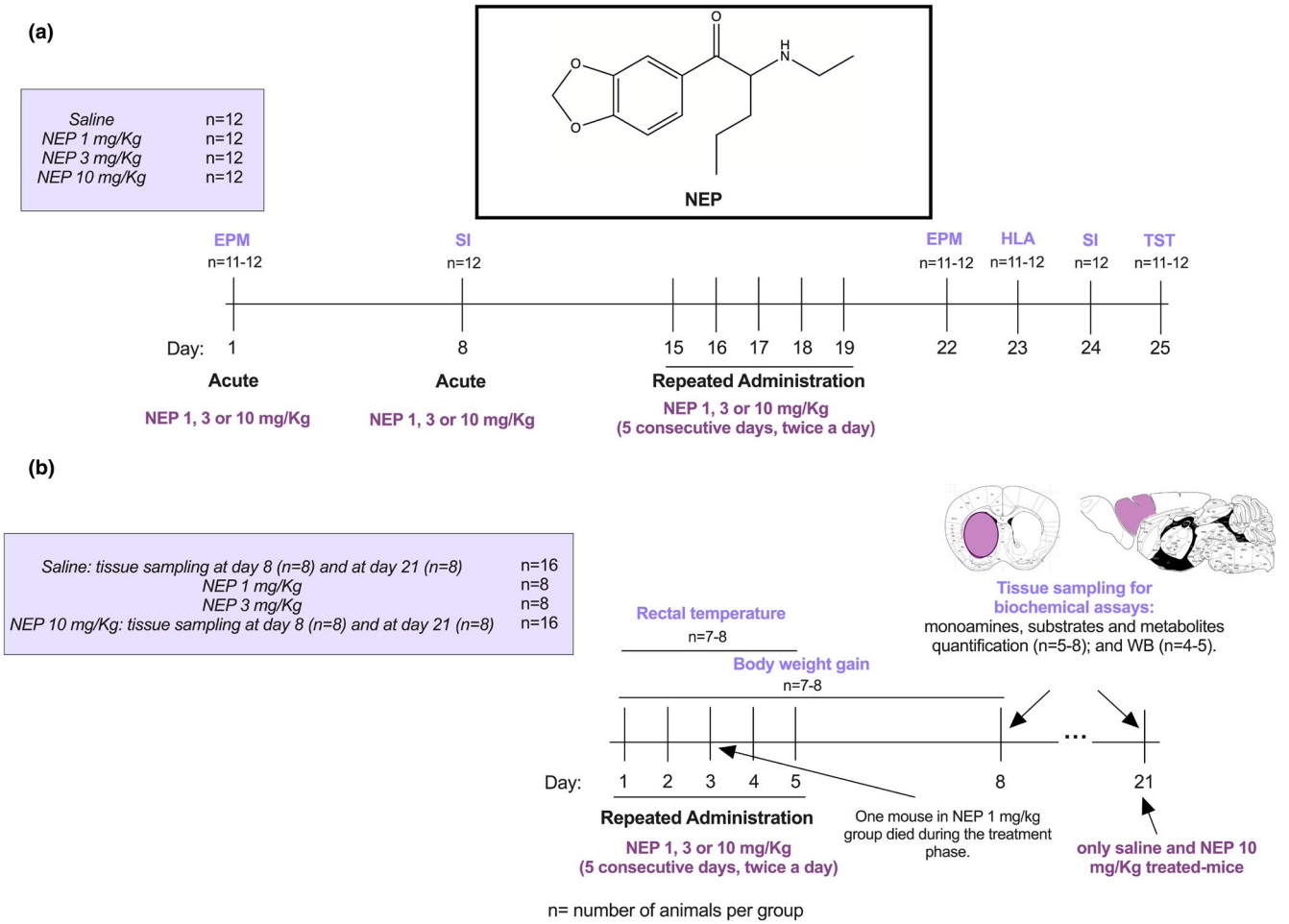

FIGURE 1 Drug administration schedule and experimental design: behavioural tests after both acute and repeated administration of NEP (a) and rectal temperature, body weight gain, tissue sampling and biochemical assays performed during the repeated-treatment phase or throughout the time of the subsequent abstinence (b). NEP structure is shown above, and tissue sampling is drawn as brain sections with Str (left, coronal plane) and PFC (right, sagittal plane) brain areas were coloured in pink

kg-treated mice were also euthanized by cervical dislocation 21 days after treatment, and neurotransmitters, substrates and metabolites levels were also evaluated in both areas, as well as the rest of the parameters that were altered 72 h after treatment (see Figure 1b). Biochemical, temperature and body weight gain assays were performed during mice light cycle.

2.4 | Social interaction

The social interaction test consisted of confronting an experimental animal with a standard opponent in a neutral cage (61 × 30.5 × 36 cm) for 10 min following a 1-min adaptation period. Standard opponents were rendered temporarily anosmic by intranasal lavage with a 4% zinc sulphate solution 1 day before testing (Smoothy et al., 1986). This kind of mouse induces an attack reaction in its opponent but does not outwardly provoke or defend itself, since it cannot perceive a pheromone that is present in the urine of the experimental animals and functions as a cue for eliciting aggressive behaviour in mice with a normal sense of smell (Brain, 1981). Videotapes were analysed using a custom-developed program that estimates the time devoted to different broad functional categories of behaviour, each of which is characterized by a series of different postures and elements. Particularly, social exploration (SE) and aggressive behaviours, considered as time spent both attacking and threating (A+T), were assessed after an acute administration of saline or NEP 1, 3 or 10 mg/Kg (n = 12/group), as well as 5 days after the last injection of the repeated administration schedule (n = 12/group). A more detailed description of the behaviours evaluated can be found in (Rodríguez-Arias et al., 1998).

2.5 | Elevated plus maze

The EPM test was carried out essentially following the procedure described by (Daza-Losada et al., 2009). The maze consisted of two open arms (30 × 5 × 0.25 cm) and two enclosed arms (30 × 5 × 15 cm), and a central platform (5 × 5 cm) elevated 45 cm above floor level. The floor of the maze was made of black Plexiglas and the walls of the enclosed arms were made of clear Plexiglas.

Open arms had a small edge (0.25 cm) to provide the animals with additional grip. In order to decrease experimental stress, animals were habituated to the room for 1 h prior to testing. At the beginning of each trial, experimental mice were placed on the central platform facing an open arm and were allowed to explore for 5 min. The maze was thoroughly cleaned with a damp cloth after each trial. The behaviour displayed by the mice during the test was recorded by an automated tracking system (EthoVision XT 11, RRiD: SCR_000441) that tracks the number of entries and time spent in each section of the maze (open arms, closed arms, central platform). An arm was considered to have been visited when the animal 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 NEP (1 ($n = 11$), 3 ($n = 11$) and 10 ($n = 12$) mg/kg) or saline ($n = 12$) and 72 h after the last chronic administration of NEP (1 ($n = 11$), 3 ($n = 12$) and 10 ($n = 10$) mg/kg) or saline ($n = 12$) (Blanco-Gandía et al., 2018; Bourin et al., 2007). In addition, the number of closed and total entries indicates motor activity.

2.6 | Spontaneous horizontal locomotor activity

An open-field apparatus (32 × 30 × 32 cm) made of black Plexiglas was used to evaluate motor behaviour, specifically spontaneous activity of mice 4 days after the last chronic administration of NEP (1 ($n = 11$), 3 ($n = 12$) and 10 ($n = 12$) mg/kg) or saline ($n = 12$). The light in the room created an illumination of 150 lx at the centre of the open field. Animal behaviour was tracked during a period of 10 min and analysed using EthoVision XT 11 software (RRiD: SCR_000441).

2.7 | Tail suspension test

The TST measures the behavioural variable of immobility, which is considered to represent despair (Pollak et al., 2010). It is based on the observation that rodents, after initial escape-oriented movements, develop an immobile posture when placed in an inescapable stressful situation. In the case of the TST, the stressful situation involves the hemodynamic stress of being hung in an uncontrollable fashion by their tail (Cryan et al., 2005). This has been used as a measure of behavioural depression as when antidepressant treatments are given prior to the test, the subjects engage in escape-directed behaviours for longer periods of time than after treatment with the vehicle (Pollak et al., 2010). Possible alterations in time spent in immobile positions in the TST induced by NEP (1 ($n = 12$), 3 ($n = 12$) and 10 ($n = 12$) mg/kg) or saline ($n = 12$) repeated administration during the abstinence period (after 6 days of the last repeated administration) were evaluated. Following the protocol described by (Vaugeois et al., 1997), mice were suspended by the tail, using adhesive tape, from a hook connected to a strain gauge that recorded their movements during a 6-min test period. The behaviour displayed by the mice was video recorded and later analysed by a 'blind' observer using a computerized method. The parameter considered for the statistical analyses was the total time spent immobile.

2.8 | Neurotransmitters, substrates and metabolites quantification

The extraction procedure of the target neurotransmitters in mouse brain was as follows: Briefly, 300 µl of a cold extractant solvent (Acetonitrile (ACN): H_2O 90:10 + 1% formic acid (FA)) was added to the samples, which were placed in Eppendorf tubes. All tested samples were spiked with Internal standard mixture (ISM). Then, three stainless steel beads (3 mm diameter) were introduced in each sample. Eppendorf tubes were placed in a bead mill (TissueLyser LT, RRiD:SCR_020428) to homogenize them, at 50 osc/min for 90 s. After, samples were centrifuged at 15 870 *g* during 20 min, at 4°C. Supernatant was filtered using 0.22 µM nylon filters (Scharlab, Barcelona, Spain; cat. no. NYL2521000) and kept in chromatographic vials at −80°C until the LC-MS/MS analysis.

LC-MS/MS analysis was based on previous reported procedures (Gómez-Canela et al., 2018; Mayol-Cabré et al., 2020). Briefly, an Acquity UPLC BEH Amide column (150 × 2.1 mm ID, 1.7 µm particle size; cat. no. 186004802) provided with an Acquity UPLC BEH Amide pre-column (5 × 2.1 mm ID, 1.7 µm particle size; cat. no. 186004799) was used to achieve neurochemicals separation, both purchased from Waters. To ensure elution, a binary mixture was employed with a gradient program. Solvent A consisted of 100 mM NH_4COOH dissolved in ultra-pure water and ACN (95:5), while solvent B contained 30 mM NH_4COOH in ultra-pure water and ACN (15:85). The pH of both solvents was acidified to pH 3, by adding FA. Regarding the gradient program, mobile phase was set at 100% B, decreased at 80% B in 4 min and held for 1 min. From 5 to 7 min, solvent B increased to 100%. Finally, initial conditions were re-equilibrated in 3 min, resulting in a total run time of 10 min. Flow rate was set at 250 µl min^{-1}, and 10 µl was injected in all cases. The column worked at 30°C, while chromatographic vials were kept at 10°C in the autosampler. Analytes were measured under electrospray ionization source in positive mode (ESI+). Nitrogen (N_2) was used as desolvation and cone gas, set at 900 L h^{-1} and 150 L h^{-1} respectively. Moreover, the desolvation temperature was set at 350°C and the source was set at 100°C. Capillary voltage of 2.0 kV was applied. The acquisition was performed in SRM mode, following the two most intense fragments of each precursor ion. The first transition was used as the quantifier ion, whereas the second was the qualifier ion. Finally, data were acquired and processed using MassLynx® Software v 4.1 (RRiD:SCR_014271). Quality assurance and parameters are provided in supplemental material.

2.9 | Western blotting

TH, TPH and ΔFosB levels were analysed using a general western blotting protocol (n = 4-5/group). Briefly, 10 µg of protein was mixed with

sample buffer (Tris-HCl 1 M, pH = 6.8; SDS 10% (w/v), glycerine 10%, 2-β-mercaptoethanol 5% (v/v), 0.05% bromophenol blue) and denatured boiling at 100°C for 5 min. Samples were electrophoresed onto a 10% acrylamide gel and transferred to polyvinilidene fluoride (PVDF) membranes (Immobilon-P; Millipore). Following the transfer, membranes were blocked with 5% defatted milk in Tris-buffer plus 0.05% (v/v) Tween-20 for 1 h at room temperature and incubated overnight at 4°C with the corresponding primary antibodies (Anti-TH 1:10000, RRID:AB_396356; Anti-TPH2 1:10000, RRID:AB_11212793; Anti-ΔFosB 1:5000, RRID:AB_303870). After a 30-min washing, membranes were incubated with the corresponding peroxidase-conjugated anti-IgG antibody for 45 min at room temperature (ECL™ Anti-Mouse IgG 1:2500, RRID:AB_794585 and Anti-Rabbit IgG 1:2000, RRID:AB_2540016). Immunoreactive protein was detected using a chemiluminescence-based detection kit (Immobilion Western, Millipore) and a BioRad ChemiDoc XRS system (BioRad, Inc.). To normalize the expression levels of the target proteins, immunodetections of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:5000, RRID:AB_2107445), β-actin (1:2500, RRID:AB_476692) and β-tubulin (1:2500, RRID:AB_477556) were used as loading controls. The bands of the target proteins were analysed 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%).

2.10 | Data analysis

Biochemical assays and body weight gain data were analysed using one-way ANOVA and post hoc Dunnett's test or with a two-tailed Student's *t*-test, accordingly. Two-way ANOVA of repeated measures was used to examine increases of body temperature respect saline, followed by Tukey's multiple comparisons test, to reveal statistical differences not only between treated and non-treated mice but also between NEP consecutive administrations. All sets of data were tested performing Grubbs' test (extreme studentized deviate method), using the QuickCalcs' calculator of GraphPad software, to identify significant outlier values. Moreover, Kolmogorov–Smirnov normality test with Dallal–Wilkinson–Lilliefor P value was applied to find out if every set of data fitted a Gaussian distribution. Data obtained from spontaneous locomotor activity, TST, SE and EPM tests were analysed by one-way ANOVA and subsequent Dunnett's post hoc test. Aggressive behaviour, considered as A+T, was examined using a non-parametric test (Kruskal–Wallis test, followed by Dunn's multiple comparisons test). 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 $p < 0.001$ (***). Body temperature values and neurotransmitters, substrates and metabolites levels are expressed as mean ± standard error of the mean (SEM). The rest of the results was expressed using box and whisker plots to visually represent the data distribution through their quartiles: the box dimension represents the interquartile range (IQR), between 25th percentile (Q1, lower extreme) and 75th percentile (Q3, upper extreme), the line represents the median value and whiskers represent the minimum (Q1-1.5*IQR) and the maximum (Q3+1.5*IQR). Statistical analyses were performed using GraphPad Prism 8.0 (RRiD:SCR_002798) and IBM SPSS Statistics v.26 (RRiD:SCR_019096) softwares.

3 | RESULTS

3.1 | Behavioural tests after a single acute dose of NEP

3.1.1 | Social interaction

Concerning SI test after an acute administration with NEP, one-way ANOVA of the results showed that the variable Treatment significantly affected SE ($F_{3,44} = 6.693$; $p < 0.001$). Subsequent post hoc test revealed that mice treated with NEP 10 mg/kg spent less time exploring other animals than those treated with saline. Moreover, Kruskal–Wallis analysis of the results also demonstrated that NEP had a significant effect on aggressive behaviours (A+T: $H_{(4)} = 16.48$; $p < 0.001$). Multiple comparisons test revealed that the highest dose of NEP increased the accumulated time that mice spent attacking and threating (Figure 2a). Furthermore, a Pearson correlation test was performed between the time spent in SE and aggressive behaviours (time spent in A+T). The analysis demonstrated an inverse correlation (Pearson's correlation coefficient = −0.331; $p < 0.05$) between both parameters.

3.1.2 | Elevated plus maze

When evaluating the results after an acute NEP administration on EPM performance, the ANOVA revealed an effect of the variable Treatment ($F_{3,42} = 4.909$; $p < 0.01$) and percentage of time spent in OA of the maze ($F_{3,42} = 5.922$; $p < 0.01$). 3 and 10 mg/kg NEP-treated mice spent more time in OA (Figure 2b, c) than saline-treated animals. For further information on EPM parameters after a single acute dose of NEP and statistical results, see Table S1.

3.2 | Effects during withdrawal after a repeated administration of NEP

3.2.1 | Hyperthermia and weight gain

To evaluate the ability of chronic treatment with NEP (5 days, twice a day: 1, 3 or 10 mg/kg) to affect weight gain, mice were weighted immediately before the first injection of the day. One-way ANOVA of the results revealed that the variable Treatment significantly affected weight gain during the treatment phase ($F_{3,27} = 36.53$; $p < 0.001$). A subsequent multiple comparisons test showed that animals treated with NEP 1 and 3 mg/kg gained less weight than saline controls. Moreover, mice injected with NEP 10 mg/kg lost weight

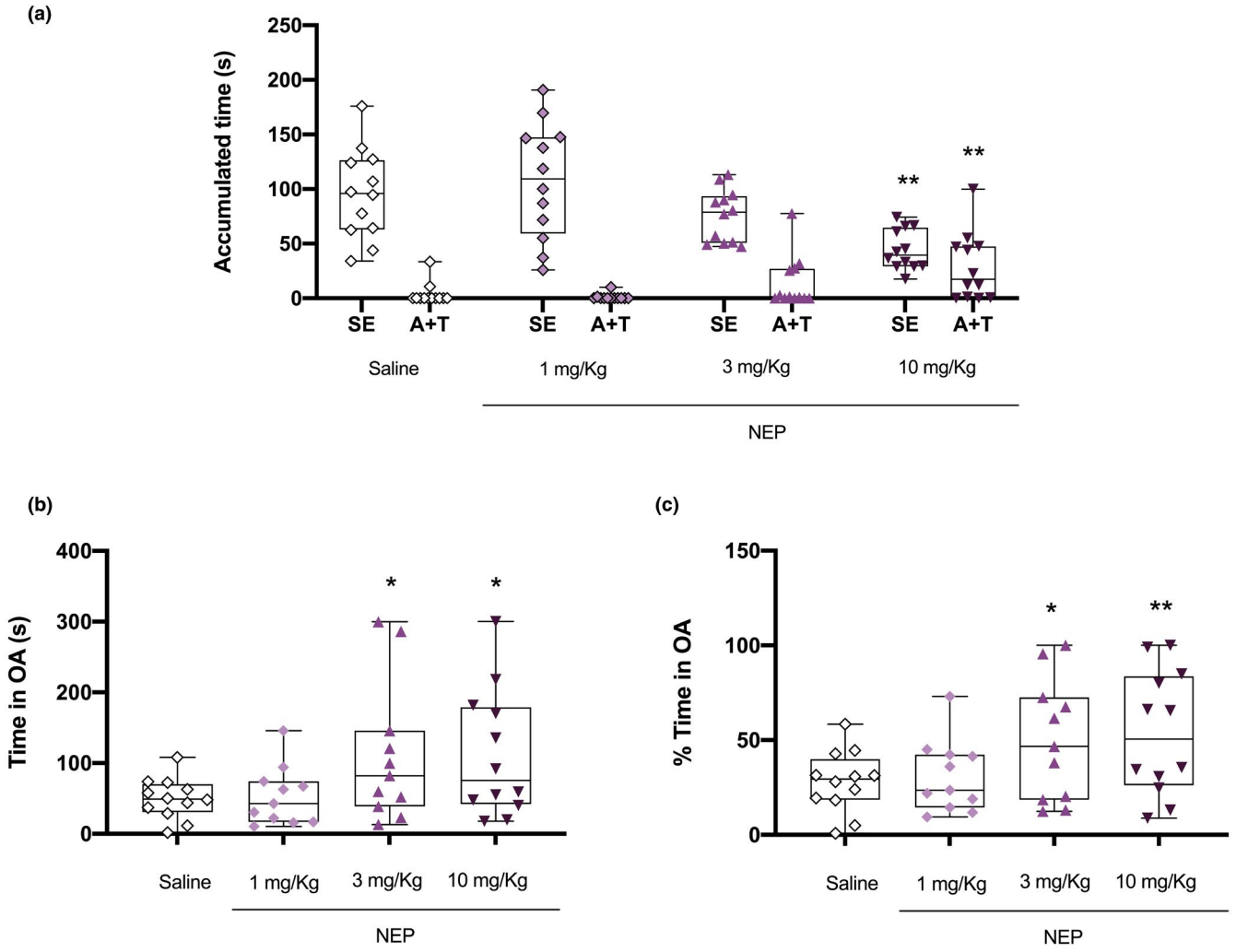

FIGURE 2 Alterations in SI (a) and anxiolytic-like effects (b and c) induced by a single acute administration of NEP (1, 3 and 10 mg/kg). SI data are represented as box and whisker plots of accumulated time spent in SE and attacking and threating (A+T) ($n = 12$ mice/group). Anxiolytic-like effect is represented as box and whisker plots of time spent in OA (b) and percentage of time spent in OA (c) during the EPM test ($n = 11$–12 mice/group). *$p < 0.05$ and **$p < 0.01$ vs. corresponding saline

during treatment (Figure 3a). Furthermore, weight gain was also assessed 72 h after the last drug injection ($F_{3,27} = 5.931$, $p < 0.01$) and post hoc test still revealed a lower weight gain in animals treated with NEP 3 and 10 mg/kg in comparison to the saline group (Figure 3b).

Concerning rectal temperature results, two-way ANOVA of repeated measures revealed that body temperature was significantly affected not only by the variables Treatment ($F_{1,14} = 66.55$, $p < 0.001$) and Time ($F_{4,56} = 2.663$, $p < 0.05$), but also a significant interaction Treatment × Time was obtained ($F_{4,56} = 2.663$, $p < 0.05$). Subsequent multiple comparisons test showed that NEP induced hyperthermia each day of the chronic treatment phase, although this increase was more pronounced on days 2, 3 and 4 (Figure 3c).

3.2.2 | Behavioural tests

The following behavioural tests were performed with the aim of finding out if repeated administration of NEP could alter SI, basal HLA, depression-like and anxiety-like related behaviours during withdrawal period.

Social interaction

Chronic treatment with NEP significantly affected SE 5 days after the chronic treatment phase ($F_{3,44} = 15.22$; $p < 0.0001$). Post hoc test demonstrated that treatments with 3 and 10 mg/kg reduced SE time in comparison to saline. Although a tendency to increase A+T was observed in all NEP-treated mice, Kruskal–Wallis test did not reveal a significant effect of the variable Treatment (Figure 4a). Moreover, and as performed for acute administration experiments, a Pearson correlation test was carried out between the time spent in SE and aggressive behaviours (time spent in A+T). The analysis also demonstrated an inverse correlation (Pearson's correlation coefficient = −0.454; $p < 0.01$) between both parameters.

Elevated plus maze

When EPM test was performed 72 h after chronic treatment, no significant effect of the variable Treatment was found ($F_{3,41} = 0.4551$;

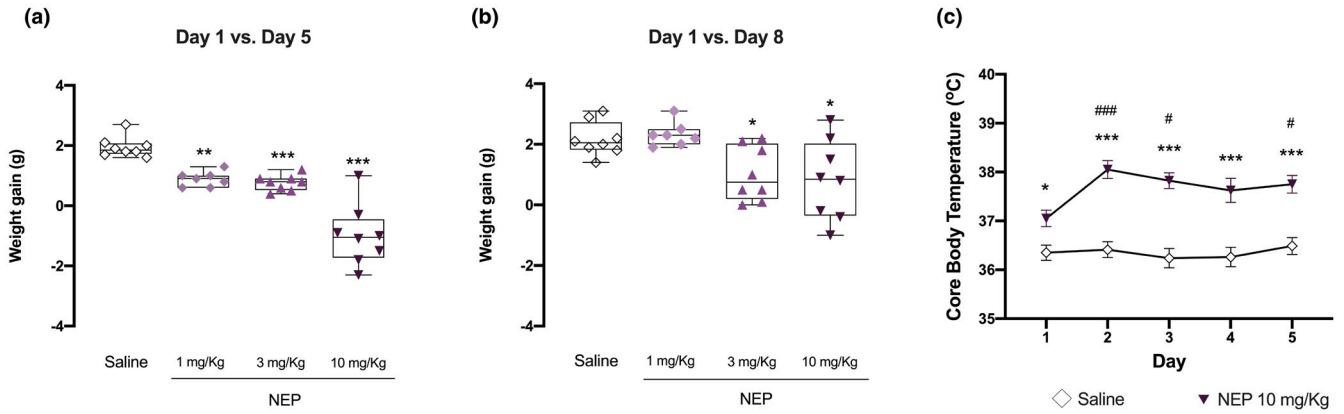

FIGURE 3 Body weight alterations and hyperthermia induced by repeated administration with N-ethyl-pentylone (1, 3 or 10 mg/kg; 5 consecutive days/ twice a day) in adolescent mice: weight gain during the treatment phase (a), weight gain 72 h after treatment (b) and increase in body temperature caused by NEP (c). Weight gain data are represented as box and whisker plots of the increase in body weight and hyperthermia is expressed as mean ± SEM of body temperature (n = 7–8 mice/group). $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$ and vs. saline. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. first day body temperature of mice treated with the 10 mg/kg dose

$p > 0.05$) and the percentage of time spent in OA ($F_{3,41} = 0.7694$; $p > 0.05$) (Figure 4b, c respectively). As a consequence of the increase in spontaneous HLA, the ANOVA showed an effect in the number of total entries of the variable Treatment ($F_{3,41} = 4.081$; $p < 0.05$), due to an enhanced effect evidenced for NEP 1, 3 and 10 mg/kg. Additionally, all NEP-treated mice increased the number of entries to OA in comparison to saline-treated animals. For further information on EPM parameters 72 h after repeated administration of NEP and statistical results, see Table S2.

Open field: spontaneous horizontal locomotor activity

One-way ANOVA of the results yielded an effect, on the distance travelled, of the variable Treatment ($F_{3,43} = 6,047$; $p < 0.01$). Particularly, animals treated with NEP at the dose of 10 mg/kg exhibited a significant increase in the distance travelled in comparison to saline group, 4 days after the chronic treatment (Figure 5a).

Tail suspension test

One-way ANOVA of the results demonstrated that the variable Treatment significantly affected immobility time during the TST ($F_{3,44} = 3.698$; $p < 0.05$). Subsequent post hoc test revealed that only the animals treated with NEP 10 mg/kg remained immobile more time than the saline-treated 6 days after the chronic treatment, although a tendency to increase immobility time was observed in all NEP-treated mice (Figure 5b).

3.2.3 | Neurotransmitters

Considering the aim of the present study, DA, NA and 5-HT concentrations were assessed both in Str and PFC 72 h after the chronic treatment to evaluate possible alterations in these monoaminergic levels induced by NEP (1, 3 and 10 mg/kg) that may explain the behavioural effects observed. In addition, their metabolites and precursors were also analysed. Since most notable changes in their levels were observed in mice treated with the highest dose, protein expressions of rate-limiting enzymes were also assessed in both brain areas (Str and PFC) of saline and 10 mg/kg-treated mice. Moreover, and with the purpose of figuring out whether the undergoing changes are reversible, the parameters that were altered at 72 h after treatment were also determined 21 days after treatment in animals treated with the 10 mg/kg dose.

Dopamine

DA concentration in Str was significantly affected by the variable Treatment. Subsequent post hoc test showed a decrease in DA levels in Str only after NEP 10 mg/kg. In contrast, one-way ANOVA of the results revealed that the variable Treatment also affected DA concentrations in PFC and, although a tendency to increase was observed in all NEP-treated groups, multiple comparisons test showed that only the animals treated with NEP 1 mg/kg and 10 mg/kg significantly increased their DA concentrations. However, DA levels, both in Str and PFC, returned to basal values 21 days after NEP-treatment at the dose of 10 mg/kg. (Table 1 and see Table S3 for statistical analysis information).

Although changes in striatal DA levels were observed after NEP treatment at the dose of 10 mg/kg, no significant differences were evidenced 72 h after treatment neither in Tyr concentrations (Table 2 and see Table S4 for statistical analysis information) nor in TH expression ($t_8 = 0.056$; $p > 0.05$).

Consistent with increased cortical DA levels, a significant increase in Tyr concentrations in PFC 72 h after NEP treatment at the dose of 10 mg/kg was observed (Table 2 and see Table S4 for statistical analysis information). Besides the increase in Tyr levels, Student's t-test also showed significantly higher levels of TH in comparison to saline-treated mice ($t_8 = 3.158$; $p < 0.05$) (Figure 6a). Both parameters, Tyr and TH ($t_8 = 0.2489$; $p > 0.05$) in PFC, were recovered 21 days after treatment (Table 2 and Figure 6b, respectively, and see Table S4 for statistical analysis information).

In addition, levels of DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT) and homovanillic acid

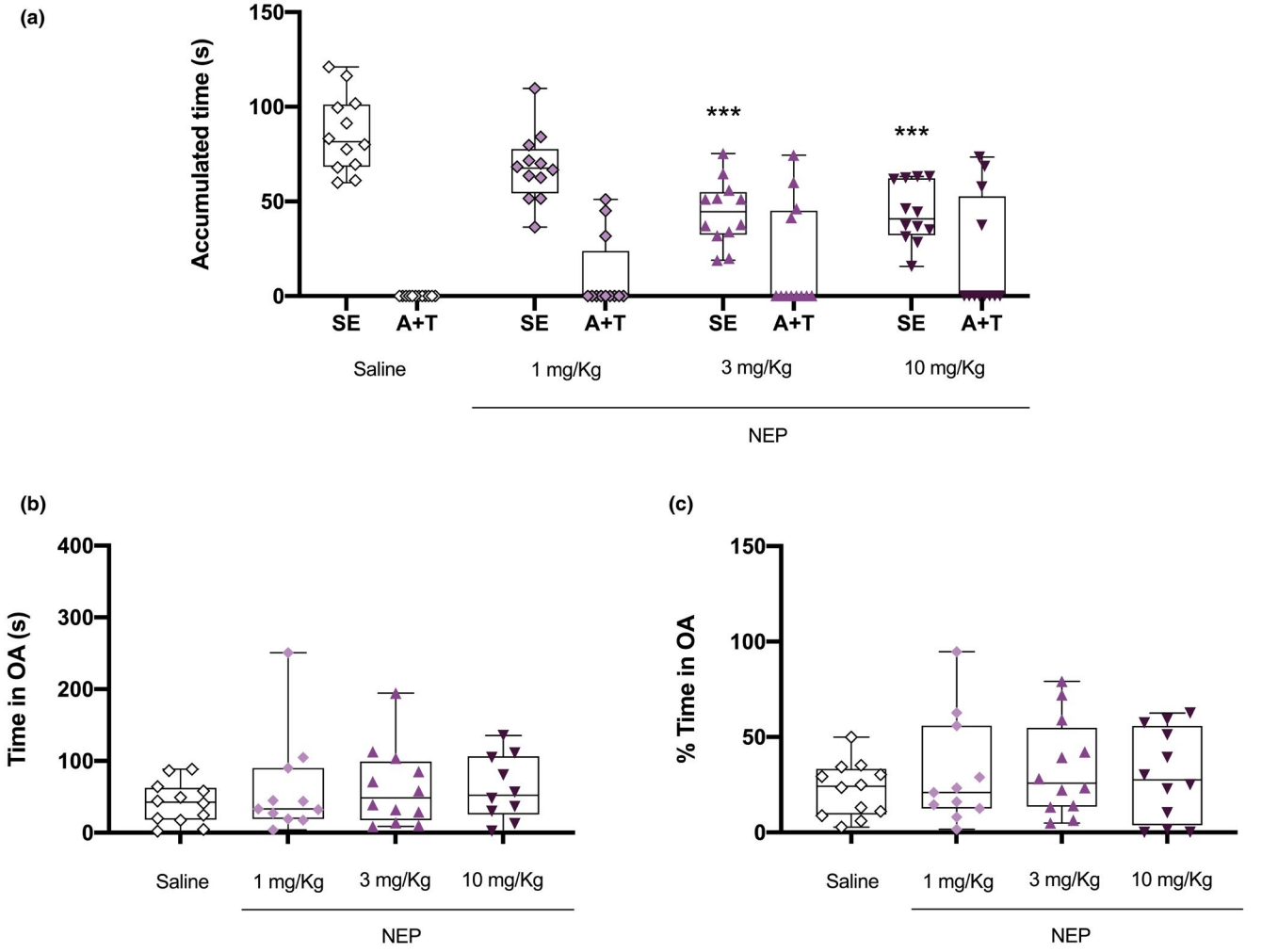

FIGURE 4 Alterations in mice SI (a) and anxiolytic-like effects (b and c) induced by a repeated administration of NEP (1, 3 and 10 mg/kg; 5 consecutive days/twice a day). SI data are represented as box and whisker plots of accumulated time spent in SE and attacking and threating (A+T) ($n = 12$ mice/group). Anxiolytic-like effect is expressed as box and whisker plots of time spent in OA (b) and percentage of time spent in OA (c) during the EPM test ($n = 11$–12 mice/group). $^{***}p < 0.001$ vs. saline

(HVA) were quantified and results are summarized in Table 2. Regarding Str and 72 h after withdrawal of chronic treatment, it is worth pointing out that NEP 10 mg/kg treatment significantly decreased levels of DOPAC but increased HVA concentrations. Concerning PFC, the same treatment also showed an increase in 3-MT and HVA levels. No alterations in none of these metabolites were found 21 days in PFC after the chronic treatment phase. (Table 2 and see Table S4 for statistical analysis information).

Noradrenaline

In line with that observed with DA, NEP chronic treatment significantly affected NA levels in Str and PFC 72 h after withdrawal. However, unlike DA, the highest dose of NEP decreased NA concentrations in both areas. NA concentrations returned to basal levels 21 days after treatment. (Table 1 and see Table S3 for statistical analysis information).

Serotonin

One-way ANOVA of the results showed that NEP significantly affected striatal and cortical concentrations of 5-HT 72 h after the chronic treatment phase. In accordance with previous results on DA and NA levels, a significant decrease in 5-HT levels was observed in both brain areas after NEP 10 mg/kg treatment. However, no changes in 5-HT levels were evidenced 21 days after treatment. (Table 1 and see Table S3 for statistical information).

Furthermore, the 5-HT synthesis rate-limiting enzyme (TPH) and its substrate Tryptophan (Tryp) concentration were also assessed. Regarding the precursor of 5-HT, Tryp, no significant differences were found in Str. However, in PFC, small but significant decrease in Tryp levels were obtained (Table 2). Moreover, no significant differences in TPH expression were found in any brain area 72h after withdrawal (Str: $t_8 = 1.283$; $p > 0.05$; PFC: $t_8 = 0.05012$; $p > 0.05$). Therefore, TPH expression was not determined 21 days after treatment.

In addition, the main metabolite of 5-HT, 5-hydroxyindoleacetic acid (HIAA), was also assessed, showing no significant differences in any of the studied areas (Table 2 and see Table S4 for statistical analysis information).

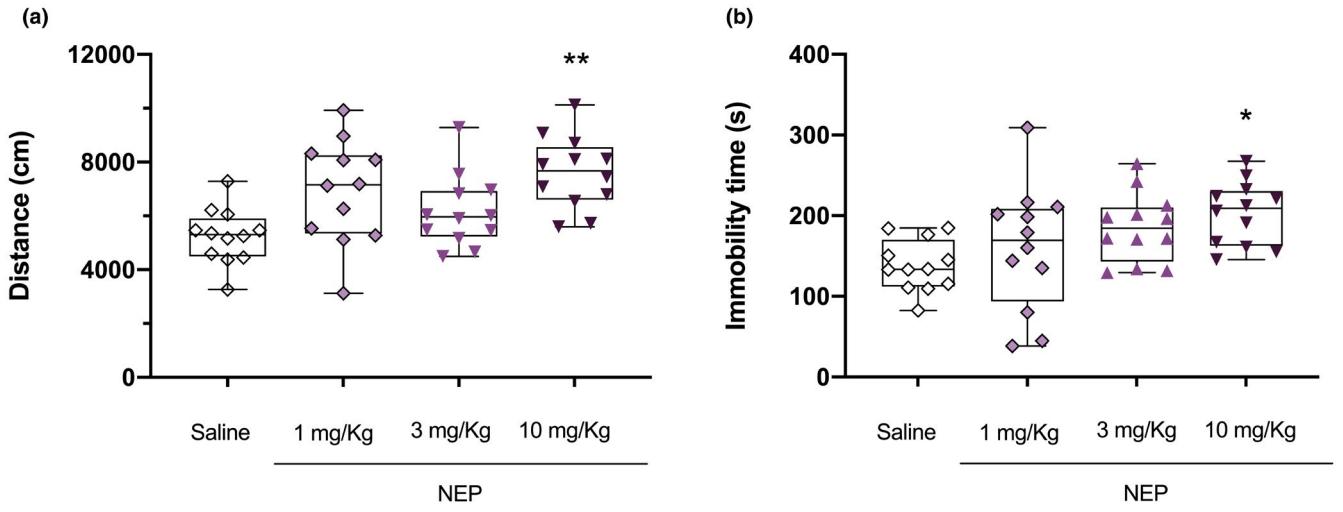

FIGURE 5 Effects on basal horizontal locomotor activity (a) and depression-like behaviour (b) induced by chronic treatment with NEP (1, 3 and 10 mg/kg; 5 consecutive days/twice a day) in adolescent mice. Locomotor activity data are represented as box and whisker plots of travelled distance and depression is represented as box and whisker plots of immobility time during TST (n = 11–12 mice/group). $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. saline

TABLE 1 Alterations in monoamine neurotransmitters after a repeated exposure to NEP (1, 3 or 10 mg/kg; 5 consecutive days/twice a day) in Str and PFC

	72 h			21 days		
	DA	NA	5-HT	DA	NA	5-HT
Str						
Saline	23.70 ± 0.74	0.21 ± 0.02	0.78 ± 0.07	18.38 ± 2.25	0.24 ± 0.04	0.51 ± 0.05
1 mg/Kg	22.01 ± 0.69	0.21 ± 0.02	0.71 ± 0.03	N.A.	N.A.	N.A.
3 mg/Kg	20.57 ± 1.00	0.25 ± 0.01	0.73 ± 0.03	N.A.	N.A.	N.A.
10 mg/Kg	↓14.91 ± 2.63***	↓0.10 ± 0.01**	↓0.51 ± 0.06**	16.61 ± 1.74	0.18 ± 0.02	0.47 ± 0.03
PFC						
Saline	0.08 ± 0.03	0.64 ± 0.15	0.63 ± 0.14	0.12 ± 0.05	0.75 ± 0.27	0.43 ± 0.11
1 mg/Kg	↑0.14 ± 0.06*	0.61 ± 0.08	0.60 ± 0.06	N.A.	N.A.	N.A.
3 mg/Kg	0.13 ± 0.03	0.64 ± 0.09	0.65 ± 0.16	N.A.	N.A.	N.A.
10 mg/Kg	↑0.51 ± 0.06***	↓0.37 ± 0.09***	↓0.42 ± 0.13*	0.08 ± 0.04	0.62 ± 0.21	0.47 ± 0.07

Data are expressed as mean ± SEM of neurotransmitter concentrations (ng DA, NA or 5-HT/ mg tissue) and statistically analysed with one-way ANOVA and Dunnett's multiple comparisons test (72 h) or Student's t-test (21 days). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. saline group (n = 5–8 mice/group).

3.2.4 | ΔFosB expression

Due to its implication in addictive disorders, protein expression of ΔFosB was determined in Str and PFC after NEP 10 mg/kg chronic treatment. NEP 10 mg/kg induced an over-expression of ΔFosB in Str at both 72 h (t_8 = 6.310; $p < 0.001$) and 21 days (t_7 = 5.881; $p < 0.001$) after the chronic treatment phase (Figure 6e, f respectively). In PFC, we also found a significant increase in ΔFosB expression 72 h after treatment (t_8 = 3.158; $p < 0.05$) but 21 days after, ΔFosB protein expression returned to basal values (t_6 = 0.3888; $p > 0.05$) (Figure 6c, d respectively).

4 | DISCUSSION

As mentioned before, NEP is a popular recreational drug that recently emerged in the illicit drug market after the schedule of other popular synthetic cathinones (i.e. mephedrone, methylone, MDPV, pentedrone, pentylone among others). The identification of NEP in fatal intoxications, its likelihood of inducing dependence as well as a lack of any currently accepted medical use have triggered to propose the placement of NEP in Schedule I of the Controlled Substances Act by the DEA in 2020 (Drug Enforcement Administration (DEA) 2021). However, little is known about the short- and long-term effects after

TABLE 2 Alterations in substrates and metabolites of monoamine neurotransmitters levels after a repeated exposure to NEP (1, 3 or 10 mg/kg; 5 consecutive days/twice a day) in Str and PFC

	72 h						21 days					
	Tyr	DOPAC	3-MT	HVA	Tryp	HIAA	Tyr	DOPAC	3-MT	HVA	Tryp	HIAA
Str												
Saline	18.20 ± 0.53	2.31 ± 0.28	1.34 ± 0.06	16.30 ± 0.65	5.77 ± 0.31	0.50 ± 0.01	23.85 ± 1.86	1.24 ± 0.13	1.20 ± 0.13	24.32 ± 1.21	7.77 ± 0.52	0.42 ± 0.03
1 mg/Kg	21.51 ± 0.81	2.29 ± 0.15	1.18 ± 0.07	17.61 ± 0.73	5.56 ± 0.26	0.44 ± 0.03	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
3 mg/Kg	19.90 ± 1.25	2.74 ± 0.15	1.14 ± 0.04	17.48 ± 1.04	6.38 ± 0.27	0.53 ± 0.03	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
10 mg/Kg	21.17 ± 2.29	↓1.17 ± 0.12**	1.10 ± 0.15	↑25.21 ± 2.09***	5.50 ± 0.71	0.43 ± 0.05	26.65 ± 1.27	↑1.92 ± 0.22*	1.50 ± 0.12	28.56 ± 1.95	6.99 ± 0.39	0.52 ± 0.06
PFC												
Saline	16.37 ± 0.47	0.62 ± 0.10	0.18 ± 0.03	14.66 ± 0.48	5.01 ± 0.35	0.44 ± 0.05	24.32 ± 1.63	0.71 ± 0.06	0.32 ± 0.05	25.78 ± 1.57	7.97 ± 0.35	0.57 ± 0.03
1 mg/Kg	19.40 ± 0.67	0.55 ± 0.08	0.16 ± 0.02	17.30 ± 0.55	4.76 ± 0.18	0.34 ± 0.02	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
3 mg/Kg	18.27 ± 1.41	0.74 ± 0.10	0.17 ± 0.01	18.13 ± 1.47	5.53 ± 0.21	0.42 ± 0.03	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
10 mg/Kg	↑24.95 ± 2.07***	0.52 ± 0.03	↑0.43 ± 0.02***	↑34.09 ± 3.79***	↑6.70 ± 0.58*	0.50 ± 0.06	26.50 ± 1.03	0.78 ± 0.10	0.35 ± 0.04	29.05 ± 1.02	↓6.62 ± 0.07**	0.53 ± 0.02

Data are expressed as mean ± SEM of concentrations (ng analyte/ mg tissue) and statistically analysed with one-way ANOVA and Dunnett's multiple comparisons test (72 h), or Student's *t*-test (21 days).
$^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. saline group (n = 5–8 mice/group) (N.A.: not assessed).

repeated exposure to this novel synthetic cathinone. Thus, the aim of this study was to investigate the neurochemical and behavioural changes induced by acute and repeated administration of NEP in mice. Our results demonstrated changes in anxiety-like behaviour, aggressiveness and SI short after a single NEP dose. Additionally, alterations of SI were also evidenced after a repeated NEP treatment, as well as anorectic and depressive symptoms and changes in monoaminergic neurotransmitters levels. Moreover, an over-expression of ΔFosB, a biomarker related to addiction points to a risk of inducing drug dependence.

The initial consumption of psychoactive drugs can provoke pleasant or aversive effects, such as anxiolytic- or anxiogenic-like effects, that might influence their further abuse. In fact, a variety of drugs of abuse has been found to be able to acutely modify anxiety-related behaviours. For example Lin et al. (1999) clearly demonstrated that MDMA was able to induce an anxiogenic-like effect at low doses and an anxiolytic-like effect at high doses (Lin et al., 1999). Similarly, Pail et al. (2015) also reported an anxiolytic-like effect after mephedrone administration at high doses (30 mg/kg) in mice (Pail et al., 2015), while others demonstrated an anxiogenic-like effect after a lower dose injection of this synthetic cathinone (10 mg/kg) (Budzynska et al., 2015). Our results showed that acute NEP administration was able to induce a dose-dependent anxiolytic effect similar to other amphetamines and cathinone derivatives, which may contribute to the effect of pleasure pursued by consumers. Moreover, our results are partially in accordance with the study recently reported by Li et al. (2019), in which they demonstrated that NEP was able to induce acute anxiolytic-like effects in rats at high doses (20 mg/kg) as well as immediately after repeated exposure (20 mg/kg, i.p. for 7 consecutive days) (Li et al., 2019). However, we did not observe changes in the anxiety-related behaviour during NEP withdrawal, suggesting a lack of long-term effects on anxiety behaviour.

The use and abuse of synthetic cathinones has also been associated with a range of acute psychiatric disturbances including aggressive, violent and bizarre behaviour (James et al., 2011; Murray et al., 2012; Penders et al., 2012). In accordance with these precedent data, in the present study, acute administration of NEP at the highest dose tested (10 mg/kg) induced an increase in both the time spent by mice attacking and threating (aggressive behaviour), revealing the violent behaviour produced under the acute effects of this novel synthetic cathinone. This feature contrasts with those reported for other psychostimulants. Chronic cocaine administration leads to a decrease in aggressive behaviour in mice at higher doses, although an increase is observed at low doses (Darmani et al., 1990). Moreover, when isolation housing is applied, acute administration of methamphetamine produces a dose-dependent inhibition of aggression in aggressive mice (Machalova et al., 2012). Therefore, induction of this aggressive behaviour may be a common effect for some synthetic cathinones since, in a previous study, we also evidenced an increased territorial aggressive behaviour shortly after MDPV repeated administration (Duart-Castells et al., 2019). It is well known that there is a high incidence of frontal lobe dysfunction in aggressive and criminal population, thereby strengthening the relationship between prefrontal

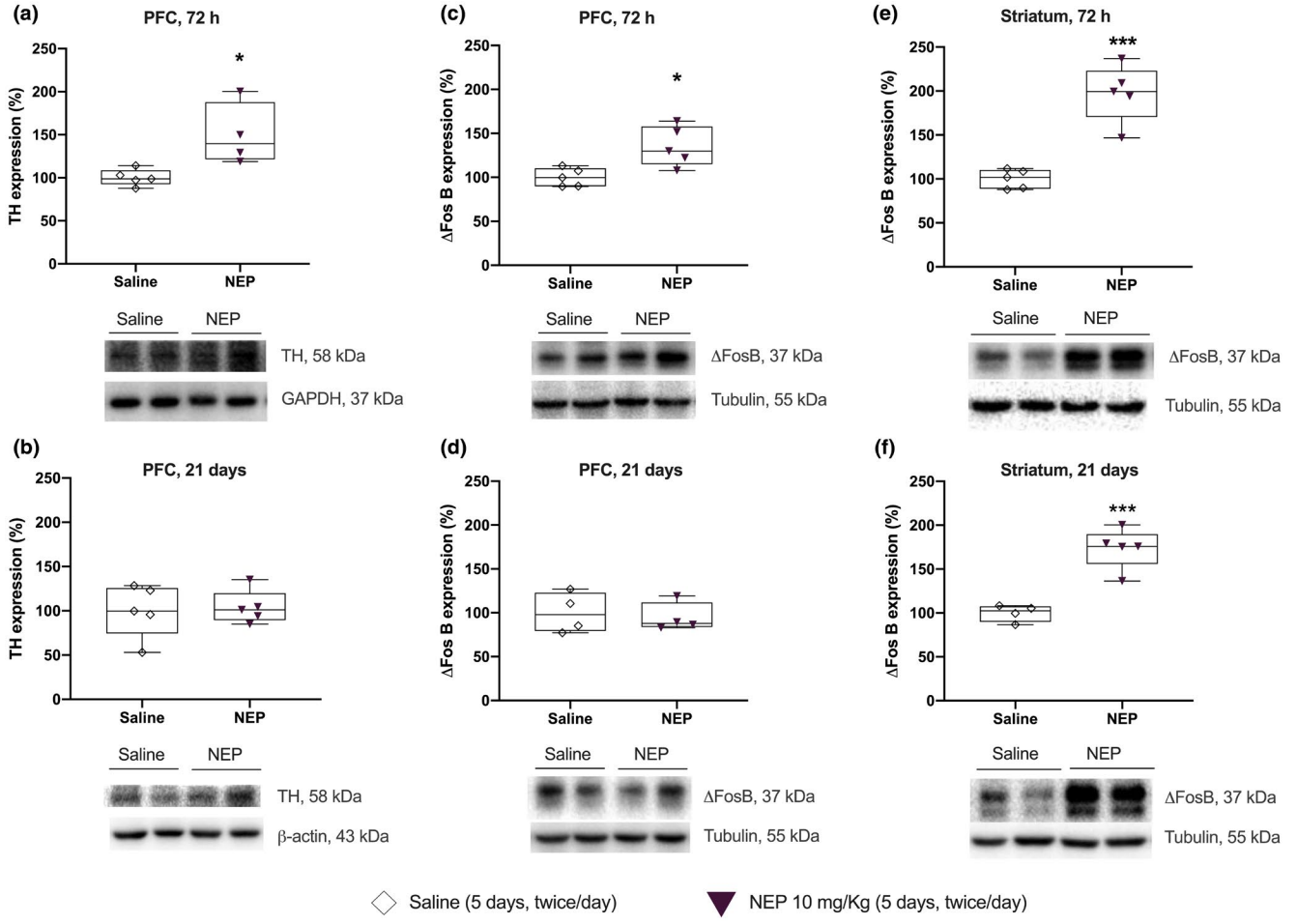

FIGURE 6 Effects on TH and ΔFosB expression induced by a chronic treatment of NEP (10 mg/kg; 5 consecutive days/twice a day) in adolescent mice Str and PFC areas, 72 h and 21 days after the treatment phase. TH and ΔFosB expression data are represented as box and plots of protein expression percentage vs. saline ($n = 4–5$ mice/group). $^{*}p < 0.05$ and $^{***}p < 0.001$ vs. saline

cortex impairment and abnormal social behaviour (Brower & Price, 2001); although these abnormalities are not inescapable for all aggressive and antisocial behaviours. Moreover, it must be pointed out that the increases in aggression observed were not uniformly distributed across the cohort of mice, suggesting that only certain vulnerable mice may be susceptible to this side effect.

Furthermore, and although psychostimulant and entactogen drugs are consumed to facilitate social relations, our study demonstrates that NEP induces a decrease in SE which was not only observed after an acute dose but also 5 days after repeated exposure. Such long-lasting effects have also been described in cocaine- (Wang et al., 2014) and heroin- (Felip et al., 2000; Piccin & Contarino, 2020) abstinent mice. Finally, after both acute and repeated exposure, our study revealed an inverse correlation between the time that the animals spent in SE and the time spent in aggressive behaviour (A+T), which means that when aggressive behaviour increases, the social non-aggressive interaction (SE) diminishes.

The rise in core body temperature is a typical and life-threatening side effect of amphetamine's abuse in humans (Prosser & Nelson, 2012; da Silva et al., 2014). Thus, we monitored the changes in body temperature during NEP chronic exposure at a dose of 10 mg/kg, using a rectal probe. Our results not only demonstrated that NEP was able to induce hyperthermia in mice during the treatment, but also that such hyperthermic response was higher from the second day of treatment in comparison to the first day of exposure, suggesting a rapid sensitization to this effect. However, in our study, the hyperthermic response induced by NEP cannot be considered as an exacerbated hyperthermia (40–41°C), which might have led to a high rate of lethality.

Some cathinone derivatives were mainly synthetized in the 60s for clinical use as antidepressants and/or anorectic agents (Lafon, 1964; Wellman, 2012). It is known that the weight loss induced by some psychostimulants is largely due to reduced food intake (Samanin & Garattini, 1993) which is associated with the dopaminergic and serotonergic mechanism involved in NPY/CART-mediated hypothalamic control of appetite (Chu et al., 2018). In accordance, NEP induced a decrease in the weight gain during chronic exposure, especially after the highest dose tested (10 mg/kg), which induced weight loss measured the last day of treatment. These effects tended to revert on withdrawal.

Moreover, during NEP withdrawal, mice were also subjected to the TST. This test is a behavioural paradigm useful for assessing

depressive-like symptoms in mice. The present study demonstrates that NEP causes an increase in the immobility time, pointing to a depressive-like symptoms induced by drug-withdrawal. Furthermore, NEP-induced depressive-like behaviour may be related to the decrease in 5-HT and NA levels observed in Str and PFC 3 days after the same NEP exposure. In fact, the serotonergic pathway is considered one of the most important pathways in depressive-like behaviours (Rajkowska et al., 2017). For example, McGregor et al. (2003) demonstrated that MDMA, a synthetic drug that can cause 5-HT depletion, was able to induce depressive-like behaviours (McGregor et al., 2003). Moreover, our research group also reported that mephedrone, a synthetic cathinone able to induce some serotonergic deficits after repeated dosing, also produces depressive-like symptoms (Martínez-Clemente et al., 2014). However, depression is more complex than just an alteration in the levels of 5-HT and, probably, also the NA depletion and the dopaminergic imbalance detected in these animals could represent a trigger stimulus leading to this mood alteration (Delgado & Moreno, 2000). Furthermore, NEP chronic exposure (10 mg/kg) also induced an increase in the basal locomotion 4 days after treatment. Therefore, the increased locomotion together with the depressive-like symptoms observed during withdrawal underline the possibility of a deprivation-like syndrome induced by repeated NEP exposure.

Monoamine neurotransmitters, their metabolites and precursors are known to play an important role in the CNS circuit. In fact, changes in monoamine levels are related to several CNS-related disorders such as drug-induced neurotoxicity (Hirata et al., 1996) and drug-dependence/withdrawal (Berridge et al., 2009). In the present study, the content of DA, 5-HT, NA, as well as their metabolites (DOPAC, 3-MT, HVA, 5-HIAA) and precursors (Tyr and Tryp) was determined by HPLC coupled to MS 3 and 21 days after NEP chronic exposure. Regarding 5-HT, and as mentioned before, chronic exposure to NEP 10 mg/kg induced a significant decrease in its levels in Str and PFC 3 days after treatment. However, no changes in 5-HT levels 21 days after treatment were found, ruling out long-term 5-HTergic deficits. Moreover, a similar pattern at the same dosing schedule was observed for NA levels, with a significant loss in Str and PFC, which turned back to normal levels 21 days after treatment.

Special mention should be made regarding the dopaminergic pathway. On the one hand, after NEP chronic exposure (10 mg/kg), no changes in TH expression were evidenced in mice, although a striatal depletion of DA and NA levels were observed. This leads us to suggest that dopaminergic terminals were not injured, which correlates with a lack of significant changes in DA and NA levels 3 weeks after treatment. On the other hand, NEP-treated mice, at the same dosing schedule, showed a significant increase in DA and Tyr levels as well as an over-expression of TH in PFC 3 days after treatment, resulting in a dopaminergic imbalance between the two brain areas studied (Str and PFC). These transient changes in monoamine markers, followed by a return to normal levels, indicate that NEP does not cause long-term neurotoxic effects on monoaminergic system at the doses employed in the present study, which is in contrast with the long-term neurotoxic effects induced by amphetamine-like (monoamine releasers) compounds, such as MDMA and methamphetamine (for a review see Cadet et al., 2007; Carvalho et al., 2012; Halpin et al., 2014; Moratalla et al., 2017). Moreover, recent studies have demonstrated that MDPV, a synthetic cathinone and a potent reuptake inhibitor, is able to increase DAT (Lopez-Arnau et al., 2019) and VMAT-2 function (Magee et al., 2020) when measured ex vivo shortly after administration and followed by drug washout. Therefore, we cannot rule out that NEP might induce a transient increase in DAT and VMAT-2 function, which may mitigate persistent dopaminergic deficits due to the aberrant DA transport (Magee et al., 2020).

In the present study, we have also analysed the monoamine metabolites DOPAC, HVA, 3-MT and 5-HIAA, yielding changes especially in the NA and DA products 3 days after NEP chronic exposure at a dose of 10 mg/kg. At this dosing schedule, a different metabolite production was observed, inducing a decrease in DOPAC together with an increase in HVA levels, suggesting an imbalance between the intraneuronal and the extraneuronal metabolism of DA. In fact, we cannot rule out that the decrease in DOPAC levels we observed might also be due to an inhibition of the monoamine oxidase (MAO) produced by NEP, as it occurs with other amphetamine-like compounds (Mantle et al., 1976; Matsumoto et al., 2014; Scorza et al., 1997).

Different research performed during this last decade indicates that synthetic cathinones have rewarding and reinforcing properties and activate brain reward circuitry, as cocaine and other classical psychostimulants do, suggesting a potential for abuse and addiction in humans, for a review see (Riley et al., 2020). Moreover, there is growing evidence for the 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 phenomenon. Therefore, it is reasonable to suggest that NEP can induce strong rewarding and reinforcing effects due to its mechanism of action and to the long-lasting increased levels observed of ΔFosB in mouse Str. Additionally, the long-term deficits in social behaviour observed in this study after repeated exposure of NEP, together with the depressive behaviour, may contribute to the establishment of an addictive-like behaviour (Orben et al., 2020). However, more studies must be carried out in order to fully elucidate the addictive potential of this novel synthetic cathinone.

5 | CONCLUSIONS

In summary, acute administration of NEP can induce anxiolytic effects but also an aggressive behaviour and SE deficits in mice. Moreover, NEP induced hyperthermia, which increased after repeated administrations and was accompanied by an anorectic effect. During withdrawal, NEP-treated mice showed deficits in SE, hyperlocomotion and depressive-like symptoms that support the

possibility of a deprivation-like syndrome. In fact, the decrease in 5-HT and NA levels found out in Str and PFC may contribute to the negative affective state associated. However, all neurotransmitter deficits observed returned to normal levels 3 weeks after exposure, ruling out long-term terminal damage. Finally, the significant long-term increase in ΔFosB levels in Str after NEP chronic exposure points to a high risk of dependence of this novel synthetic cathinone. Altogether indicates that acute and/or repeated consumption of NEP are able to induce different neurological and neuropsychiatric disorders, posing a threat to public health.

ACKNOWLEDGEMENTS

This study was supported by Ministerio de Economía y Competitividad (grant number SAF2016-75347-R), Ministerio de Ciencia e Innovación (PID2019-109390RB-I00) and Plan Nacional sobre Drogas (2020I051). JC, DP, RLA and EE belong to 2017SGR979 from Generalitat de Catalunya.

CONFLICTS OF INTEREST

All authors declare no conflicts of interest.

AUTHORS' 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., P.P., X.B. and M.B.; data curation, D.P.; writing—original draft preparation, M.E.V., E.E., R.L.A. and M.D.R.; writing—review and editing, R.L.A., E.E., M.R.A.; project administration, R.L.A., E.E., M.R.A.; funding acquisition, R.L.A., E.E. All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

The data are available from the authors upon request.

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How to cite this article: Espinosa-Velasco, M., Reguilón, M. D., Bellot, M., Nadal-Gratacós, N., Berzosa, X., Puigseslloses, P., Gómez-Canela, C., Rodríguez-Arias, M., Pubill, D., Camarasa, J., Escubedo, E., & López-Arnau, R. (2022). Behavioural and neurochemical effects after repeated administration of N-ethylpentylone (ephylone) in mice. *Journal of Neurochemistry*, 160, 218–233. https://doi.org/10.1111/jnc.15542