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RESEARCH ARTICLE

Sex differences in the effects of *N*-ethylpentylone in young CD1 mice: Insights on behaviour, thermoregulation and early gene expression

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Abstract

Background and Purpose: New psychoactive substances such as *N*-ethylpentylone (NEP) are continuously emerging in the illicit drug market, and knowledge of their effects and risks, which may vary between sexes, is scarce. Our present study compares some key effects of NEP in male and female mice.

Experimental Approach: Psychostimulant, rewarding and reinforcing effects were investigated by tracking locomotor activity, conditioned place preference (CPP) paradigm and through a self-administration (SA) procedure, respectively, in CD1 mice. Moreover, the expression of early genes (*C-fos*, *Arc*, *Csnk1e*, *Pdyn*, *Pp1r1b* and *Bdnf* in addiction-related brain areas) was assessed by qPCR. Finally, serum and brain levels of NEP were determined by UHPLC-MS/MS.

Abbreviations: ACN, acetonitrile; Arc, activity-regulated cytoskeleton-associated protein; Bdnf, brain-derived neurotrophic factor; CPP, conditioned place preference; DARPP-32, dopamine- and cAMP-regulated phosphoprotein, molecular mass 32 kDa; DAT, dopamine transporter; DEA, Drug Enforcement Administration (USA); DS, dorsal striatum; FR, fixed ratio; FST, forced swim test; HLA, horizontal locomotor activity; IEGs, immediate early genes; MDMA, 3,4-methylenedioxy-methamphetamine; MDPV, methylenedioxypyrovalerone; mPFC, medial prefrontal cortex; MTBE, methyl tert-butyl ether; NEP, *N*-ethylpentylone; NPS, new psychoactive substances; OF, open field; Pdyn, prodynorphin; Post-C, postconditioning phase of CPP; PR, progressive ratio; Pre-C, preconditioning phase of CPP; qPCR, quantitative real-time PCR; SA, self-administration; UHPLC-MS/MS, ultra-high-performance liquid chromatography tandem mass spectrometry; VS, ventral striatum.

Raúl López-Arnau and David Pubill contributed equally to this work

Key Results: NEP-treated males experimented locomotor sensitisation and showed higher and longer increases in locomotion as well as higher hyperthermia after repeated administration than females. Moreover, while preference score in the CPP was similar in both sexes, extinction occurred later, and reinstatement was more easily established for males. Female mice self-administered more NEP than males at a higher dose. Differences in early gene expression (*Arc*, *Bdnf*, *Csnk1e* and *Ppp1r1b*) were found, but the serum and brain NEP levels did not differ between sexes.

Conclusion and Implications: Our results suggest that male mice are more sensitive to NEP psychostimulant and rewarding effects. These differences may be attributed to different early gene expression but not to pharmacokinetic factors. Moreover, males appear to be more vulnerable to the hyperthermic effects of NEP, while females might be more prone to NEP abuse.

KEYWORDS
addiction, ephylone, *N*-ethylpentylone, new psychoactive substances, sex differences, synthetic cathinones

1 | INTRODUCTION

N-ethylpentylone (NEP, also known as ephylone and *N*-ethylnorpentylone) is a beta-keto-methylenedioxyamphetamine that belongs to the cathinones family, one of the most prevalent of the new psychoactive substances (NPS). Cathinone derivatives emerged as alternatives to classical controlled psychostimulant drugs such as **amphetamine**, **cocaine** or **3,4-methylenedioxy-methamphetamine (MDMA)**. Among synthetic cathinones, NEP was the most encountered NPS around 2018 (Drug Enforcement Administration [DEA], 2018), leading to its federal scheduling and banning (DEA, 2021). Although it was quickly replaced by non-controlled analogues (Krotulski et al., 2021), NEP is still present in current cathinone trend reports and in countries where it has not been scheduled yet. In fact, some fatalities and acute intoxications after NEP consumption, sometimes as a counterfeit of MDMA or other psychostimulants, have been reported (Blanco et al., 2021; Eiden et al., 2019; Ikeji et al., 2018; Krotulski et al., 2018).

The strong psychostimulant effects of NEP are due to its ability to inhibit the reuptake of **dopamine**, **5-HT** and **noradrenaline** (Eshleman et al., 2019; Nadal-Gratacós et al., 2021). In fact, NEP has been described as one of the cathinones with higher inhibitory potency at the **dopamine transporter (DAT)** (Eshleman et al., 2019; Nadal-Gratacós et al., 2021), increasing levels of dopamine in the nucleus accumbens, 10-fold higher than those of 5-HT (Lin et al., 2020). NEP increases locomotor activity in male rodents similarly to **methamphetamine** and can fully substitute for the discriminative stimulus effects of methamphetamine and **cocaine** (Gatch et al., 2019; Li et al., 2019). Also, NEP induces conditioned place preference (CPP) in male mice, pointing to strong rewarding properties (Nadal-Gratacós et al., 2021) as well as lasting increases in ΔFosB levels in striatum, which suggest a high dependence potential (Espinosa-Velasco et al., 2022). Moreover, acute administration of NEP induces aggressive behaviour and social exploration deficits in male mice. Also, hyperthermia, aggressiveness and social exploration deficits were observed after repeated administration, and behavioural despair symptoms appeared during the subsequent withdrawal (Espinosa-Velasco et al., 2022). To our knowledge, no experiments with NEP in females have been reported yet.

Synthetic cathinones, including NEP, cause changes in the expression of immediate-early genes (IEGs) (Nadal-Gratacós et al., 2021; Wojcieszak et al., 2019), which codify for inducible transcription factors that play a role in the transition from a recreational to a compulsive drug use (Lanahan & Worley, 1998). However, little is known about sex differences in the expression of IEGs induced by synthetic cathinones.

What is already known

- *N*-ethylpentylone (NEP) is a synthetic cathinone with high abuse and risk potential.

What does this study add

- In CD1 mice, some key NEP effects differ significantly between the sexes.

What is the clinical significance

- Men and women may respond differently to the addictive and undesirable effects of NEP.

Substance use disorder is a psychiatric disease with clearly evident sex differences (Castro-Zavala et al., 2021; Lynch, 2008). A revision from the National Institute of Drug Abuse (NIDA, 2022) reports that, among humans, males may be more prone than females to use almost every kind of illegal drugs. However, after initial consumption, females have the same probability to develop a substance abuse disorder than males (Anthony et al., 1994) but may be more susceptible to craving and relapse, which are key steps in the addiction cycle. For these reasons, studying the effects of drugs in both sexes is as relevant as ever. Unfortunately, most preclinical assays with NPS, including those studying NEP effects, have been performed in male subjects. Nonetheless, a recent review by Fattore et al. (2020) compiled the existing evidence of sex differences in the effect of the different families of NPS. For cathinones, King et al. (2015) reported that methylenedioxypyrovalerone (MDPV) induces similar conditioning in both sexes, but females showed a lower taste avoidance response than males, suggesting that females might have an increased sensibility to use and abuse MDPV. A study by Nelson et al. (2019) investigating the cathinone α-PVP in rats reported that males display greater rewarding and aversive effects (indexed by taste avoidance, hyperthermia and stereotypies) than females, suggesting that males may be more likely to use the drug. Thus, because NEP possesses structural similarities and shares not only the mechanism of action but also some of the behavioural responses with these synthetic cathinones, it may also exhibit similar sex differences regarding their addictive and thermoregulatory effects. Moreover, some pharmacodynamic and/or pharmacokinetic properties of NEP may explain such sex differences.

Therefore, the aim of this study is to investigate the possible sex differences in the psychostimulant, rewarding and reinforcing effects of acute and repeated NEP administration. For that, the effects of NEP were assessed in behavioural paradigms such as locomotor activity, sensitisation, CPP and self-administration (SA). Also, the induction of hyperthermia after repeated exposure and IEGs expression involved in addiction was explored. Among these IEGs, we focused on the following: (1) *C-fos*, a neuronal activity marker whose expression is related to memory formation and neuroplasticity, among others processes (Gallo et al., 2018; Kovács, 2008); (2) *Arc* (activity-regulated cytoskeleton-associated protein), whose expression is considered a reliable marker of synaptic modifications associated with neuroplasticity and addiction (Fumagalli et al., 2006; Robinson & Kolb, 2004); (3) ***Bdnf*** (brain-derived neurotrophic factor), which is implicated in neuroadaptive processes that manage lasting functional changes in neuronal synapses (Ghitza et al., 2010); (4) prodynorphin (*Pdyn*), whose post-translational product is **dynorphin**, that binds to **κ-opioid receptors** and plays an important role in drug dependence (Butelman et al., 2012); (5) ***Csnk1e***, a genetic regulator of sensitivity to psychostimulants (Bryant et al., 2012); and, finally, (6) *Ppp1r1b*, encoding dopamine- and cAMP-regulated phosphoprotein, molecular mass 32 kDa (DARPP-32), which regulates synaptic plasticity as well as many other biological and behavioural responses driven by drugs of abuse (Gould & Manji, 2005; Svenningsson et al., 2004).

2 | METHODS

2.1 | Animals

Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). All animal care and experimental procedures complied with the European Community Council guidelines (2010/63/EU) as amended by Regulation (EU) 2019/1010 and were approved by the Animal Experimentation Ethics Committees of the Universities of Barcelona, Valencia and Pompeu Fabra which were, in turn, under supervision of the corresponding local Autonomous Governments (Catalonia and Valencia). Every effort was made to minimise animal suffering and discomfort and the number of animals used. Welfare of the animals was ensured by regular supervision for signs related with discomfort or suffering such as immobility, abnormal vocalisations or postures, piloerection, self-mutilation and extreme weight loss, defined as a weight loss exceeding 20% of initial body weight. If these signs were observed, the affected animal was humanely killed by cervical dislocation.

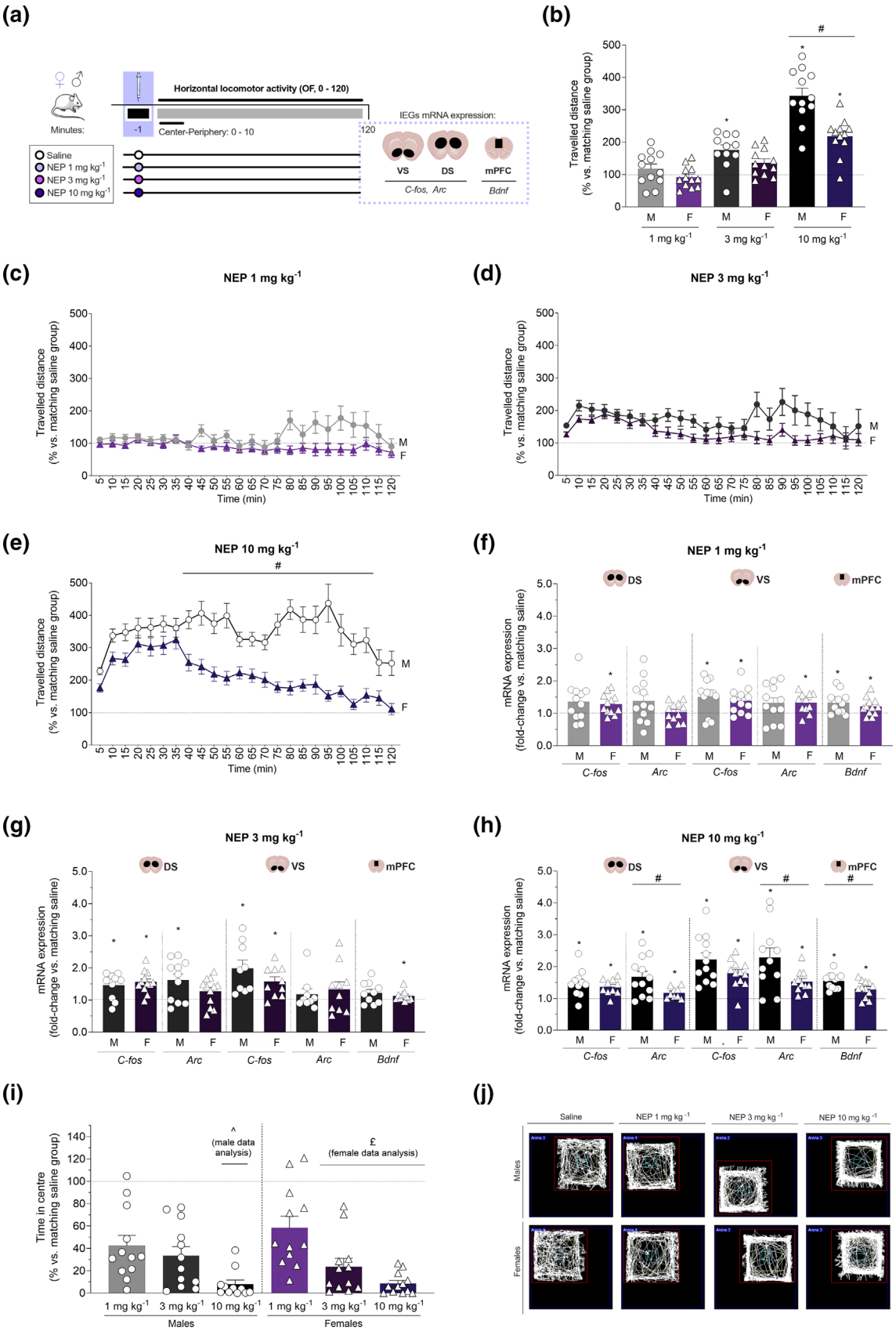
Male and female Swiss CD1 mice (Janvier, Le Genest, France) aged 8 weeks at the beginning of the experiments were used. They were housed in stable groups of four to six individuals of the same sex in plastic cages under a 12-h light/dark cycle, regulated room temperature of 22 ± 1°C and humidity of 65%. All the experiments were performed at the same conditions of temperature and humidity as in housing. They had ad libitum access to both drinking water and standard laboratory diet. In the beginning of the procedures, the mice were 8-week-old and weighed 36–41 g (males) and 27–34 g (females). The choice of the Swiss CD1 mouse strain is due to its widespread use in neuropsychopharmacology experiments. Also, this strain has been previously used for studying the effects of cathinones by our group (Duart-Castells et al., 2021) and others (El Yacoubi et al., 2000).

2.2 | Experimental groups and design

Sample size for each experiment was calculated according to a factorial design, with treatment and sex as variables for an ANOVA *F* test, using the GPower software (Version 3.1.9.6) (Faul et al., 2007). We also considered data from previous studies supporting that such group sizes provide sufficient power to obtain statistically significant differences (Castro-Zavala et al., 2021; Daza-Losada et al., 2009; Duart-Castells et al., 2021).

The animals were distributed in blocks according to their body weight, and treatment was assigned using the Random Allocation Software v.1.0 (developed by M. Saghaei). The number of animals and their distribution in the different experiments is shown below:

- Locomotor activity after acute administration: A total of 96 mice (12 males and 12 females for each of the four groups, namely saline, NEP 1 mg·kg^{-1}, NEP 3 mg·kg^{-1}, NEP 10 mg·kg^{-1}) were used. All the mice were humanely killed after the 2-h activity



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recording and brain areas of interest were removed and stored frozen for further mRNA extraction. The whole procedure is shown in Figure 1a and explained in the corresponding section.

- Locomotor sensitisation: A total of 48 mice (12 males and 12 females assigned to each of the two groups, saline and NEP) were used. All these mice underwent the entire sensitisation procedure, explained in the corresponding section and shown in Figure 2a. The NEP group received the 3 mg·kg^{-1} (i.p.) dose for priming and challenge and 10 mg·kg^{-1} (i.p.) during the sensitisation phase. Mice were killed immediately after the 2-h activity recording after the final challenge dose to obtain the brain areas of interest used for further mRNA extraction. In order to reduce the number of animals used, the mRNA expression induced by the priming dose (3 mg·kg^{-1}) was determined in samples from the mice of the acute administration experiments that had received this dose, as they had undergone the same procedure until this point. For more details on the procedure, please refer to the corresponding section.
- Hyperthermia during repeated administration: 48 mice were used (12 males and 12 females assigned to each of the two groups, saline and NEP 10 mg·kg^{-1}). All the mice were implanted with temperature transponders and underwent the whole procedure shownin Figure 3a, ending with the forced swim test (FST), as explained in the corresponding methods section.
- CPP acquisition, extinction and reinstatement: A total of 119 mice (59 males and 60 females) were used after being randomly assigned to one of the four treatment groups: saline (12 males and 12 females), NEP 1 mg·kg^{-1} (16 males and 16 females), NEP 3 mg·kg^{-1} (15 males and 16 females) and NEP 10 mg·kg^{-1} (16 males and 16 females). The procedure is shown in Figure 4a and explained in the corresponding section.
- SA experiments: A total of 72 mice were used (36 males and 36 females). Because we had little information on the effect of the 0.75 mg·kg^{-1} dose when self-administered, we used a slightly higher number of animals to anticipate possible side effects; thus, 22 males and 22 females were used. Of these, three males and one female died during surgery. Moreover, three more males and two females showed adverse effects during the SA schedule and were killed. This left us with 16 males and 19 females for the final analyses. In the case of the low dose (0.25 mg·kg^{-1}), 14 males and 14 females were used, and all of them survived the whole experiment. The procedure is illustrated in Figure 5a and detailed in the corresponding section.
- Pharmacokinetics experiment: A total of 70 mice were used, from which five males and five females were randomly assigned to every one of the seven chosen time points (5, 10, 30, 60, 90, 120 and 180 min after drug administration). The entire procedure is explained in the corresponding section.

2.3 | Assessment of horizontal locomotor activity and thigmotaxis in the open field

The open field (OF) was used to study both locomotor activity and thigmotaxis after an acute drug administration. Increased locomotor activity (hyperlocomotion) is characteristic of psychostimulant drugs and can be assessed in most mice strains (Simon et al., 1996). When placed in an open field arena, rodents tend to remain close to the walls, a phenomenon known as thigmotaxis. Mice previously treated with classical anxiolytic drugs spend more time in the centre of the arena (Simon et al., 1994). Experiments were conducted according to previous studies (Duart-Castells et al., 2021; Nadal-Gratacós et al., 2021, 2022). Twelve males and 12 females were used per treatment group. The experimental room was equipped with warm low-light conditions (150 lx) and white noise.

Briefly, an apparatus made of black Plexiglas, comprising four open field arenas each measuring 25 (l) × 25 (w) × 40 (h) cm, was used. A habituation phase was performed for 2 days, in which mice were injected with saline (i.p.) and placed in one arena. On the test day, they received the assigned treatment (saline or NEP 1, 3 or 10 mg·kg^{-1} [i.p.]) and were immediately placed in a randomly assigned arena, where the travelled distance was recorded for 120 min with a video camera connected to a computer running the tracking software Smart 3.0 (PanLab SLU, Spain). Also, during the first 10 min after the injection, the time spent in the centre and the periphery of the arena was measured to assess thigmotaxis.

2.4 | Locomotor sensitisation

Drug sensitisation is defined as an increased response to a drug after a period of withdrawal that followed repeated exposure. This phenomenon in laboratory animals has been related to the ability of a drug to induce relapse (Robinson & Berridge, 1993). In the case of psychostimulants, locomotor sensitisation has been demonstrated for

FIGURE 1 Acute effects of NEP (1, 3 and 10 mg·kg^{-1}) in male (M) and female (F) CD1 mice on locomotor activity, expression of immediate early genes (IEGs) and permanence in the centre of the open field. Panel a shows the time course of the procedures. Locomotor activity results are expressed as percentage of the area under the curve (b) or as percentage of the distance travelled in 5 min blocks (c–e), relative to the matching control group (saline-treated). Panels f–h show the expression of IEGs. Panel i shows the percentage of time, with respect to the matching saline-treated group, spent by the mice in the centre of the open field during the first 10 min after drug administration. Panel j shows a representative tracking of the ambulation of one representative mouse for each experimental group. Data are expressed as mean ± SEM, with individual values in (b, f-i), from nine to 12 animals for each experimental condition (initially 12 males and 12 females per group). *P < 0.05, significantly different from corresponding saline group; #P < 0.05, males significantly different from females; ^P < 0.05, significantly different from saline group (males); £P < 0.05, significantly different from saline group (females).

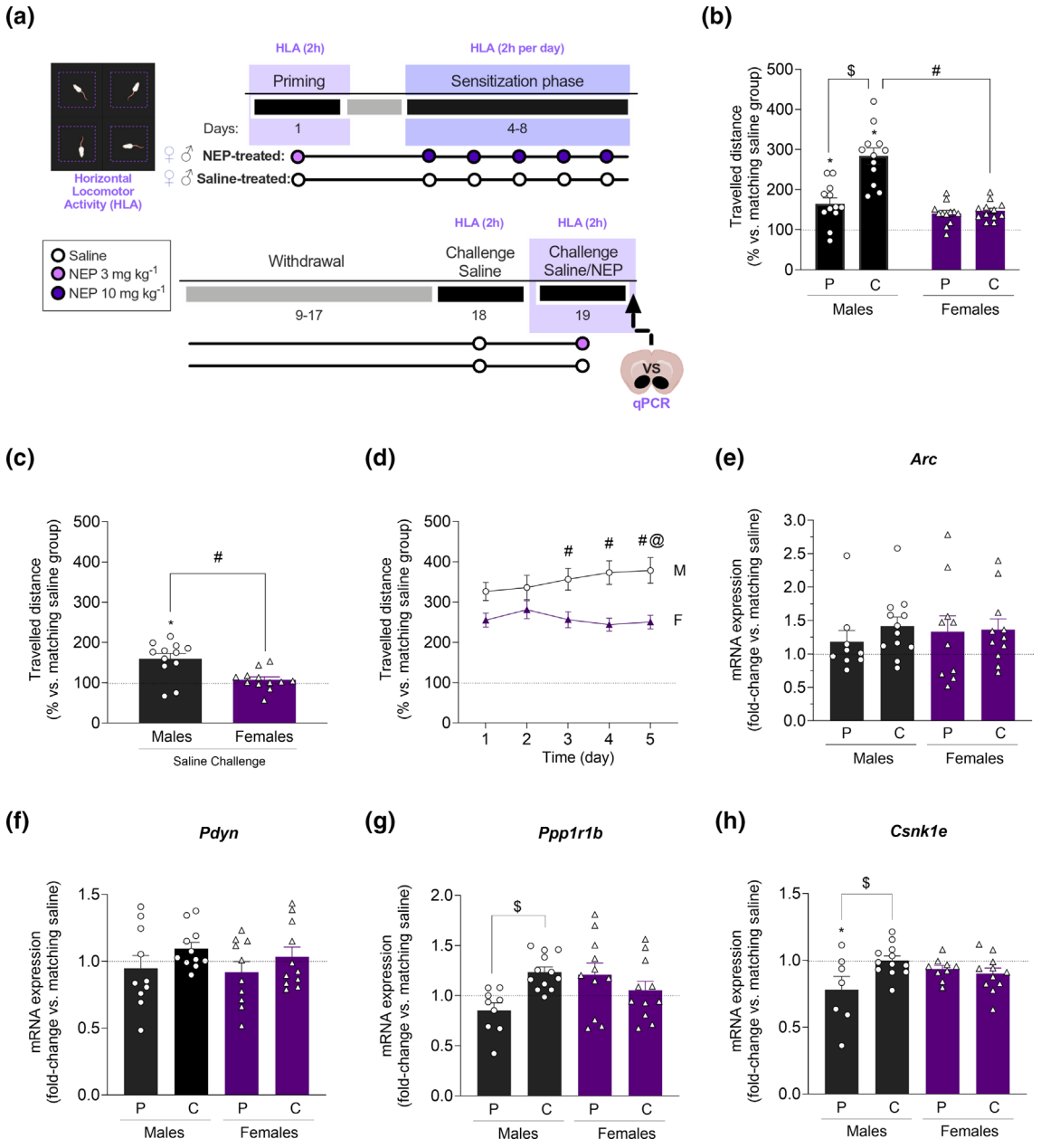

FIGURE 2 Locomotor sensitisation by NEP. Male and female CD1 mice were subjected to the administration procedure described in the Methods section and illustrated in panel a. Panel b shows the locomotor activity measured after the priming (P) dose (to drug-naïve mice) and the challenge (C) (after the sensitisation procedure), expressed as the percentage of that shown by the matching saline-treated group. Panel c shows the effect of a challenge with saline to the mice that received NEP during the sensitisation phase, as the percentage of that shown by the matching group that received saline. Panel d depicts the distance travelled by the NEP-treated animals along the five sensitisation days. Panels e–h show the mRNA expression of selected genes after a priming dose (obtained from the animals of the acute effects experiments) and after the challenge with respect to the expression assessed in the matching saline-treated mice. Data are expressed as mean ± SEM,,with individual values in (b, c, e-h), from 12 animals for each experimental condition (12 males and 12 females per group). *$P < 0.05$, significantly different from the matching saline-treated group; #$P < 0.05$, males significantly different from females at the same time point; $$P < 0.05$, significantly different between indicated groups; @$P < 0.05$, significantly different from day 1.

amphetamine-like drugs (Pierce & Kalivas, 1997) and for cathinones such as MDPV (Duart-Castells et al., 2019), among others.

The sensitisation procedure consisted of five phases over 21 days: habituation, priming, sensitisation, withdrawal and challenge (saline or NEP). A habituation phase was performed as described above for locomotor activity. After this phase, the priming (day 1) consisted of the i.p. injection of saline (5 ml·kg^{-1}) or NEP (3 mg·kg^{-1}). The sensitisation phase was carried out on days 4–8, with a daily injection of saline or NEP 10 mg·kg^{-1}. Thereafter, a 10-day withdrawal period began (days 9–17). On day 18, all the mice were challenged with saline and locomotor activity was recorded to assess conditioning to the injection procedure. Finally, at day 19, the mice

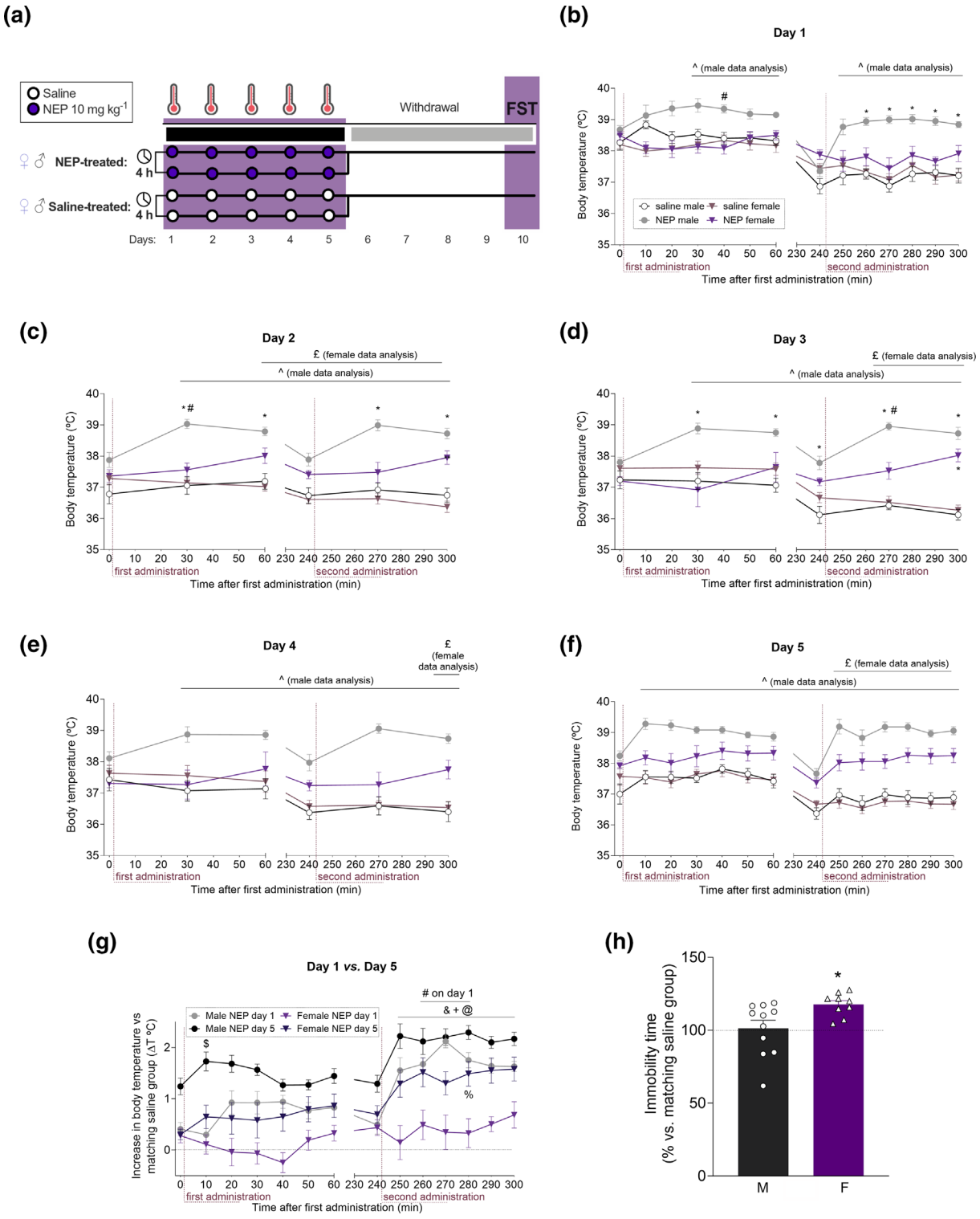

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FIGURE 3 Effect of repeated NEP administration on body temperature and on behavioural despair (FST) after withdrawal, in male and female CD1 mice. Mice with implanted temperature transponders received two daily NEP injections (10 mg·kg^{-1}, i.p.) during five consecutive days as shown in panel a. Temperature of each mouse was recorded every 10 min for 1 h after the first and second daily administration and data were plotted (panels b–f). Panel g compares the time-course of temperature increments of NEP-treated mice at day 1 with day 5. Panel h shows the percentage of immobility time in the FST in males and females after 5 days of drug withdrawal. Data are expressed as mean ± SEM, with individual values in (h), from 12 animals for each experimental condition (12 males and 12 females per group). After analysing separately males and females (two-way ANOVA), ^ and £ indicate statistically significant differences between NEP-treated and its time and sex-matching saline-treated mice for males and females, respectively (i.e., hyperthermia). The rest of symbols derive from the three-way ANOVA analysis. *P < 0.05, significantly different from its sex and time-matching saline group; #P < 0.05, significantly different from its time and treatment-matching female group; &P < 0.05, males on day 1 significantly different from males day 1 at time = 0 (basal temperature); +P < 0.05, T at a given time significantly different from T at time 0 of males at day 5, @P < 0.05, T at a given time significantly different from T at time 0 of females at day 5; $$P$ < 0.05, for males, T at a given time at day 5 significantly different from T at same time of day 1; %P < 0.05, for females, T at a given time at day 5 significantly different from T at same time of day 1.

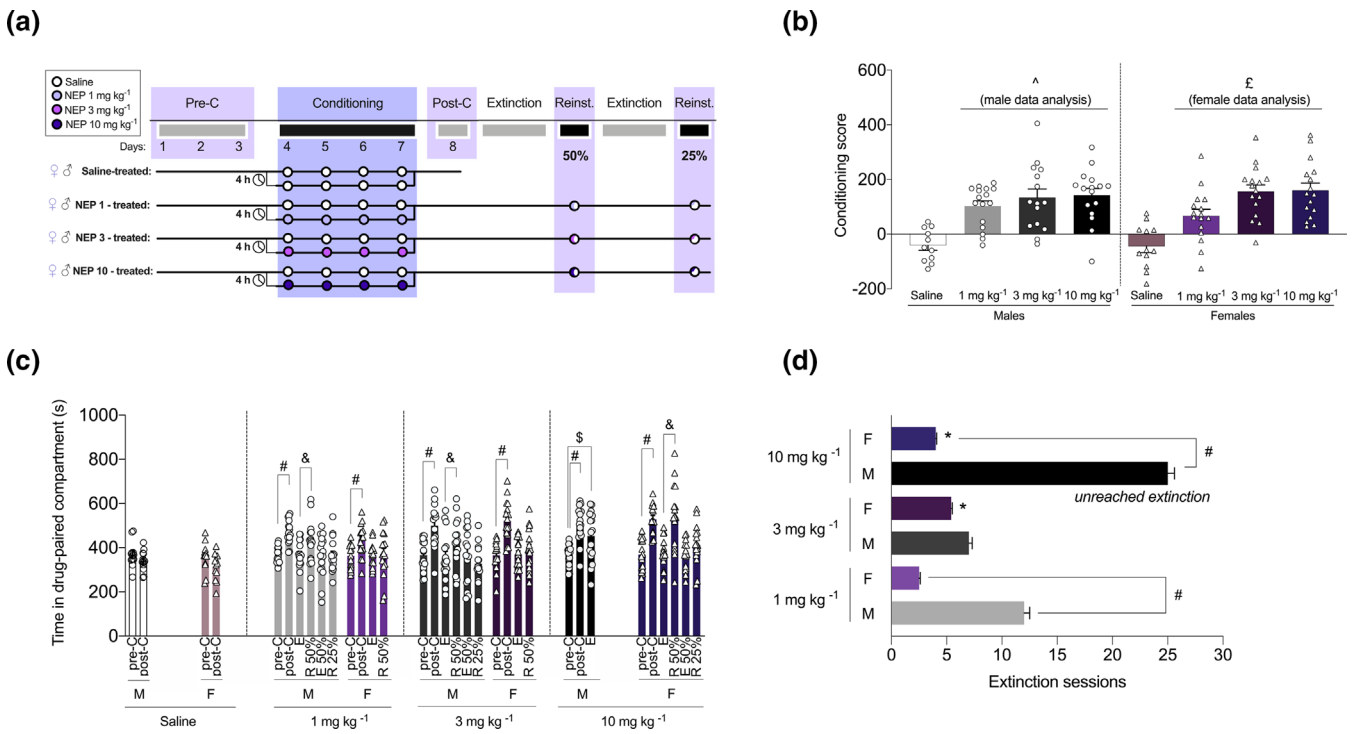
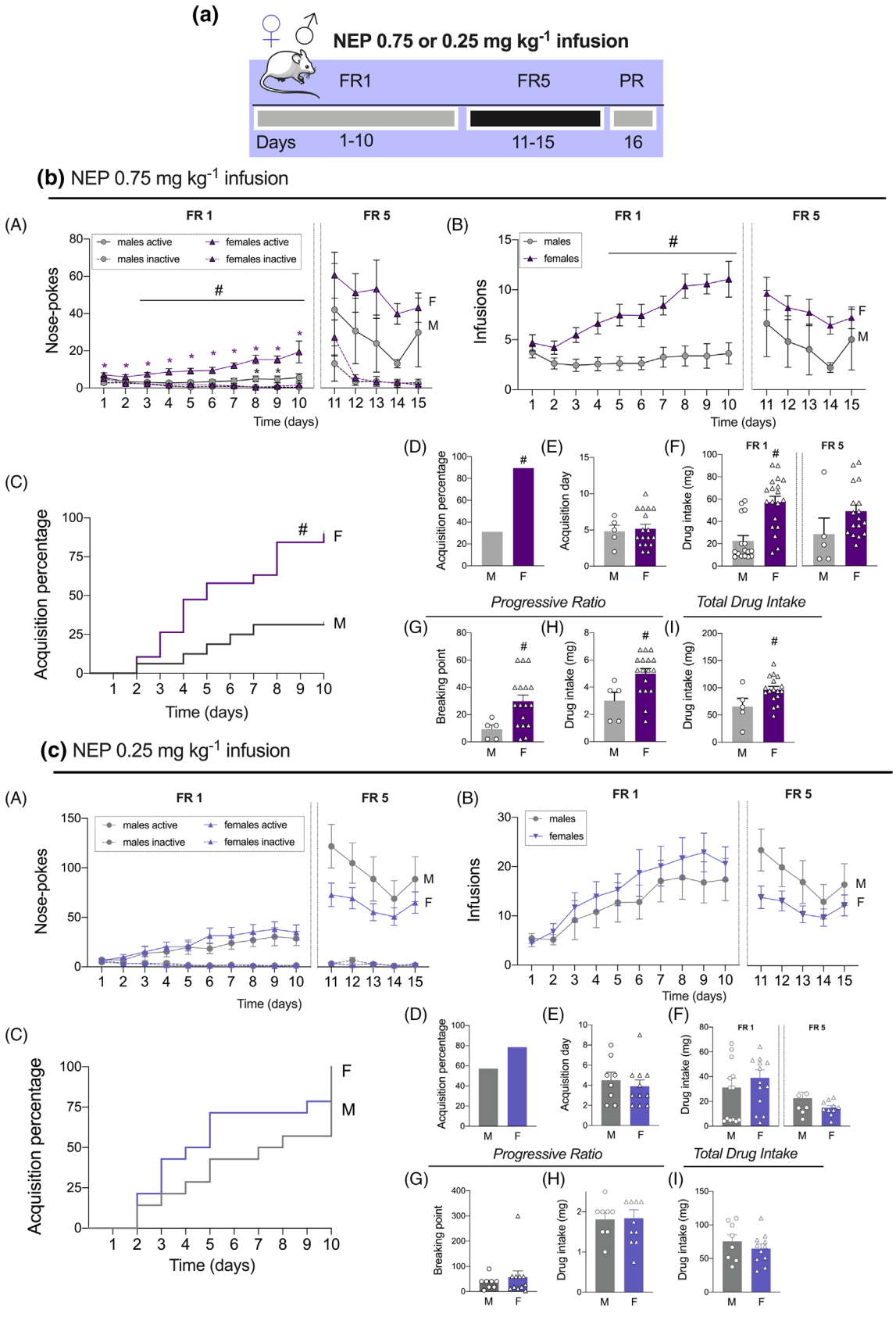


FIGURE 4 Rewarding effects of NEP (1, 3 and 10 mg·kg^{-1}, i.p.) in male (M) and female (F) CD1 mice. A CPP procedure, followed by extinction and reinstatement sessions was carried out according to that described in the Methods section and illustrated in panel a. Panel b shows the CPP scores in males and females. Panel c shows, for each treatment group, the mean times spent in the drug-paired compartment before conditioning (pre-C), after conditioning (post-C), after extinction (E) and after reinstatement (R) with half (50%) or quarter (25%) of the dose used for conditioning. Panel d shows the number of sessions needed to reach the extinction of the conditioning. Data are expressed as mean ± SEM, with individual values in (b, c), from 12 to 16 animals for each experimental condition. *P < 0.05, significantly different from the matching saline-treated group; @P < 0.05, significantly different from the matching 1 mg·kg^{-1} NEP-treated group; # and &P < 0.05, significantly different as indicated; ^P < 0.05, significantly different from saline, one-way ANOVA analysis of male subjects; £P < 0.05, significantly different from saline, one-way ANOVA analysis of male subjects.

received a challenge of either saline or NEP (3 mg·kg^{-1}) according to their assigned treatment. During priming, sensitisation and challenge phases, activity counts were recorded as previously described. The locomotor activity was normalised to the corresponding sex- and time-matching saline-treated group. For each group, the travelled distance on the day of the challenge with NEP or saline was compared with that of the day of the priming to assess the degree of locomotor sensitisation and conditioning, respectively.

2.5 | Quantitative real-time PCR (qPCR)

After the 2-h open field test, mice were killed by cervical dislocation at the established time points. Ventral striatum (VS), dorsal striatum (DS) and medial prefrontal cortex (mPFC) were dissected according to Paxinos and Franklin (2008) (see Figure 1a for the experimental design). After acute administration, we tested the RNA expression levels of selected immediate early genes (IEGs) by qPCR. *C-fos* and *Arc*



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mRNA were assessed in DS and VS, whereas *Bdnf* expression was determined in mPFC. Moreover, the levels of mRNA coding for the proteins Arc, Prodynorphin *(Pdyn)*, DARPP-32 (encoded by the gene *Ppp1r1b*), and *Csnk1e* were assessed in VS of the mice killed 2 h after administration of the NEP challenge dose and compared with the expression found in mice treated acutely with the 3 mg·kg^{-1} dose (equivalent to the NEP priming).

Total RNA isolation of these samples was performed following a TRIsure-chloroform standard protocol. RNA concentrations in sample extracts were assessed at 260 nm in a NanoDropTM ND-1000 spectrophotometer (ThermoFisher Scientific). Afterward, reverse transcription polymerase chain reaction (RT-PCR) (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems) was performed to obtain cDNA with 1 µg of RNA and appropriate volumes of each reagent to a final volume of 20 µl. Subsequent qPCR was carried out with the Step One PlusTM Real-Time PCR System (Applied Biosystems) and employing TaqMan probes (Mm01204954_g1 for *Arc*, Mm00487425_m1 for *C-fos*, Mm04230607_s1 for *Bdnf*, Mm00457573_m1 for *Pdyn*, Mm00454892_m1 for *Ppp1r1b*, and Mm01300216_m1 for *Csnk1e*). Samples were tested in duplicate. Possible alterations in mRNA expression of these genes were assessed using a comparative Cycle threshold (Ct) method (ΔΔCt), with expression was previously normalised by using Ct values of the housekeeping gene *β-actin* (Mm00607939_s1).

2.6 | Hyperthermia assessment during repeated administration

Repeated cathinone administration has been related to the development of hyperthermia (Espinosa-Velasco et al., 2022; Grecco & Sprague, 2016). For this study, the changes in body temperature were measured by means of subcutaneously implanted temperature transponders (IPTT-300; Bio Medic Data Systems, Inc., DE, USA), so that the stress and discomfort of animals when measuring temperature is considerably reduced compared with, for example, rectal temperature measurement.

Five days before the beginning of the treatment phase, mice were briefly anaesthetised using **isoflurane**, and the transponders were subcutaneously placed in the interscapular zone, by means of the provided transponder injector; 10% iodine antiseptic solution was applied to the injection point to prevent infections, and mice were placed back in their home cages, being supervised by the experimenters until complete cicatrisation. The following days, the mice were treated with saline (i.p.), and their body temperatures were recorded to test the transponder and ensure their habituation to the experimenters and the assay.

For the hyperthermia assay, animals were previously placed every day at 8 AM in the experimental room and were allowed to habituate for 1 h. The repeated treatment schedule consisted of two daily injections of saline or NEP 10 mg·kg^{-1} (i.p.), 4 h apart, for five consecutive days. To measure the temperature, a DAS-8007 Wireless Reader System (Bio Medic Data Systems, Inc) was approached for approximately 5 s to each mouse, allowing the transponder to send the data to the reader, thus reducing manipulation and consequent stress to the mice. On days 1 and 5, temperature assessments were done every 10 min during 1 h after each administration. On days 2, 3 and 4, temperature was assessed every 30 min during the hour after each injection. The temperatures at 0 and 240 min were measured right before each injection.

2.7 | FST

The FST is a widely used method to assess antidepressant effectiveness (Petit-Demouliere et al., 2005; Porsolt et al., 1977), measuring a single stress-induced behaviour as immobility time. Increased immobility after drug administration, often interpreted as behavioural despair, might suggest an initial depressive state in mice. However, the validity of this test for directly modelling human depressive states is controversial. In this sense, to study possible behavioural despair symptoms during subsequent abstinence period, the FST was performed 5 days after the end of the repeated administration schedule described above (saline or NEP 10 mg·kg^{-1} (i.p.), twice a day, 4 h apart, for five consecutive days).

Mice were placed in the experimental room for 1 h prior to starting the test. Every single mouse was placed in a vertical glass cylinder (24 cm height and 11.5 cm diameter) previously filled to 17 cm with water at 25 ± 1°C and was allowed to swim for 6 min, while recording with a camera. After the test, mice were immediately dried and returned to their home cages. Then, the videos were analysed by an experimenter blinded to the treatment. The first 2 min of each trial were considered habituation, so the immobility time was counted during the last 4 min of the test. Mice were considered to be immobile when remaining floating passively in the water, including those movements that allowed a floating posture.

FIGURE 5 Self-administration of NEP by male (M) and female (F) CD1 mice. The procedure, starting with fixed ratio FR1, followed by FR5 and progressive ratio (PR) is described in the Methods section and outlined in panel a. Panels b and c correspond to the two infusion doses assayed (0.75 and 0.25 mg·kg^{-1}, respectively). Subpanels A and B show the number of nose pokes and infusions administered under FR1 and FR5, respectively. Subpanels C show the time course of self-administration behaviour acquisition as the percentage of mice that accomplished the requirements. Subpanel D shows the final percentage of mice that acquired self-administration behaviour after FR1, whereas subpanel E represents the mean acquisition day for the two sexes. Subpanel F shows the mean total drug intake after FR1 and FR5. Panels G and H report the breaking point and the total drug intake under the progressive ratio period, and subpanel I shows the mean total drug intake after all the stages of the self-administration procedure. Data are expressed as mean ± SEM, with individual values, from initially 16 males and 19 females for the 0.75 mg·kg^{-1} dose and 14 males and 14 females for the 0.25 mg·kg^{-1} dose. #P < 0.05, males significantly different from females.

2.8 | Conditioned place preference acquisition, extinction and reinstatement

The rewarding effects of NEP were assessed using the CPP paradigm. CPP is a gold standard in basic research to study addictive drugs and it has proved to be a reliable model to study drug-induced reward in several strains of mice, including CD1 (Daza-Losada et al., 2009; Golden et al., 2019).

Male and female CD1 mice were randomly assigned to the different treatment groups (saline or NEP 1, 3 or 10 mg·kg^{-1}), The experiments were carried out during the dark phase of the light/dark cycle.

For place conditioning, we used 12 identical Plexiglas boxes with two equally sized compartments (30.7 cm length × 31.5 cm width × 34.5 cm height) separated by a grey central area (13.8 cm length × 31.5 cm width × 34.5 cm height). The compartments had different coloured walls (black vs. white) and distinct floor textures (fine grid in the black compartment and wide grid in the white one). Four infrared light beams in each compartment of the box and six in the central area allowed the recording of the position of the animal and its crossings from one compartment to the other. The equipment was controlled by two computers using MONPRE 2Z software (CIBERTEC S.A., Spain).

The procedure of place conditioning was unbiased in terms of initial spontaneous preference and consisted of three phases. In the first phase (preconditioning phase, Pre-C), mice were allowed access to both compartments of the apparatus for 15 min (900 s) per day for 3 days. On day 3, the time spent in each compartment over a 900-s period was recorded, and animals showing a strong unconditioned aversion (<33% of the session time) or preference (more than 67%) for any compartment were excluded from the rest of the experiment. The procedure of assignment was unbiased, assigning half of the animals in each group to the drug or vehicle in one compartment (e.g., white), and the other half in the other compartment (e.g., black). Additionally, half of the animals were assigned to the initially preferred compartment and the other half to their non-preferred compartment.

After assigning the compartments, no significant differences were detected between the time spent in the drug-paired and vehicle-paired compartments during the Pre-C. In the second phase (Conditioning), which lasted 4 days, the animals received an injection of saline immediately before being confined to the vehicle-paired compartment for 30 min. After an interval of 4 h, they received an injection of NEP immediately before being confined to the drug-paired compartment for 30 min. Control groups received saline in both compartments of the CPP boxes.

Confinement was made possible in both cases by closing the guillotine door that separated the two compartments, rendering the central area inaccessible. The third phase, called the post-conditioning phase (Post-C), was performed the day after and consisted of allowing the untreated mice to freely move between the compartments by removing the guillotine door while recording the time spent by in each compartment during a 15 min observation period. The difference in seconds between the time spent in the drug-paired compartment during the Post-C test and the Pre-C phase is a measure of the degree of conditioning induced by the drug. If this difference is positive, then the drug has induced a preference for the drug-paired compartment, while the opposite indicates aversive behaviour.

When preference for the drug-paired compartment had been established, all groups underwent two extinction sessions per week (on Mondays and Thursdays), in which they were placed in the apparatus (without the guillotine doors separating the compartments) for 15 min. Results were checked every week for each group to confirm if criteria was met. Extinction was achieved when there was a lack of significant differences between the time spent in the drug-paired compartment during the extinction session and Pre-C test values for two consecutive sessions.

Twenty-four hours after extinction had been confirmed, the effects of a priming dose of NEP were evaluated. Reinstatement tests were the same as those for the Post-C (free ambulation for 15 min), except for the fact that mice were tested 15 min after administration of a priming dose (half of the dose used for conditioning). Priming injections were administered in the vivarium, which constituted a non-contingent place to that of the previous conditioning procedure. If animals reinstated the preference, the extinction sessions continued in time, and when the criteria were met again, the next half-dose was administered. If they did not reinstate the preference, then the experiment was finished. Therefore, each group could have finished the procedure at different times.

The schematic timeline organisation of these experiments is shown in Figure 4a.

2.9 | SA experiments

The reinforcing effects of NEP were investigated using the SA paradigm. This procedure is a well-established method to assess reinforcement induced by drugs of abuse (Spealman & Goldberg, 1978) including cathinones (Watterson & Olive, 2017).

NEP was dissolved in sterile saline solution to yield a dose of 0.25 mg·kg^{-1} or 0.75 mg·kg^{-1} when administered as an i.v. infusion of 20 µl during 2 s.

The SA experiments were conducted as previously described (Castro-Zavala et al., 2020, 2022). Briefly, a jugular-vein catheter was implanted. The surgery was done following anaesthesia with an i.p. injection of a mixture of **ketamine** hydrochloride (75 mg·kg^{-1}; Imalgène 1000, Lyon, France) and **medetomidine** hydrochloride (1 mg·kg^{-1}; Medeson®, Barcelona, Spain), administered in a volume of 10 ml·kg^{-1}. Mice received analgesic treatment before and after surgery. In both cases, **meloxicam** (0.5 mg·kg^{-1}) was administered subcutaneously. The schedule of administration was 30 min before surgery and after surgery every 24 h for 72 h. At the end of surgery, mice also received a single administration of enrofloxacin (7.5 mg·kg^{-1}, i.p. in a volume of 30 ml·kg^{-1}). After surgery, the animals were housed individually, placed on electric blankets and allowed to recover.

The SA experiments were carried out in mouse operant chambers (Model ENV-307A-CT, Medical Associates, Cibertec S.A., Madrid,

Spain) containing two holes; one was defined as active and the other as inactive. Nose poking into the active hole produced a reinforcer delivery (drug infusion) paired with two stimulus lights, one placed inside the nose poke and the other above the active hole. Mice received a maximum of 150 reinforcers, and each reinforcer was followed by a 15-s time-out period in which no reinforcers were delivered. Nose poking into the inactive hole had no consequences. The side on which the active/inactive hole was placed was counterbalanced. At the beginning of each session, the house light was ON for 3 s and OFF for the rest of the experiment. The session started with a drug priming infusion and 4-s presentation of the light cue, situated above the active hole. Infusions were delivered in a volume of 20 µl·2 s^{-1}.

At least 3 days after surgery, animals were trained on a fixed ratio 1 (FR1), to self-administer NEP (0.25 or 0.75 mg·kg^{-1} per infusion). During 10 consecutive days, the mice were subjected to 2-h daily sessions, where we counted the responses in the *time in* (drug infusions) and the responses in the *time in* + *time out* (nose pokes). The *Hole* effect was observed when mice discriminated between the active and the inactive hole. Mice were considered to have acquired stable SA behaviour when the following criteria were met on two consecutive days: ≥5 responses in the active hole and ≥65% of responses in the active hole. All animals accomplished the 10 sessions independently on the day of acquisition.

Mice that met acquisition criteria were moved to FR5, where five nose pokes are required before receiving a drug infusion. Five days later, these animals were shifted to one progressive ratio (PR) session, in which the drug delivery requirement to earn an infusion escalated according to the following series: 1-2-3-5-12-18-27-40-60-90-135-200-300-450-675-1000.

The schematic timeline organisation of these experiments is presented in Figure 5a.

2.10 | Brain and serum NEP levels after i.p. administration

To assess whether the observed differences between males and females were related to relevant differences in the disposition of NEP, a pharmacokinetic assay was performed to determine the time-course of NEP levels in serum and brain after an i.p. administration. As pharmacodynamic differences were more noticeable at 10 mg·kg^{-1} NEP, this was the chosen dose for the assay. Because serum and brain concentration values for each experimental observation were obtained at the same data point, data of both biological fluids were fitted simultaneously with the objective of describing the kinetic behaviour of the drug in central (blood samples) and peripheral (brain samples) compartments.

Mice were injected with 10 mg·kg^{-1} of NEP (i.p.). Then, at the assigned time points (5, 10, 30, 60, 90, 120 and 180 min) after administration, they were anaesthetised with isoflurane and blood was extracted by intracardiac puncture, followed by decapitation and brain removal. Brains were weighed and frozen in dry ice. Blood samples were collected in Serum-Gel Z micro tubes (SARSTEDT AG & Co. KG, Germany) and centrifuged at 1000 × *g* at room temperature for 10 min to isolate serum. Serum and brain samples were preserved at −80°C until analysis.

For analysis, acetonitrile (1 ml) was added to brain tissue samples, and the mixture was homogenised using Homogenizer 150 (Fisher Scientific, Loughborough, UK), kept on ice for 30 min and centrifuged at 15,870 x *g* for 5 min at 4 °C, and the supernatant was kept at −20°C till UHPLC-MS/MS analysis. For serum samples, aliquots of 100 µl were mixed with 100 µl of a saturated solution of sodium tetraborate and 900 µl of methyl *tert*-butyl ether, centrifuged at 15,870 × *g* for 5 min and evaporated to dryness under nitrogen stream. Samples were reconstituted with 500 µl of methanol and kept at −20°C till UHPLC-MS/MS analysis. UHPLC-MS/MS analysis was performed using an Acquity UPLC H-Class liquid chromatograph coupled to a triple quadrupole mass spectrometer (Waters, Milford, MA, USA). Information about bioanalysis, quantification by UHPLC-MS/MS and suitability of the analytical method are included in "Chemical analysis supplementary material" and was adapted from previous studies (Fabregat-Safont et al., 2020).

Results were expressed as µg NEP·L^{-1} for serum or as ng NEP·g^{-1} for the brain.

2.11 | Data analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018).

All sets of data were previously tested for normality (Kolmogorov–Smirnov normality test), sphericity (Mauchly's test), homoscedasticity (Levene's test) and outliers (Grubbs' test). All results are expressed as mean ± SEM, except in the pharmacokinetic analysis, where means ± SDxx were plotted. Statistical analyses were performed using GraphPad Prism 8.0 and IBM SPSS Statistics v.28. In all the experiments, a P value <0.05 was considered statistically significant.

Results of all the statistical analyses have been tabulated and provided as Supporting Information to simplify manuscript reading and comprehension.

2.11.1 | Analysis of locomotor activity and open field assessments

Female mice showed higher basal locomotion than males in the open field. Similar observations were made by other authors (Borbélyová et al., 2019; Cailhol & Mormède, 1999). Therefore, the horizontal locomotor activity of the different NEP-treated groups was analysed and expressed as a percentage of travelled distance with respect to the value from the matching saline-treated group, set to 100%.

Two-way ANOVA, one-way ANOVA or Student's *t* test were used according to the number of factors. Two variables (*Treatment*

and *Sex*, *Sex and Time or Time and Treatment*) data were analysed by using a two-way ANOVA followed by Bonferroni's post hoc test when the interaction between both variables reached statistical significance. When the variable *Sex* had no significant effect, as in the case of the time in the centre of the open field, we analysed each sex separately by a one-way ANOVA and subsequent Bonferroni's test.

The time spent by mice in the centre of the arena was recorded during 10 min after the injection and expressed as the percentage with respect to that of the matching saline-treated group (100%).

2.11.2 | Analysis of RNA levels

For the analysis of RNA expression levels, data were expressed as a percentage of expression in the matching saline group (set to 100%). Student's *t* tests were used to compare results of the acute administration experiment. For comparing RNA expression levels after the priming with NEP with those after the challenge, a two-way ANOVA was applied, followed by Bonferroni's post-hoc test if applicable.

2.11.3 | Analysis of temperature assay data

Temperature data were analysed using a two-way ANOVA for each sex (*Treatment* and *Time* variables), followed by Bonferroni's post-hoc tests to assess the occurrence of hyperthermia in each sex along the treatment days, whereas a three-way ANOVA (mixed-effects analysis) was used to investigate the possible sex differences. Only when the triple factor interaction (*Treatment* × *Sex* × *Time*) reached $P < 0.05$, Tukey's multiple comparisons test was applied. To compare the increases in temperature on day 1 versus on day 5, as mice were more habituated to the experiment and their body temperatures were lower on day 5 in comparison with day 1, data were expressed as the increase in temperature with respect to the sex and time-matching saline group. These data were analysed with a two-way ANOVA and, when *Sex* × *Time* interaction was $P < 0.05$, Bonferroni's post-hoc test was applied.

2.11.4 | FST

The values of immobility time obtained from the analysis of this experiment were expressed as the percentage of immobility time with respect of that of the sex-matching saline-treated group (set to 100%), due to differences between the behavioural of the sexes in this test. Then, a Student's *t* test was applied.

2.11.5 | Conditioned place preference

The conditioning scores were calculated for each mouse as the increase in time spent in the drug-paired compartment on the Post-C and on the Pre-C tests, in seconds. Data were analysed using a two-way ANOVA, and as the interaction *Sex* × *Treatment* did not reach $P < 0.05$ and no effect of the variable *Sex* was observed, data were collapsed by the factor *Sex* and subsequent one-way ANOVA and Bonferroni's post-hoc test were applied. Data related to extinction and reinstatement values in the groups showing CPP were analysed by means of Student's *t* test. The time required for the preference to be extinguished in each animal was analysed by means of the Kaplan–Meier test, with Breslow (generalised Wilcoxon) comparisons when appropriate (Daza-Losada et al., 2009). Although the mean of the group, as a whole, determined the day on which extinction was considered to have been achieved, preference was considered to be extinguished when a mouse spent 365 s or less in the drug-paired compartment on two consecutive days. We chose this time based on the values of all the Pre-C tests performed in the study (mean = 365 s). When the preference was not extinguished in an animal, it was assigned the number of days required for extinction for the whole group.

2.11.6 | SA paradigm

Repeated measures three-way or two-way ANOVA were applied to analyse nose pokes and infusions data, respectively. Bonferroni's post-hoc test was applied when the all-factors interaction (*Sex* × *Time* × *Hole* for nose pokes and *Sex* × *Time* for infusions) reached $P < 0.05$. The percentage of acquisition, the acquisition day, breaking point and drug intake were analysed using Fisher's exact test or Student's *t* test, accordingly. The acquisition FR1 curve was analysed using the Log-rank test.

2.11.7 | Pharmacokinetic analysis

Data fitting was performed with the aggregates of different experimental time points (data pooling from 71 mice) to estimate a unique set of parameters in males and females. NEP concentrations in serum and brain obtained after i.p. administration were described by an open two-compartmental model and fit to the following equation:

$$Cp = A^{-\alpha t} + B^{-\beta t}$$

where C_p is the total serum drug concentrations at time t (ng·mL^{-1}), A and B are the extrapolated zero intercepts and α and β represent the apparent first-order elimination rate constants. NEP half-life ($t_{1/2}$) was calculated as $t_{1/2} = 0.693 \cdot k_{10}{}^{-1}$ where k_{10} represents elimination rate from the central compartment and the irreversible compound loss from the body. Transfer coefficients k_{12} and k_{21} are transfer rates of the amounts of NEP between central (V_c) and peripheral (V_p) compartments. Serum and brain NEP concentrations were linked to the central (V_c) and peripheral (V_p) volumes of distribution respectively.

The areas under the curve from zero to the last sample time (AUC_{0_last}) concentrations, maximum concentration in serum (ng·mL^{-1}·kg^{-1}) CL_p (total serum clearance) and V_{dss} (steady state

apparent volume of distribution) were calculated by a standard method (Gibaldi & Perrier, 1982). Model fitting was built with all male and female data and are expressed as means and their corresponding standard deviations, corrected by body weight, and CI 95% was obtained by correcting by the body weight range.

Assessment of goodness of fit of the proposed models to the observed data was based on the objective function value, Akaike's information criteria (AIC) and the weighted residual plot analysis. Pharmacokinetic analysis was achieved by using the compartmental modelling SAAM II software system (SAAM II Institute, Seattle, WA, USA) (Barrett et al., 1998).

2.12 | Materials

Racemic NEP was synthesised as the hydrochloride salt and chemically identified according to Nadal-Gratacós et al. (2021). NEP solutions for intraperitoneal (i.p.) administration were freshly prepared in sterile saline solution (0.9% NaCl, pH 7.4). Sodium tetraborate (Borax), and methyl tert-butyl ether (MTBE) were supplied by Sigma-Aldrich (St. Louis, USA). Acetonitrile and methanol (MeOH), both LC-MS grade, were purchased from VWR chemicals Prolabo (Leuven, Belgium), whereas LC-MS grade formic acid (FA) was provided by Fisher Scientific (Loughborough, UK). The rest of the chemicals used and their sources are either mentioned in the corresponding section of the methods or were purchased from recognised laboratory suppliers, always being of analytical grade or superior.

2.13 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/24 (Alexander, Christopoulos, Davenport, Kelly, Mathie, Peters, Veale, Armstrong, Faccenda, Harding, Davies, et al., 2023; Alexander, Fabbro, Kelly, Mathie, Peters, Veale, Armstrong, Faccenda, Harding, Davies, Amarosi, et al., 2023; Alexander, Fabbro, Kelly, Mathie, Peters, Veale, Armstrong, Faccenda, Harding, Davies, Annett, et al., 2023).

3 | RESULTS

3.1 | Male mice show higher and prolonged increase in locomotion than females after acute NEP administration

We tested the doses of 1, 3 and 10 mg·kg^{-1} (i.p) of NEP for their effects on locomotor activity. Concerning cumulative horizontal locomotor activity (HLA) during the 2 h after administration, two-way ANOVA reported statistically significant effects for *Sex*, *Treatment*, and an interaction between them. Post-hoc analyses showed that NEP induced statistically significant increases in locomotion at the doses of 3 and 10 mg·kg^{-1} in males, whereas in females, only the 10 mg·kg^{-1} dose was effective. Moreover, the increase in locomotion induced by the 10 mg·kg^{-1} dose was significantly much higher (near twice) in males than in females. The lowest dose (1 mg·kg^{-1}) did not induce significant changes in locomotion in either sex (Figure 1b).

Moreover, locomotion profiles in 5-min blocks for both sexes were compared for each dose. Statistically significant differences were also observed with the 10 mg·kg^{-1} dose, which induced higher and prolonged increases in locomotor activity in males than in females (Figure 1c–e).

3.2 | NEP-induced increases in *Bdnf* and *Arc* RNA expression are higher in male than in female mice

Expression of IEGs related to drug addiction was assessed shortly after activity recording (Figure 1a) in selected brain areas. Increases in IEGs expression, 2 h after NEP acute administration, were found for the three tested doses.

The 1 mg·kg^{-1} dose of NEP modestly increased the expression of *Arc*, *C-fos* and *Bdnf* in most of the studied brain areas, and no statistically significant differences between sexes were found (Figure 1f). Similarly, the dose of 3 mg·kg^{-1} also increased the expression levels of these IEGs, but no significant sex differences were found (Figure 1g).

Particularly, in ventral striatum, the 10 mg·kg^{-1} dose induced increases in *C-fos* and *Arc* mRNA in both males and females. It was also with this dose that males showed statistically significant higher increases in *Arc* and *Bdnf* in mPFC compared with females, but not in *C-fos* expression (Figure 1h).

To investigate the link between hyperlocomotion and increased IEGs expression, we performed a correlation analysis with the HLA values from the mice treated with saline and NEP (10 mg·kg^{-1}) as well as the expression of *Arc* (in dorsal and ventral striatum) and *Bdnf*. These genes were chosen as they had shown differential expression between males and females. In both cases, we found a statistically significant positive correlation between HLA and gene expression (Table 1). Notably, the slopes of the regression lines were similar, indicating that the animals showing greater increases in locomotion (males) also showed higher gene expression (see Figure S3 for graphs).

3.3 | NEP induces similar thigmotactic effects in male and female mice

The analysis of the percentage of time spent in the centre of the open field did not report either effect of the variable *Sex* nor of the interaction *Treatment* × *Sex* (Figure 1i). However, separate analyses of male and female data revealed a different pattern. One-way ANOVAs for each sex reported a significant effect of *Treatment*, with a dose-dependent reduction in the percentage of time spent in the central area of the arena. This suggests that NEP induced dose-dependent thigmotactic effects in male and female mice (Figure 1i,j). Interestingly,

TABLE 1 Correlation analysis between HLA (X) and gene expression (Y) in the indicated brain areas. Equations of the regression lines are shown, followed by the statistical significance signs of the correlation: *$P < 0.05$, significant differences between the slopes of the lines from males and females for a given gene: n.s., non-significant; Student's *t* test.

	Males	Females	*P* value (*t* test)
Bdnf (mPFC)	Y = 0.001952*X + 0.8508 (*)	Y = 0.001723*X + 0.8907 (*)	n.s.
Arc (DS)	Y = 0.002307*X + 0.8247 (*)	Y = 0.001495*X + 0.8385 (*)	n.s.
Arc (VS)	Y = 0.004844*X + 0.5859 (*)	Y = 0.003210*X + 0.7819 (*)	n.s.

females showed significant reductions in the time spent in the centre starting at the 3 mg·kg^{-1} dose, whereas males only exhibited a significant reduction at the highest dose (10 mg·kg^{-1}).

3.4 | NEP only induces locomotor sensitisation in male mice

When studying locomotor sensitisation induced by NEP, we chose the 10 mg·kg^{-1} dose for the sensitisation phase, because it elicited differential acute locomotor effects in males and females. When comparing HLA induced by priming and challenge doses of NEP, a significant interaction between *Sex* and *Time* was observed. Male mice that received repeated NEP doses displayed a significant increase in HLA (almost threefold) after the challenge dose compared with the priming dose, which is indicative of locomotor sensitisation (Figure 2b). In contrast, females pretreated with NEP showed a similar increase in HLA after the challenge dose, compared with the priming dose, which did not reach statistical significance. Therefore, only males exhibited sensitisation after this treatment. Moreover, those treated with NEP also showed increased locomotion when challenged with saline on day 18 (Figure 2c).

During the 5 days of sensitisation, the increased locomotion induced by NEP in males was above the activity of females from day 3 to 5 (Figure 2d). Moreover, the distance travelled by males increased along the sensitisation days, showing a significantly higher locomotion at day 5 with respect to day 1, whereas it remained similar in females.

No differences between the expression levels of *Arc* and *Prodyn* after priming and challenge with NEP were found. Nevertheless, the expression of *Ppp1r1b* and *Csnk1e* after the challenge with NEP was significantly higher than after priming only in males (Figure 2e–h).

3.5 | Males suffer stronger NEP-induced hyperthermia after repeated administration

On the first day of treatment (Figure 3a), NEP induced hyperthermia only in males (Figure 3b), especially from 10 min after the second daily administration, and this effect was present along the treatment days (Figure 3c–f). The tracing of temperatures from NEP-treated males always remained above those from females. However, females started showing significant hyperthermia around 1 h after the second daily dose from day 2 but, at day 5, it already manifested 10 min after this administration (Figure 3f).

As it is shown in Figure 3c–f, the larger differences between sexes in the ability of NEP to increase body temperature decreased along the treatment, as females started showing hyperthermia after the second dose, so that from day 4 the triple factor interaction did not reach statistical significance. In fact, it can be observed that, from day 4, female mice exhibited higher temperatures after receiving NEP than in the previous days.

In addition, when comparing the increases in temperature on day 1 versus day 5 after NEP administration, those reached on day 5 were always above those of day 1 (Figure 3g), suggesting that there is no tolerance to the hyperthermia after repeated dosing. Even some time points showed significantly higher increases on day 5 versus on day 1, rather pointing to a certain sensitisation to the hyperthermic effects of NEP.

3.6 | Females show higher increase in immobility time in the FST after repeated administration withdrawal

To investigate the residual effects of NEP following repeated exposure and withdrawal (procedure depicted in Figure 3a), we performed the FST. This test revealed that females showed an increase in immobility time 5 days after treatment with respect to saline-treated controls, whereas males did not show any changes (Figure 3h).

3.7 | NEP induces similar CPP in both sexes, but males are more resistant to extinction and reinstate the preference with lower doses

Analysis of the CPP conditioning scores reported no significant effect of the variable *Sex* or a *Treatment* × *Sex* interaction. When analysing separately both sexes, one-way ANOVAs showed that NEP induced CPP at all the tested doses (Figure 4b).

For male mice, reinstatement of place preference was exclusively obtained by a priming dose of 0.5 mg·kg^{-1} NEP (R 50%) in the group conditioned with 1 mg·kg^{-1}. Lower priming doses did not induce reinstatement. Similarly, in the group conditioned with 3 mg·kg^{-1}, reinstatement of preference occurred only with a priming dose of 1.5 mg·kg^{-1} (R 50%). The group conditioned with 10 mg·kg^{-1} was not tested for reinstatement of the preference because conditioned behaviour was not extinguished (Figure 4c).

In the case of female mice, reinstatement of the preference was only obtained in the group conditioned with 10 mg·kg^{-1} and only after a priming dose of 5 mg·kg^{-1} (R 50%) (Figure 4c).

Regarding the number of extinction sessions needed for each treatment group, Kaplan–Meier analysis of the data revealed sex differences at the doses of 1 and 10 mg·kg^{-1}. Females conditioned with the doses of 3 and 10 mg·kg^{-1} of NEP required a higher number of extinction sessions compared with the dose of 1 mg·kg^{-1}. Males conditioned with 10 mg·kg^{-1} of NEP (this group had not reached extinction after 31 sessions) needed significantly more sessions than the group conditioned with 1 mg·kg^{-1} and 3 mg·kg^{-1}. With respect to *Sex*, males conditioned with 1 or 10 mg·kg^{-1} NEP required more extinction sessions than the matching female group (Figure 4d).

3.8 | NEP at a high dose (0.75 mg·kg^{-1}) facilitates acquisition and consumption in the SA paradigm in females

During the FR1 schedule of reinforcement phase, NEP (0.75 mg·kg^{-1}) enhanced the number of active nose pokes and infusions in female mice compared with males mice from day 3 until day 10 (Figure 5b(A,B)). Consequently, females performed higher active nose pokes and increased the drug intake during the FR1 than males (Figure 5b(F)).

Likewise, female mice showed a higher level of acquisition of SA behaviour than males (89.47% of females vs. 31.25% of males). According to the log-rank test, the level of acquisition differed between the sexes and, while males did not achieve the 50% during the 10 days of SA, females reached this percentage on day 4. Indeed, only five males accomplished the acquisition criteria (representing 16.6% of experimental male mice) and could be subjected to FR5 schedule of administration, whereas 17 females accomplished the criteria (Figure 5b(C,D)). No sex differences were found for the day of acquisition (Figure 5b(E)).

Notwithstanding the effect of *sex* observed during FR1, no significant differences were found between male and female performance during the FR5 schedule of reinforcement (Figure 5b(A,B,F)), although a significant *hole* effect indicated that mice discriminated between the active and the inactive hole during this phase. Accordingly, no significant differences in the overall drug intake during FR5 were found.

After FR5, mice accomplished one PR session in which females reached a higher breaking point and self-administered more drug than males (Figure 5b(G,H)).

Total drug intake during all the phases (FR1, FR5 and PR) showed that females consumed more NEP than males along the SA procedure (Figure 5b(I)).

3.9 | A low dose of NEP (0.25 mg·kg^{-1}) induces similar reinforcing effects in both sexes

Concerning the infusion dose of 0.25 mg·kg^{-1}, no significant sex differences were found neither for nose pokes nor for infusions during the FR1 phase (Figure 5c(A,B)). In the same vein, no significant divergences were detected regarding the acquisition of SA behaviour (78.57% of females vs. 57.14% of males, Fisher's exact test. n.s.). Similarly, the acquisition day and the acquisition curve did not significantly differ between sexes (Figure 5b(C–E)). Also, no statistically significant differences were found between the SA curves of both sexes during the FR5 phase (Figure 5c(A,B), right panels).

Moreover, no significant differences were revealed when analysing drug intake during FR1, FR5 and PR, total drug intake or breaking point (Figure 5c(F–I)).

3.10 | NEP pharmacokinetics showed no relevant differences between sexes

Pharmacokinetic analysis showed no relevant sex differences in central and peripheral distribution nor in transfer rates of NEP between compartments and elimination from compartments (see Table 2). Observed and predicted pharmacokinetic profiles obtained in females and males in serum and brain are shown in Figure 6.

Higher C_0 concentrations in females versus males were obtained in the model because of the higher amount of extrapolated A in the female group at time 0 in the central compartment (see Supporting Information).

4 | DISCUSSION

In this study, we investigated sex differences in the effects of NEP. We found that male mice were more sensitive to NEP psychostimulant effects than females and developed motor sensitisation at the dose tested, whereas females did not. Attempting to compare our results with those from previous reports, we reviewed the literature dealing with sex differences in the effects of MDMA in rodents, due to its similar structure bearing a methylenedioxy group and because NEP has been reported to be sometimes used as a MDMA counterfeit (Eiden et al., 2019). Overall, Palenicek et al. (2005) and Walker et al. (2007) reported that MDMA elicits greater locomotor activation in female rats than in males, whereas McNamara et al. (1995) found similar locomotor activation but with earlier onset in males and longer duration in females. However, one study in pubescent Long–Evans rats (Koenig et al., 2005) reported that MDMA induces higher hyperlocomotion in males, whereas the other studies were carried out in other rat strains. This suggests that not only the species but also the strain may play a role in the sex-based pharmacological differences.

On the other hand, a previous study from our group (Espinosa-Velasco et al., 2022) revealed that male Swiss CD-1 mice acutely administered with NEP spent more time in the open arms in the Elevated Plus Maze (EPM) test, a common behavioural response to anxiolytic drugs. However, in the present study, the analysis of the behaviour in the OF demonstrated that, after acute administration of NEP, both male and female Swiss CD-1 mice showed thigmotaxis, a common behavioural response to anxiogenic drugs in the OF,

TABLE 2 Predicted pharmacokinetic parameters in male and female mice after administration of 10 mg·kg^{-1} (i.p.) of NEP. Relationships between brain and serum disposition are shown with AUC obtained from observed data. Microconstants (k_{10}, k_{12} and k_{21}), macroconstants (α and β) and *A* and *B* values corresponding to the fitted bicompartmental model are included in a table shown in "Pharmacokinetic data analysis" in the Supporting Information.

		Males			Females		
Parameter	**Units**	**Mean value**	**SD**	**CI 95%**	**Mean value**	**SD**	**CI 95%**
C_0	ng·L^{-1}·kg^{-1}	109.6	2.9	89.6–127.1	194.66	12.69	164.3–222.0
V_c	L·kg^{-1}	1.6	0.1	1.3–2.0	1.54	0.10	1.3–1.7
V_p	L·kg^{-1}	4.6	0.3	3.9–5.3	5.35	0.43	4.3–6.2
V_{dss}	L·kg^{-1}	80.8	6.6	68.1–96.6	65.92	4.30	55.6–75.2
CL_p	L·min^{-1}·g^{-1}	8.1	0.4	5.8–8.1	6.94	0.45	5.8–7.9
$t_{1/2}$	min^{-1}	8.1	0.4	7.1–9.1	6.59	0.38	5.8–7.3
Serum							
$AUC_{0\text{-last}}$	ng·min·L^{-1} ·kg^{-1}	2281	631.7	2039.6–2530.2	2690.23	987.79	2335.2–3038.9
Brain							
$AUC_{0\text{-last}}$	ng·min·L^{-1} ·kg^{-1}	879.9	159.1	815.5–947.0	1043.31	202.85	950.7–1429.8
Ratio B/S $_{obs}$		0.38		0.37–0.39	0.39		0.37–0.41

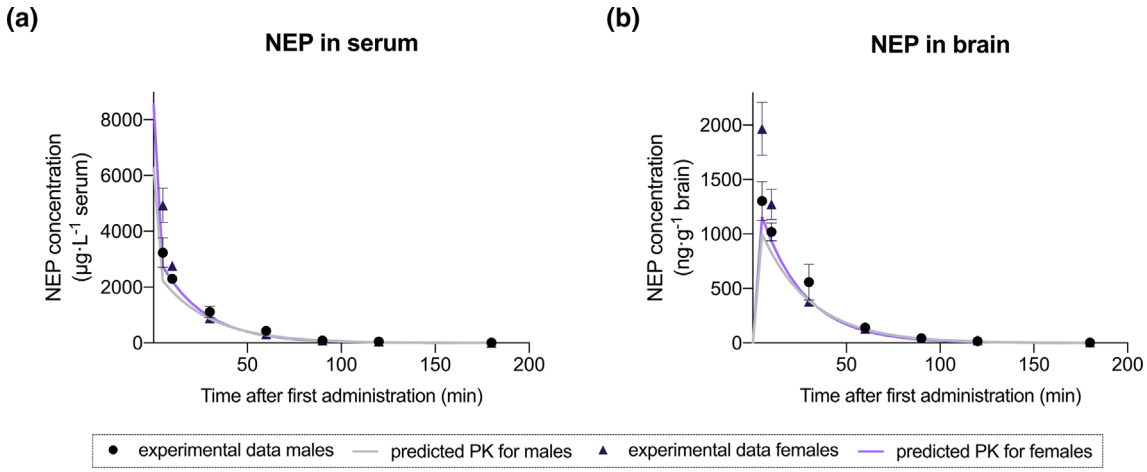

FIGURE 6 Time course of NEP levels in serum (a) and brain (b) after an IP administration of 10 mg·kg^{-1}. Experimental points are the means ± SEM of the values originating from five animals each.

although this does not necessarily mean the presence of anxiety. Such discrepancies could be explained by the different protocols (EPM vs. OF) and experimental conditions used (i.e., habituation, light), as demonstrated by other studies (Carola et al., 2002).

Studies in rodents (Costa & Gołembiowska, 2022) coincide in the observation that males are more sensitive than females to the hyperthermic and neurotoxic effects of MDMA. This is in line with our results in which hyperthermia appeared sooner and was higher in male mice after acute and repeated administration of NEP. Moreover, the hyperthermia after the second daily dose with respect to the controls was higher than after the first, pointing to a persistent effect on thermoregulatory mechanisms within the same day.

On the other hand, the occurrence of hyperthermia along the five treatment days suggests a lack of tolerance to these effects, but instead a trend to sensitisation, further supported by the higher temperature increases on day 5 compared with day 1. Overall, such hyperthermic effects raise concerns about an additional health risk because they may enhance the acute toxic potential of NEP, as it occurs with other structurally related amphetamine derivatives such as MDMA (Green et al., 2003). Such increased toxicity can lead to emergencies and even fatalities.

However, we must consider our previous study (Espinosa-Velasco et al., 2022) including the measurement of monoamine content (dopamine, 5-HT and noradrenaline) in striatum and prefrontal cortex 72 h and 21 days after a 5-day treatment with two daily injections of 1, 3 or 10 mg·kg^{-1} of NEP. The most significant changes included decreases in these monoamines 72 h after receiving the highest dose (10 mg·kg^{-1}), but all these alterations had disappeared 21 days after

the treatment, suggesting that NEP, at these doses, does not induce significant persistent neurotoxicity.

In the FST performed after 5 days of withdrawal following the repeated administration procedure, only females showed increased immobility time. An increase in this parameter is considered a sign of behavioural despair, which is commonly associated to depressive behaviour (Porsolt et al., 1977), as it is normalised by treatment with antidepressants. However, caution must be taken with this assumption, especially when translating these findings to humans. This observed sex difference may be a consequence of the neurochemical alterations described in our previous paper (Espinosa-Velasco et al., 2022), which might not have fully reversed even 5 days after treatment. Further investigation is needed to understand this phenomenon completely.

To assess the rewarding and reinforcing effects of NEP, CPP and SA experiments were performed, respectively. Although CPP showed no significant differences between males and females, these appeared when assessing extinction and reinstatement. Females extinguished CPP produced by the three tested doses of NEP, and only those receiving the highest dose reinstated this behaviour. By contrast, males needed more extinction sessions than females to extinguish CPP caused by 1 and 3 mg·kg^{-1} and reinstated the behaviour in both cases. Moreover, males had not reached extinction of the CPP induced by the 10 mg·kg^{-1} dose after 31 extinction sessions. These results suggest that, concerning drug reward and conditioning, NEP induces more persistent effects in male than in female mice.

In addition, SA experiments revealed that NEP, at the highest dose tested, induced stronger responses in females than in males. Also, females progressively increased the number of infusions per day and nearly 100% acquired the SA behaviour, whereas males maintained the number of daily infusions at a lower level and only 30% accomplished the SA requirements by the end of FR1. However, no sex differences were observed with the low dose (0.25 mg·kg^{-1}). Such differences, taken together with the other effects mentioned above, could be explained by the fact that males seem to be more sensitive to the effects of NEP, which may induce unpleasant experiences, leading to a reduction in drug intake. In line with this, we previously reported that, in male mice, the injection of a high dose of NEP (30 mg·kg^{-1}) induced a lower increase in HLA than a 10 mg·kg^{-1} dose (Nadal-Gratacós et al., 2021). On the other hand, enhanced hyperthermia might also contribute to the reduced SA assessed with the 0.75 mg·kg^{-1} NEP dose in males. Overall, our data suggest that NEP induces similar reinforcing effects in males and females but, from certain doses upwards, the higher sensitivity of males to some of the adverse effects may prevent them from increasing SA.

We initially considered that pharmacokinetics of NEP might account for the observed differences. For this reason, we determined the time course of the levels of NEP in the brain and serum after an acute dose. However, the results clearly showed that there are no relevant differences in the kinetics of NEP between sexes, ruling out a pharmacokinetic cause.

We then investigated potential pharmacodynamic differences by assessing the expression of specific genes related to addiction. Acute and repeated administration of addictive drugs triggers the expression of IEGs, which will be responsible for activating and reinforcing the neuronal pathways leading to drug addiction (Nestler, 2012).

C-fos is an IEG whose expression is used as an indirect indicator of recent neuronal activation, as it is rapidly and transiently expressed in neurons after receptor activation and depolarisation (VanElzakker et al., 2008). Thus, the magnitude of the increase in *C-fos* mRNA is indicative of the acute potency of a drug at stimulating neurons. Increases in *C-fos* mRNA have already been reported by our group after acute administration of 10 mg·kg^{-1} of NEP to male mice (Nadal-Gratacós et al., 2021). In the present work, we found similar increases in males and females at the 10 mg·kg^{-1} dose, with lower doses inducing lower increments, suggesting a dose-effect relationship. The differences between the increases in males and females did not reach statistical significance although we observed a tendency towards higher increases in ventral striatum of males.

On the other hand, we assessed *Arc* mRNA, which is increased in dendritic processes and involved in neuronal plasticity procedures that follow dopaminergic activity (Gallo et al., 2018; Kovács, 2008). In the present work, the *Arc* increases found in males treated with 10 mg·kg^{-1} NEP were higher than those found in females. This suggests that NEP could induce stronger neuroplastic effects in males than in females, and this may underpin the long-lasting conditioning effects observed in males in the CPP experiments. In line with this result, *Bdnf* mRNA was also increased after NEP (10 mg·kg^{-1}) and the overexpression in males was significantly higher than in females. The increase in this neurotrophin may explain the more sustained Arc levels over time induced by NEP and other synthetic cathinones such as MDPV (Duart-Castells et al., 2019; Nadal-Gratacós et al., 2021) because BDNF is able to enhance the synthesis of Arc protein and up-regulate *Arc* mRNA levels (Yin et al., 2002).

The induction of these IEGs after acute drug exposure has also previously been reported for other cathinones such as MDPV. Caffino et al. (2021) described an increase of *Bdnf* mRNA in frontal cortex of about 35% 2 h after an administration of 1 mg·kg^{-1} MDPV, whereas, in the present work, we found a 40% increase after a dose of 10 mg·kg^{-1} NEP. Regarding *C-fos*, Giannotti et al. (2017) reported increases in striatum of around 100% after 1 mg·kg^{-1} MDPV, and Wojcieszak et al. (2019) showed a 400% increase after 3 mg·kg^{-1} MDPV, whereas we report an increase around 130% with 10 mg·kg^{-1} NEP. Finally, Giannotti et al. (2017) also detail an *Arc* increase of around 100% in striatum after 1 mg·kg^{-1} MDPV, which is similar to that we found after 10 mg·kg^{-1} NEP. Overall, data suggest that the induction of IEGs expression depends on the dose and the potency of the drug. In fact, the increase in locomotor activity reported after acute injection of 0.3 mg·kg^{-1} MDPV (around 80%) (Buenrostro-Jáuregui et al., 2016) is similar to that reported in the present and previous work (Nadal-Gratacós et al., 2021) for a 3 mg·kg^{-1} dose of NEP. These comparisons suggest that MDPV is roughly 10 times more potent than NEP in inducing hyperlocomotion and IEGs expression,

despite having a slightly higher molecular weight (1.09-fold that of NEP).

This observation led us to investigate the relationship between hyperlocomotion and increase in IEGs expression by correlating HLA with the expression of both *Arc* (in dorsal and ventral striatum) and *Bdnf* from the mice treated with saline and NEP (10 $mg \cdot kg^{-1}$). In both cases, we found a significant positive correlation between HLA and gene expression. This finding corroborates that increased locomotor activation, probably due to increased dopaminergic and serotonergic activation, is associated with a higher induction of IEGs expression. Notably, the lack of differences between the slopes of the regression lines from males and females indicates that this relationship is similar in both sexes.

We also assessed the mRNA levels of some genes, which may be involved in the increased hyperlocomotion observed after the sensitisation procedure. *Arc* mRNA levels were not modified after the sensitisation procedure with respect to the priming dose of 3 $mg \cdot kg^{-1}$. This could be explained by the fact that *Arc* mRNA levels peak within 1 h after acute drug administration and return to basal levels by 6 h (Kodama et al., 1998). This implies that, after the sensitisation phase and the subsequent withdrawal, the levels of *Arc* mRNA should have returned to basal and, after the challenge, the only increase should originate from the challenge dose, which was low and non-significant. This also suggests that there does not exist sensitisation of Arc expression.

By contrast, *Csnk1e* and *Ppp1r1b* mRNA levels, assessed after the final drug challenge, were significantly increased in males but not in females. The gene product of *Csnk1e*, casein kinase-1, phosphorylates and increases the activity of the DARPP-32 protein, which is known to be a critical regulator of the locomotor response to dopaminergic drugs. DARPP-32 phosphorylates and inhibits protein phosphatase 1, an inhibitor of locomotor stimulation (Greengard, 2001). On the other hand, DARPP-32 protein (encoded by *Ppp1r1b*) is phosphorylated after **dopamine D_1 receptor** stimulation, whereas **D_2 receptor** stimulation leads to its dephosphorylation, thus modulating striatal dopamine cellular excitability and synaptic plasticity related to dopamine receptors (Gould & Manji, 2005). Overall, these molecular changes would result in increased hyperlocomotion and contribute to the locomotor sensitisation observed only in males. Unfortunately, it was not possible to relate the gene expression along the sensitisation procedure with the HLA, because the mice used for post-priming gene analysis were not the same ones that remained after the challenge dose. We also attempted to correlate the HLA after challenge with the levels of *Csnk1e* and *Ppp1r1b* but, in this case, no significant correlation was found (data not shown).

Looking for complementary upstream causes, a dopamine superfusion study (Bhatt & Dluzen, 2005) reported that female CD-1 mice exhibit higher dopamine uptake and vesicular packaging activity than males. Also, other authors have reported higher striatal DAT densities in female rats (Rivest et al., 1995) and humans (Lavalaye et al., 2000). This may account for a milder effect of NEP, as increased dopamine would be more avidly taken up before activating post-synaptic receptors.

To summarise, this work is the first to report sex differences in the effects of NEP in mice. Males seem to be more sensitive than females to its psychostimulant effects, developing locomotor sensitisation and experiencing longer CPP. These differences cannot be attributed to pharmacokinetic factors, but they may be to differential gene expression and lower DAT density in males. Additionally, males appear to be more vulnerable to the adverse effects of NEP, as suggested by the stronger hyperthermia and reduced SA at high doses. Females, in turn, might be more prone to NEP abuse as they tolerate higher SA doses and exhibit milder increases in body temperature. Although complementary studies are needed to detail the underlying mechanisms, a warning should be posted about NEP, which may differently affect men and women, leading to different abuse liability and undesirable effects.

AUTHOR CONTRIBUTIONS

D. Pubill, R. López-Arnau and E. Escubedo conceived the project, designed, analysed and supervised the locomotor activity, sensitisation, FST, temperature measurements, pharmacokinetic and qPCR experiments. M. Rodríguez-Arias and O. Valverde designed, analysed and supervised the CPP and SA experiments, respectively. M. Espinosa-Velasco carried out locomotor activity, sensitisation, FST, temperature measurements, pharmacokinetic and qPCR experiments, performed data analysis and contributed to the manuscript draft writing. O. Rublinetska contributed to temperature measurements, FST and some qPCRs experiments. A. Castro-Zavala, I. Gallego-Landin and O. Valverde carried out the SA experiments. M.D. Reguilón and M. Rodríguez-Arias conducted the CPP experiments. N. Nadal-Gratacós and X. Berzosa synthesised and purified NEP. C. Gómez-Canela and M. Bellot performed sample processing and UHPLC-MS/MS analysis of NEP in the pharmacokinetics experiment. M. Carbó performed the pharmacokinetics data analysis. J. Camarasa contributed to data analysis and curation. D. Pubill and R. López-Arnau wrote the manuscript. I.Gallego-Landin checked and corrected language and grammar of the revised version. All authors critically read and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

All data generated or analysed during this study are included in this published article (and its supporting information files) and are available from the corresponding authors upon reasonable request.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

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

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