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ORIGINAL ARTICLE

Designer Cathinones *N*-Ethylhexedrone and Buphedrone Show Different In Vitro Neurotoxicity and Mice Behaviour Impairment

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Abstract

N-Ethylhexedrone (NEH) and buphedrone (Buph) are emerging synthetic cathinones (SC) with limited information about their detrimental effects within central nervous system. Objectives: To distinguish mice behavioural changes by NEH and Buph and validate their differential harmful impact on human neurons and microglia. In vivo safety data showed the typical induced behaviour of excitation and stereotypies with 4–64 mg/kg, described for other SC. Buph additionally produced jumping and aggressiveness signs, while NEH caused retropulsion and circling. Transient reduction in body-weight gain was obtained with NEH at 16 mg/kg and induced anxiolytic-like behaviour mainly with Buph. Both drugs generated place preference shift in mice at 4 and 16 mg/kg, suggestive of abuse potential. In addition, mice withdrawn NEH displayed behaviour suggestive of depression, not seen with Buph. When tested at 50–400 μM in human nerve cell lines, NEH and Buph caused neuronal viability loss at 100 μM, but only NEH produced similar results in microglia, indicating different cell susceptibilities. NEH mainly induced microglial late apoptosis/necrosis, while Buph caused early apoptosis. NEH was unique in triggering microglia shorter/thicker branches indicative of cell activation, and more effective in increasing microglial lysosomal biogenesis (100 μM vs. 400 μM Buph), though both produced the same effect on neurons at 400 μM. These findings indicate that NEH and Buph exert neuro-microglia toxicities by distinct mechanisms and highlight NEH as a specific inducer of microglia activation. Buph and NEH showed in vivo/in vitro neurotoxicities but enhanced specific NEH-induced behavioural and neuro-microglia dysfunctionalities pose safety concerns over that of Buph.

Keywords Synthetic cathinones · Addiction potential · Microglia activation · Human cell culture models · Mice behavioural tests · Neurotoxicity

Introduction

A considerable and increasing worldwide public health challenge is related with the abuse of new psychoactive substances (NPS), a variety of novel synthetic drugs divided into several chemical group derivatives, being synthetic cannabinoids and cathinones two of the most representative and extensively used compounds (EMCDDA 2019; Schifano et al. 2019). These drugs were not included, at first, under international legislation control, which allowed an easier and wide dissemination. The European Monitoring Centre of Drugs and Drug Addiction (EMCDDA) was one of the first international organizations to put a focus on these drugs and making a close monitoring of their appearance in Europe through its Early Warning System (EWS). Sixty-six NPS were detected in 2016—a rate of over one per week (EMCDDA 2017c)—and by the end of 2018 the monitoring was covering more than 730 NPS as a result of formal

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European notifications since 1997 (EMCDDA 2018b). In spite of recent reports mentioning that fewer new psychoactive substances are now registered for the first time at European level on a yearly basis (EMCDDA 2018a), there is a growing evidence of the occurrence of harmful effects by the consumption of these drugs.

Synthetic cathinones (SC), commonly known as 'bath salts', are chemical derivatives related with cathinone, a natural stimulant drug found in the khat plant (*Catha edulis*), which were first detected in Europe in 2008. Their effects are similar to other illicit stimulants, such as cocaine, amphetamine or MDMA and stands as the second largest group of NPS generally seized and abused. It is a major concern the misuse of NPS taken in certain settings, namely by other drug abuse profiles, such as opioid users, making use of different administration procedures (injection), which seems to be associated with increased levels of physical and mental health problems (EMCDDA 2017a). These substances are ring-substituted phenylethylamines with a substitution of a ketone group at the β-carbon position. Different R-group substitutions give rise to a large list of SC, and many of them are identical except for the β-carbon ketone group (Spiller et al. 2011). Although often seen as an alternative to MDMA, amphetamines and cocaine due to their stimulant psychoactive effects, only limited information on the pharmacokinetics and pharmacodynamics of SC is available but, like amphetamines and cocaine, they are thought to act on the central nervous system (CNS), promoting release of monoamine neurotransmitters, and most likely inhibiting their reuptake. SC can elicit powerful effects such as delusions, hallucinations and potentially dangerous behaviour (Baumann et al. 2014; EMCDDA 2017a; EMCDDA 2017c; Valente et al. 2014).

The physical and psychological detrimental effects caused by NPS consumption rely mostly on data from NPS hospital emergency presentations, post-mortem toxicology examinations, fatal and non-fatal intoxications reported to EWS, user surveys, regional and national poison information services, single-case and scientific studies, as there are no clinical trials available on NPS usage (EMCDDA 2017b). Concerning SC, the pharmacological effects and the neurotoxic potential of the majority of them are still scarcely described except for mephedrone (4-methylmethcathinone or 4-methylephedrone) (Martinez-Clemente et al. 2014; Schifano et al. 2019) and methedrone (4-methoxymethcathinone or methoxyphedrine) (Pail et al. 2015; Soares et al. 2019). In 2015, a new SC, N-ethylhexedrone (2-(ethylamino)-1-1phenylhexan-1-one, Hexen or NEH), has appeared in the illicit drug market, and has become popular ever since. It was first reported by the EMCDDA in February 2016 (Hungary, Slovenia and the Netherlands) (EMCDDA 2017b). NEH is the *N*-ethyl derivative of a previously notified SC, hexedrone (β-propylmethcathinone). It has a structural similarity to alpha-PHP (α-pyrrolidinohexanophenone) and was first mentioned in a patent on aminoketone derivatives in the 1960s (Herbert et al. 1965). According to users, NEH has fast-acting but short-lived euphoric stimulant effects, which are comparable with those produced by crack-cocaine, particularly when insufflated or vaporized. Therefore, it is expected that this SC will lead to compulsive redosing and addictive behaviours on its users, while inducing paranoia, anxiety, various delusional states and stimulant psychosis (Erowid 2018). Buphedrone (2-(methylamino)-1-phenylbutan-1-one, α-methylamino-butyrophenone, MABP or Buph) was first synthesized in 1928 for studies with synthetic homologues of ephedrine. Like many other novel substances, Buph reappeared on the drug market as a result of scientific and patent literature searches for failed pharmaceuticals by manufacturers of 'legal high' preparations. In recent years, it has been produced to be sold for its psychoactive properties and used as a recreational drug (Zuba et al. 2013).

International drug agencies (FDA, EMA, EMCDDA) suggest that abuse-related animal behavioural studies should be performed up on observation of a test drug or any of its major metabolites that interferes with CNS-function. These in vivo studies evaluate whether those substances produce any animal behavioural changes that may suggest abuse potential in humans. Safety studies for drug affecting general behaviour (including motor performance and Irwin test) show if it produces abuse-related signals (e.g. stimulation including hyperactivity or anxiogenic effects). Specific abuse-related studies, namely conditioned place preference test, are important to evaluate whether a drug has rewarding or reinforcing properties (FDA 2017).

In vitro studies are important tools to perform the first screening of NPS-induced toxicity, providing consistent data in terms of cellular function. Behavioural alterations have been related to the underlying neurobiological mechanisms (Annau and Cuomo 1988), but a better understanding of the toxicity of NPS, namely cathinones, needs to consider both behavioural and neurobiological assays. However, little is known about their neurotoxic effects and even less on the neuroinflammatory processes that they may trigger. Indeed, several studies relate the existence of glial activation and the release of pro-inflammatory mediators with neurodegeneration (Brites and Vaz 2014; Ugolini et al. 2018). Additional studies on SC-induced neuroinflammation are required, as well as on the neuroimmune mechanisms underlying their neurotoxic effects. In this regard, human cell lines can provide an important tool to collect additional information in terms of mechanisms that can be species specific, such as the pathways involved in the neuroinflammatory processes (Kodamullil et al. 2017), emphasizing the need of systematic comparisons between human samples and the animal model, as well as possible factual findings in rodents (either mice or rats). For neurotoxicity, it is only known that cathinone derivatives are inducers of oxidative stress and mitochondrial dysfunction

(den Hollander et al. 2014; den Hollander et al. 2015; Wojcieszak et al. 2016). Lately, new results were obtained for 13 different SC, including Buph, in differentiated human SH-SY5Y neuronal cells, where pro-apoptotic effects and autophagic potential were demonstrated (Soares et al. 2019). However, authors do not provide data for the cathinone NEH, as we will cover here. More importantly, alterations on immunoregulatory properties of microglia, the immunocompetent cells in the CNS, by SC are sparse and only scarcely documented (Angoa-Perez et al. 2017). Therefore, it is important to unveil NEH effects in terms of its general neurotoxicity, mechanisms of action and induced dysfunction on neuronal and non-neuronal cells like microglia. Moreover, to the best of our knowledge, no reports were made to date on the neuroinflammatory potential of either NEH or Buph. In this study, we used multiple in vivo and in vitro assays to evaluate the behaviour and the neurotoxicological/neuroinflammatory effects of NEH when compared with other SC as Buph, known for its rewarding and reinforcing properties (Oh et al. 2018).

Materials and Methods

Compounds

Buphedrone (Buph) [2-(methylamino)-1-phenylbutan-1-one;hydrochloride] and N-ethylhexedrone (NEH) [2-(ethylamino)-1-phenyl-1-hexanone;hydrochloride] were synthesized as described below (Fig. 1). Methedrone [1-(4-methoxyphenyl)-2-(methylamino)propan-1-one;hydrochloride] was previously characterized by us using a battery of analytical methods including Fourier transform infra-red (FTIR), GC–MS, NMR and wavelength dispersive X-ray fluorescence (Zancajo et al. 2014). Each tested SC was dissolved either in phosphate buffer saline (PBS) for in vitro studies or in sterile 0.9% saline for intraperitoneal administrations (i.p., 10 mL/kg). All tested solutions were daily prepared.

Synthesis and Characterization

General Procedures Reagents and solvents were bought from commercial sources and used without further purification. HCl was generated by slow dropping of sulphuric acid on hydrochloric acid and dried by bubbling on sulphuric acid. Proton and carbon nuclear magnetic resonance spectra (^{1}H and ^{13}C NMR) were recorded on a Bruker Avance II 300, operating at 300 and 75 MHz, respectively. Chemical shifts are quoted on the δ scale, in ppm, using residual solvent peaks as internal standard. Coupling constants (J) are reported in hertz with the following splitting abbreviations: s = singlet, br. s = broad singlet, d = doublet, t = triplet, q = quartet, p = quintet, m = multiplet. Thin layer chromatography (TLC) was carried out using Merck aluminium backed sheets coated with 60 F254 silica gel. Visualization of the silica plates was achieved under UV light. Flash column chromatography was performed using Merck 60 (230–400 mesh ASTM) silica gel. High-resolution ESI positive mode mass spectra of buphedrone and N-ethylhexedrone were performed in a Waters Acquity I-Class UPLC system (Waters, Milford, MA, USA), interfaced to a VION IMS-QTOF mass spectrometer, using an electrospray ionization (ESI) interface, operating in positive mode. The chromatographic separation was performed using a Cortecs C18 2.1 × 100 mm, 2.7 μm (Waters) at a flow rate of 0.3 mL/min. Gradient elution was performed using mobile phases of A = H_2O and B = MeOH, both with 0.01% HCOOH. The initial percentage of B was 10%, which was linearly increased to 90% for 14 min, followed by a 2-min isocratic period, then, immediately returned to initial conditions for 2 min. The total run time was 18 min. Nitrogen was used as the drying gas and nebulizing gas. A capillary voltage of 0.7 kV and cone voltage of 40 V were used. The desolvation temperature was set to 550 °C, and the source temperature to 120 °C. The cone gas flow was 250 L/h and desolvation gas flow of 1000 L/h. The column temperature was set to 40 °C and sample temperature at 10 °C. MS data was acquired using the

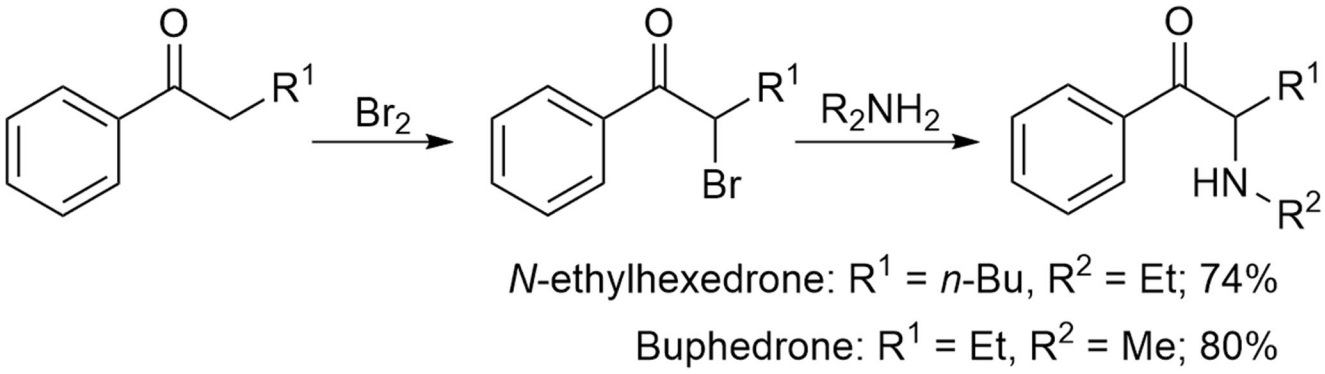


Fig. 1 Synthesis of buphedrone and N-ethylhexedrone

VION in HDMSe mode, in the range 50–1000 m/z, with N2 as the drift gas. Two independent scans with different collision energies were acquired during the run: a collision energy of 6 eV for low energy (LE) and a ramp of 28–56 eV for high energy (HE). The LE and HE functions settings were for both a scan time of 0.3 s. Nitrogen was used as collision-induced dissociation (CID) gas. All data was examined using an accurate mass screening workflow within UNIFI informatics platform from Waters Corporation.

General Procedure for Phenone Brominations Bromine (0.16 mL, 3.0 mmol) was added to a solution of phenone (3.0 mmol) in dichloromethane (5 mL). After stirring for 2 h, dichloromethane was added (20 mL), and the mixture washed with sat. Na_2CO_3 (20 mL), sat. $Na_2S_2O_3$ (20 mL) and pumped to dryness, affording the respective brominated phenone. When necessary, products were purified by flash chromatography, using n-hexane/AcOEt mixtures as eluent.

General Procedure for Amination of Brominated Phenones Amine aqueous solution (12 mmol) was added to brominated products (3 mmol). After stirring overnight at r.t., the mixture was pumped to dryness, affording the respective aminated phenone. When necessary, products were purified by flash chromatography, using CH_2Cl_2/MeOH mixtures as eluent. HCl was bubbled for 2 min in ethanol solutions of the pure cathinones. Solvent was removed under reduced pressure and products were washed with ethyl ether, affording the respective hydrochlorides.

N-Ethylhexedrone hydrochloride: white solid; $\eta = 74\%$. ^{1}H NMR (400 MHz, DMSO-*d6*) δ 9.51 (br. s, 1H, NH_2^+Et), 9.08, (br. s, 1H, NH_2^+Et), 8.10 (d, 2H, $J = 7.6$ Hz, $H_{2,6}$Ph), 7.76 (t, 1H, $J = 7.6$ Hz, H_4Ph), 7.61 (t, 2H, $J = 7.6$, $H_{3,5}$Ph), 5.32 (s, 1H, H_2), 3.03 (s, 1H, NCH_2CH_3), 2.92 (s, 1H, NCH_2CH_3), 1.85–2.00 (m, 2H, H_3), 1.02–1.29 (comp., 7H, H_5, H_4 + NCH_2CH_3), 0.74 (t, 3H, $J = 7.2$ Hz, $3H_6$); ^{13}C NMR (100 MHz, DMSO-*d6*) δ 196.4 (C_1), 135.4 (C_4Ph), 134.4 (C_1Ph), 129.7 ($C_{3,5}$Ph), 129.2 ($C_{2,6}$Ph), 61.0 (C_2), 41.7 (NCH_2CH_3), 29.8 (C3), 26.1 (C_4), 22.3 (C_5), 14.0 (NCH_2CH_3), 11.6 (C_6). ESI-HRMS (+) m/z: 220.16989 $[M+H]^+$; exact mass calcd. for $C_{20}H_{34}N_7O_6$: 220.17014.

Buphedrone hydrochloride: white solid; $\eta = 80\%$.^{1}H NMR (300 MHz, DMSO-*d6*) δ 9.82 (br. s, 1H, NH_2), 9.34 (br. s, 1H, NH_2^+), 8.04 (d, 2H, $J = 7.6$ Hz, $H_{2,6}$Ph), 7.74 (t, 1H, $J =$ 7.6 Hz, H_4Ph), 7.60 (t, 2H, $J = 7.6$ Hz, $H_{3,5}$Ph), 5.27 (s, 1H, H_2), 2.56 (s, 3H, NCH_3), 2.06–2.14 (m, 1H, H_3), 1.85–2.04 (m, 1H, H_3), 0.76 (t, 3H, $J = 7.0$ Hz, H_4); ^{13}C NMR (75 MHz, DMSO-d6) δ 196.5 (C_1), 135.2 (C_4Ph), 134.4 (C_1Ph), 129.6 ($C_{3,5}$Ph), 129.2 ($C_{2,6}$Ph), 63.2 (C_2), 31.7 (NCH_3), 23.1 (C_3), 8.6 (C_4). ESI-HRMS (+) m/z: 178.12242 $[M+H]^+$; exact mass calcd. for $C_{20}H_{34}N_7O_6$: 178.12319.

In Vivo Studies

Animals

Male CD-1 mice (Charles River, France), fed with standard laboratory chow (4RF21 LPG, Mucedola Srl, Milan, Italy) and water ad libitum, were kept at an average temperature of 21 ± 1 °C with 12-h light/dark cycle. Animal experiments were carried out in accordance with the relevant European Community and National rules on the protection of animals used for experimental and other scientific purposes (Directive 2010/63/EU; Decreto-Lei 113/2013) and were approved by the animal Ethics Committee of the Faculty of Pharmacy, Universidade de Lisboa and by the Portuguese National Authority (Directorate-General of Food and Veterinary Medicine). According to the 3R's principle, every effort was made to minimize the number and suffering of animals.

Study Procedure

Single- and repeated-dose regimens were used to study the in vivo effects of the selected SC—Buph and NEH. Mice were handled daily prior to each dosing study in order to acclimatize them to routine contact. Behaviour tests took place during the illuminated part of the cycle (between 8:00 and 16:00) in a room with low noise. For *single-dose studies*, mice were randomly divided in groups of 3 mice/dose. Dose increments were based on a double scale increase from 1 up to 128 mg/kg. Animal behaviour and clinical signs (morbidity and mortality) were monitored for 3 h and then 24 h (D2) and 7 days after dosing according to the behaviour Irwin test. For *repeated-dose studies*, mice were randomly assigned to groups of 7 mice/dose each. Animals were daily administered with 4 and 16 mg/kg of each tested synthetic cathinone, or vehicle, for 4 consecutive days. On post-dose day 1 (D5) conditioned place preference (CPP) and marble burying behaviour were assessed, the latest occurring 2 h after the CPP test. On post-dose day 3 (D7) the splash test was conducted under reduced illumination before sacrifice. Mentioned behaviour tests are described below. Individual body weights were monitored during both the single- and the repeated-dose studies. Body-weight changes were calculated with, final body weights recorded prior to animal sacrifice, and a gross necropsy was conducted. Figure 2 depicts a diagram of both single- and repeated-dose protocol.

Primary Observation (Irwin) Test

Assessment of mice behaviour was done simultaneously by two observers, blind to the treatment, using the procedure previously described (Roux et al. 2005). Briefly, for each studied dose, three mice were placed in a transparent container (26.8 × 21.5 × 14.1 cm) 24 h before administration in order to

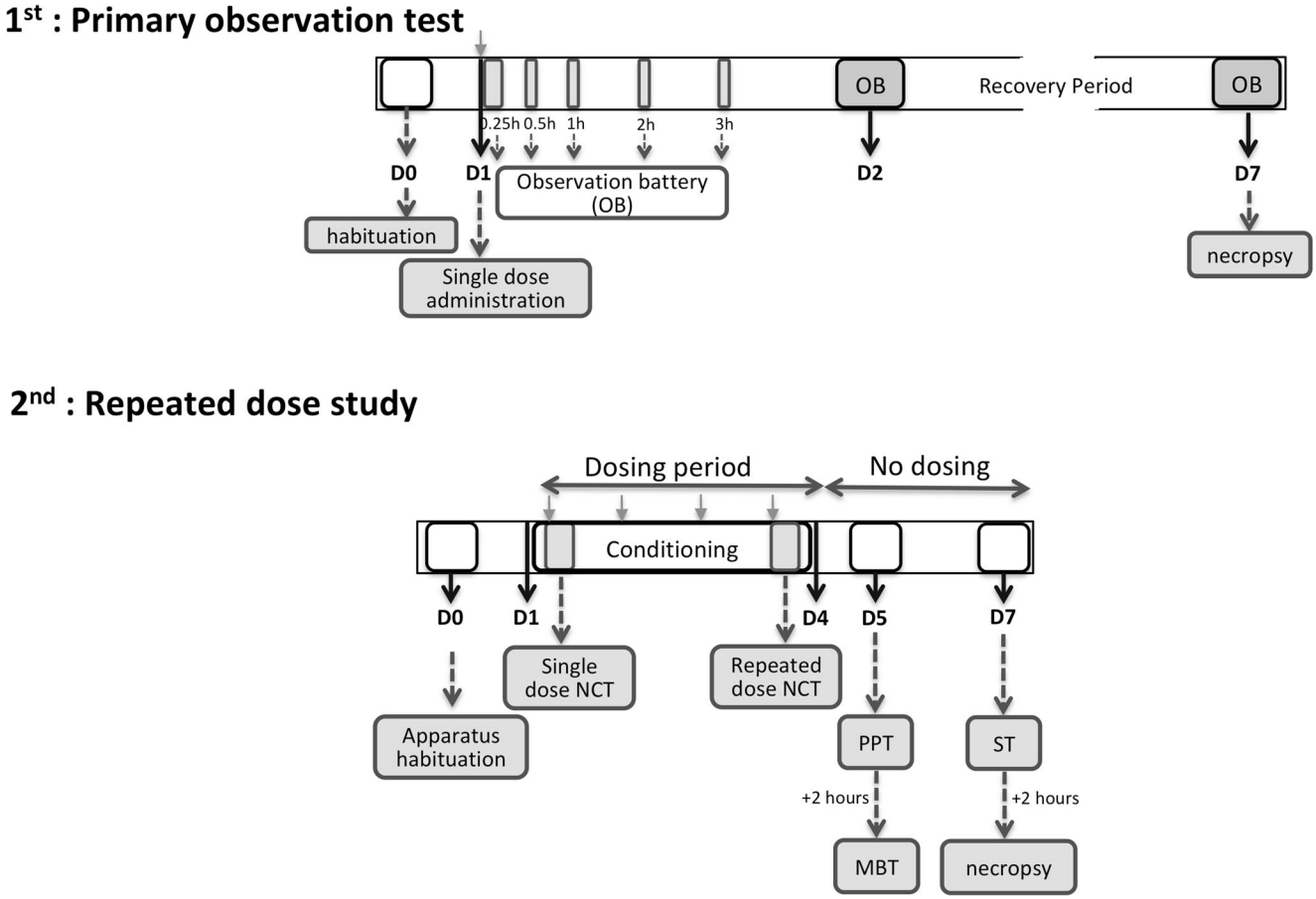


Fig. 2 Diagram of the timeline of the in vivo experimental protocol used indicating both the dosing period and the schedule of behavioural testing: OB observation battery, NCT novel cage test, PPT place preference test, MBT marble burying test, ST splash test. Animals were sacrificed on day 7 and a gross necropsy conducted

classify observable effects of the drug. Each animal group was then administered with Buph or NEH at 1, 4, 8, 16, 32, 64 or 128 mg/kg (i.p.) and observed in simultaneous comparison with a control group given vehicle (saline, non-blind conditions). As many as 2/3 treated groups were compared with one control group at every time-point in each run of observations. Observations were performed at 15, 30, 60, 120 and 180 min, 24 h and 7 days post-administration. Additionally, the symptoms that do not needed handling (lethality, convulsions, straub tail, sedation, excitation, abnormal gait, jumps, motor incoordination, abdominal writhes, piloerection, stereotypes, head twitches, scratching, respiratory movements) were also observed for its presence/absence up to 15 min immediately following administration. All trials were video-recorded and later used to confirm the observable effects at the specific time schedules and to grant no other effects outside the timings. Observations were taken on 50 signs including motor displacement, motor reflexes, stereotypies (licking, biting, head movement, head twitches), grooming, startle response, circling, retropulsion, jumps, analgesia, secretions, respiratory movements, piloerection, ptosis, muscle tone, ataxia, straub tail, tremors and convulsions. All observed signs were assessed as previously described (Roux et al. 2005). A final evaluation of excitation/sedation expresses the final interpretation of a group of signs.

Conditioned Place Preference and Novel Environment

The conditioned place preference (CPP) apparatus consisted on 2 conditioning compartments of equal size (20 × 20 × 10 cm), composed by a black one with smooth floor and a white one with rough floor, connected by a grey corridor (15 × 8 × 8 cm) that remained closed during the conditioning sessions. Four apparatus/trials were used simultaneously. The used procedure was adapted from Karlsson et al. 2014. The habituation was run on the day before first conditioning (D0), in order to reduce any effect of experimental novelty, by placing the mice in the middle corridor and allowing free circulation between compartments for 15 min. Conditioning sessions (D1–D4) were performed on 4 consecutive days; two sessions with a 4-h interval for each animal, one during mornings with saline (10 mL/kg, i.p.) and the second with 0, 4 and 16 mg/kg of tested SC. Immediately after injections, mice were confined to the assigned compartment for 15 min. Animals were

assigned to a 'drug-paired compartment' according to their non-preference on day D1. At D5, place preferences were registered by positioning the animals in the middle corridor and allowing them to circulate freely between compartments for 15 min. All trials were video-recorded on D0 and D5 to score the time spent by animals in each compartment using the 'Any-maze' software (Stoelting Co.). After each test, the apparatus were wiped thoroughly with an ethanol solution (15%) to clean and eliminate the residual odour. Additionally, the number of faecal-boli produced by each animal while being confined for 15 min in the drug compartment (squared arena of 20 × 20 cm)—conditioning phase—was registered in order to investigate the induced behaviour of being in a novel environment, other than their usual maintenance cage.

Marble Burying Test

The emotional effects induced by the repeated exposure to each drug were further evaluated in the marble burying test (MBT). For that clean cages (22 × 38 × 19 cm) filled with 4.5 cm LAB-CHIP sawdust bedding were gently overlaid with 20 glass marbles (15 mm diameter) equidistant in a 4 × 5 rows arrangement. Mice were individually placed in a cage and left undisturbed for a 30-min exploration period (Deacon 2006). A treatment-blind observer counted the number of marbles buried (at least two-thirds covered by bedding material).

Splash Test

The emotional changes induced by the repeated exposure to each drug were further studied by evaluating its effect on mice grooming motivation by using the splash test. This test consists on dirtying the mouse fur by squirting a 10% sucrose solution on its dorsal coat. Animals were placed individually in their home cage and the evaluation for the usual grooming behaviour was initiated. The total grooming activity time was manually recorded for a 6-min test, after solution spurting. Results are expressed as percentage of total test time (Smolinsky et al. 2009).

In Vitro Studies

Culture of Neuroblastoma Cell Line and Differentiation

Human neuroblastoma cells SH-SY5Y (SH) were a gift from Prof. Anthony Turner (University of Leeds, UK). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Thermo Fisher Scientific) supplemented with 10% foetal bovine serum (FBS, Merck) and 2% antibiotic/antimycotic (AB/AM, Sigma-Aldrich). Medium was changed every 2 days. Culture plates were coated with poly-D-lysine (100 μg/mL) and laminin 4 μg/mL (Gibco), before plating the cells. For the experiments, cells were seeded at a concentration of 1.3×10^4 cells/cm^2 in 12-well plates (Orange Scientific) and maintained at 37 °C in a humidified atmosphere of 5% CO_2. After 24 h proliferation, differentiation into a neuronal phenotype was induced by adding retinoic acid (RA, Sigma-Aldrich) at a final concentration of 10 μM in culture medium and maintaining cells for 7 days, as usual in our lab (Fernandes et al. 2018). RA-containing culture medium was changed every 2 days.

Culture of Human CHME3 Microglia Cell Line

Human CHME3 microglial cells were a gift from Prof. Marc Tardieu (Janabi et al. 1995). Cells were cultured in DMEM supplemented with 10% FBS, 2% AB/AM (Sigma-Aldrich) and 1% L-glutamine (Sigma-Aldrich). Medium was changed every 2 days. For the experiments, cells were seeded at a concentration of 1.3×10^4 cells/cm^2 in 12-well plates (Orange Scientific) and maintained at 37 °C in a humidified atmosphere of 5% CO_2. Cells were used 24 h after plating (Fernandes et al. 2018).

Incubation with NPS

Cells were incubated for 24 h with the NPS Buph and NEH, as well as with methedrone for comparison, in a concentration range of 50–400 μM. Non-treated cells were used as controls.

Cell Viability/Function

Cell viability was assessed using the cellular reduction of [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium] (MTS) by viable cells into an aqueous, soluble and coloured formazan product, in the presence of phenazine methosulphate (PMS). After exposure to NPS, cells were incubated for 1 h at 37 °C with the mixture MTS (PMS diluted in DMEM-F12) and the absorbance was measured at 490 nm using the microplate reader PR 2100 (Bio-Rad Laboratories). For each experiment, the mean value of absorbance obtained from non-treated cells (control) was considered as 100% of cell viability (Falcão et al. 2017).

Determination of Cell Death

Phycoerythrin-conjugated annexin V (V-PE) and 7-aminoactinomycin-D (7-AAD) mixture (Guava Nexin® Reagent, Millipore; Billerica, MA, USA) were used to determine the percentage of early apoptotic and late apoptotic/necrotic cells by flow cytometry. After incubation, adherent microglia were collected by trypsinization and added to the cells present in the incubation media. After centrifugation, the cellular pellet was resuspended in PBS containing 1% of bovine serum albumin (BSA), stained with Guava Nexin® Reagent according to manufacturer's instructions, and

analysed on a Guava easyCyte 5HT flow cytometer (Guava Nexin® Software module, Millipore). Two readings were performed for each sample (Falcão et al. 2017). Three cell populations were identified: viable cells (annexin V-PE and 7-AA negative), early apoptotic cells (annexin V-PE positive and 7-AA negative) and late apoptotic/necrotic cells (annexin V-PE and 7-AA positive). Staurosporine was used at 50 nM as a positive control for cell apoptosis.

Lysosome Labelling

To track microglial lysosomal activity, cells were labelled with LysoTracker™ Red DND-99 fluorescent probe for 30 min after incubation with NPS and fixed with 4% (w/v) paraformaldehyde in PBS. Nuclei were stained with Hoechst 33258 dye. Fluorescence was visualized using an AxioCam HR camera adapted to an AxioScope A1® microscope (Zeiss, Germany). Pairs of U.V. and red-fluorescence images of ten random microscopic fields (original magnification, × 400) were acquired per sample. Lysotracker fluorescence intensity was measured using ImageJ® software (1.49 version, NIH, USA) and results are presented as arbitrary units normalized to the total cell number (Fernandes et al. 2018). A negative control was performed by using 3-methyladenine (3-MA) at 5 mM to inhibit autophagy.

Immunocytochemistry

For immunofluorescence detection, CHME3-microglia were fixed with 4% (w/v) paraformaldehyde in PBS and a standard immunocytochemical technique was performed. Briefly, cells were incubated overnight at 4 °C with the primary antibody rabbit anti-TMEM119 (1:500, Abcam, Cambridge, UK). The secondary antibody used was goat anti-rabbit Alexa Fluor 488, (1:1000, Invitrogen CorporationTM, Carlsbad, CA, USA). Cell nuclei were stained with Hoechst 33258 dye (blue, Sigma-Aldrich). Fluorescence was visualized as described above. Merged images of UV and fluorescence of ten random microscopic fields were acquired per sample by using Zen 2012 (blue edition, Zeiss) software. Original magnifications used were × 400 and × 630. For morphological characterization of CHME3-microglia, we used the tracing analysis in ImageJ® software to measure the 2D area and perimeter of single microglia cells after TMEM119 immunostaining, as usual in our laboratory (Cunha et al. 2016). Results were obtained from 150 cells in each tested condition, for all assays.

Statistical Analysis

Results are expressed as mean ± SD. In vitro results were obtained from at least four independent experiments. Comparisons among the different groups were done by one-way ANOVA followed by multiple comparisons using Bonferroni post hoc correction. $p < 0.05$ was considered statistically significant, in both in vitro and in vivo studies. Statistical analysis was made using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, USA).

Results

Effects of N-Ethylhexedrone and Buphedrone on Body-Weight Gain and Mortality

Initial experiments were carried out at extreme dose range, 1 and 128 mg/kg, of NEH (single administration) with 3 animals per group, as an attempt to find the dosing ranges to use. At the highest tested dose of 128 mg/kg NEH, the number of fatalities reached 100% in a short time post-exposure. Apart from those fatalities, all animals survived to the studied period in all dosing regimens tested, single or repeated exposure of either Buph or NEH.

When compared with saline-treated mice, NEH and Buph decelerated the weight gain once animals were single exposed to high tested doses (from 32 mg/kg for NEH ($F(6,17) = 5.173$; $p = 0.0034$) and from 64 mg/kg for Buph ($F(5,15) = 1.920$; $p = 0.046$, Table 1)). The weight gain signal detected during the single-dose study, although using up to 3 animals/group, was later confirmed in the repeated-dose study using 7 animals/group at doses of 16 mg/kg ($F(2,18) = 4.461$; $p = 0.0218$ and $F(2,18) = 3.009$; $p = 0.0499$, respectively), but not 4 mg/kg, of each SC (Fig. 3) even immediately after first administration of NEH at such low doses as 16 mg/kg ($F(2,18) = 9.049$; $p = 0.0019$). Moreover, during the seven and the three days that followed the single and the repeated-dose administration, respectively, no signs of toxicity and no changes in animal behaviour were observed, having the animals recovered their body-weight gain, as it is depicted in Fig. 3—day 7 corresponding to 3 days post-last-repeated administration (also in single-dose study, not shown). The macroscopic examination during necropsy, as well as the relative organ weight (brain, heart, liver, kidneys, spleen), showed no differences between control and each studied animal dose group (Supplementary Table 1: Effects of N-ethylhexedrone (NEH) and of buphedrone (Buph) repeated exposure on major body organs relative weight).

Behavioural Changes Observed Following N-Ethylhexedrone or Buphedrone Exposure

Table 2 illustrates the results obtained following mice single administration of NEH (1, 4, 8, 16, 32, 64 mg/kg, i.p.) and Buph (4, 8, 16, 32, 64 mg/kg, i.p.) in the Irwin test, after having validated the real time results obtained by videos recorded for each set of trials. This procedure, included in the recommended package of safety pharmacology studies (ICH

Table 1 Effect of N-ethylhexedrone (NEH) and of buphedrone (Buph) single exposures on mice weight gain

Dose (mg/kg)						
SC	0	4	8	16	32	64
NEH	− 0.24 ± 0.24	− 0.35 ± 0.22	− 0.57 ± 0.18	− 0.85 ± 0.59	− 1.17 ± 0.60*	− 1.56 ± 0.18***
Buph	− 0.18 ± 0.22	− 0.36 ± 0.23	− 0.38 ± 0.29	− 0.47 ± 0.23	− 0.58 ± 0.16	− 0.68 ± 0.22*

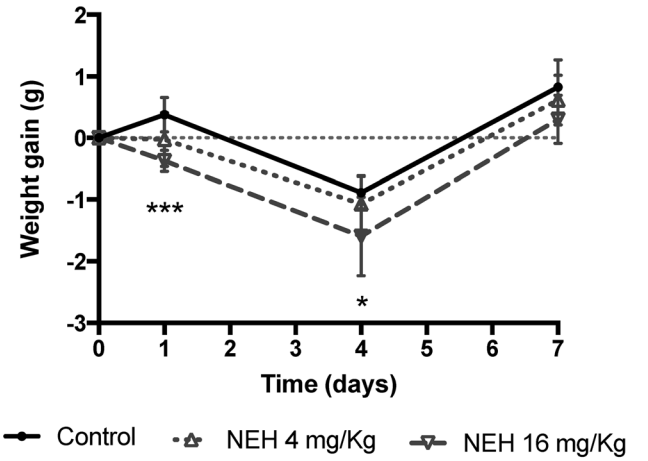
Data (mean ± SD) are expressed as the difference among the body weight (in grams) measured 24 h after the single administration and the body weight measure prior to the exposure. */***Significant difference ($p < 0.05$/$p < 0.001$) vs. saline-treated animals (0 mg/kg), $n = 3$/group

S7A), allowed profiling the overall behaviour of each tested substance and its potential safety concerns by using a reduced number of animals. NEH at 1 mg/kg had no detected effects on mice behaviour but induced dose-dependent signs of excitation from 4 mg/kg, stereotype head movement from 8 mg/kg and sniffing, head twitches and circling from 16 mg/kg. It induced clear dose-dependent signs of hypersensitivity to external stimulation (increased fear and increased reactivity to touch) from 8 mg/kg, which were lost at 64 mg/kg. Marked effects including vocalization, straub tail and clear signs of neurotoxicity (tremor and convulsions) were observed at 64 mg/kg, as well as several posture and motor signs (see Table 2). Buph also induced dose-dependent excitation and stereotypies (sniffing), though with different dose levels (from 8 to 4 mg/kg, respectively). It induced clear signs of hypersensitivity in a similar way as NEH, which were partially absent at start effect of lowest tested dose, resembling a sensory deficit effect at 64 mg/kg as observed with NEH, too. Additionally, Buph induced stereotypies (head movement) and jumping from 32 mg/kg. Head twitches, licking, biting, aggressiveness, circling, scratching, motor dysfunction and marked effects including vocalization, straub tail and signs of neurotoxicity (convulsions) were observed at the highest tested dose of 64 mg/kg. These apparent strong neurotoxic signs were reason for not testing further increased doses of Buph. In addition, effects on autonomic system were resumed to few signs occurring at 32 mg/kg of NEH or high doses of either Buph or NEH (Table 2).

The particular phenotype behaviours induced by each SC were slightly different as described, adding, exception for most of bizarre behaviours, that some of Buph-induced behaviours were of longer duration than those triggered by NEH, which disappeared rapidly with a duration no longer than 60 min in most of the cases.

N-Ethylhexedrone and Buphedrone Exposure Induces Conditioned Place Preference

The conditioned place preference (CPP) test was used to evaluate the rewarding properties of NEH and of Buph. Figure 4 shows the score of the time spent in the drug-associated compartment, post 4 days conditioning trial for NEH and for Buph. The score was obtained by computing the difference between time spent in the drug-paired compartment during the post-conditioning test and time spent on drug-paired compartment during the pre-conditioning test (Karlsson et al. 2014). Both NEH and Buph induced significant CPP at 4 and 16 mg/kg doses compared with controls ($F(2,17) = 6.749$;



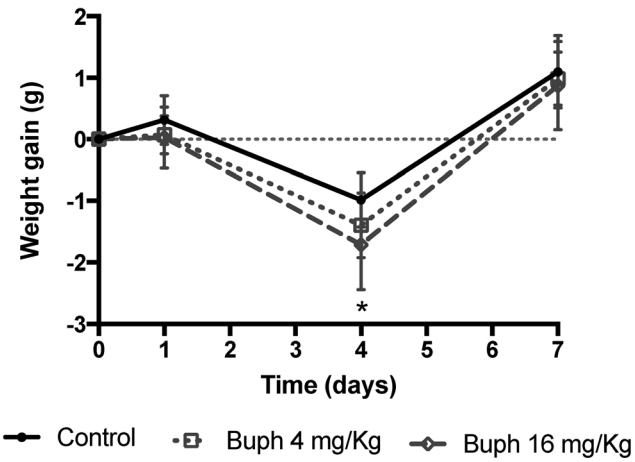


Fig. 3 Effect of N-ethylhexedrone (NEH) and of buphedrone (Buph) on mice weight gain following repeated exposure (4 days) and 3 days post-last exposure (7 days) to doses of 4 and 16 mg/kg (i.p.). Data (mean ± SD) are expressed as the difference among the body weight (in grams) measured 24 h after the last administration and that measure prior to the exposure. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. saline-treated animals (control), $n = 7$/group; Comparisons were made by one-way ANOVA followed by Bonferroni's test

Table 2 Effects of N-ethylhexedrone (NEH) and of buphedrone (Buph) in the primary observation (Irwin) test in the mice; the table resumes the neurobehavioural signs detected among 50 observed signals. Doses (mg/kg) that produced different behaviour from control (vehicle) are shown in column, preceded by the number of animals showing that effect in brackets and followed by the time points of neurobehavioural signs observation also in brackets

Observation	Single-doses tested (mg/kg)	
	Buph (4–64)	NEH (1–64)
Lethality	0/3	0/3
CNS activity		
Cage activity	+(3/3) 8–16 (→ 30–120′)	+(3/3) 4 (→ 15′)
	++(3/3) 32–64 (→ 180–120′)	++(3/3) 8–64 (→ 30–60′)
	+(2/3) 64 (180′)	+(2/3) 8, 16–32 (60,120 → 180′)
		+(2/3) 64 (120′)
	↓ (3/3) 4 (→ 10′)	
CNS excitability		
Convulsions	(3/3) 64 (*)	(2/3) 64 (*)
Tremor		(3/3) 64 (→ 15′)
Straub Tail	(3/3) 64 (→ 15′)	(3/3) 64 (→ 15′)
Jumps	(1–3/3) 32–64 (→ 15′)	(1/3) 32 (→ 30′)
Reactivity to touch	↑ (2–3/3) 4–32 (15′ → 30′)	↑ (3/3) 8–32 (→ 30–60′)
	↑ (3/3) 64 (60 → 120′)	↑ (2/3) 16–32 (120′)
	↓ (3/3) 4, 64 (60′,15 → 30′)	
Fear	↑ (2/3) 8 (15′)	↑ (3/3) 16–32 (→ 30–60′)
	↑ (3–1/3) 8–16 (30′ → 60′)	↑ (1/3) 16 (60 → 180′)
	↑ (3/3) 64 (120′)	
	↓ (3/3) 64 (→ 30′)	↓ (3/3) 32–64 (→ 30′)
Aggressiveness	(3/3) 64 (→ 15′)	
Bizarre behaviours		
Circular ambulation (circling)	↑ (3/3) 64 (→ 15′)	↑ (2–3/3) 16, 32–64 (→ 30′,→ 120′)
Stereotyped head moving	(3/3) 32–64 (→ 15′)	(3/3) 8, 16, 32 (→ 15′, → 30', → 60′)
		(2/3) 64 (→ 60′)
Stereotyped liking	(3/3) 64 (→ 15′)	(2/3) 64(→ 15′), (1/3) 64 (30′)
Stereotyped sniffing	(2/3) 4 (30 → 60′)	(1/3) 16, 64 (30 → 60′, → 120′)
	(3/3) 16–64 (30 → 180′)	(3/3) 32 (→ 120′)
Head twitches	(1/3) 64 (→ 15′)	(1/3) 16 (→ 30′)
		(2–1/3) 32–64 (→ 60′)
Biting	(2/3) 64 (→ 15′)	
Retropulsion		(2/3) 64 (→ 15′)
Grooming	↑ (1/3) 8 (→ 15′)	↑ (1/3) 32 (30′), (2/3) 32 (60 → 120′)
Algesia (sensitivity to tail pinching)	↓ (3/3) 16,32,64 (120′,15 → 60′,30′)	↓ (3/3) 64 (30 → 60′)
Scratching	(3/3) 64 (→ 15′)	
Vocalization	(3/3) 64 (→ 15′)	(2/3) 64 (→ 15′)
Muscle tone/equilibrium		
Akinesia	(3/3) 64 (→ 15′)	(1/3) 64 (→ 15′)
Rolling gait	(1/3) 64 (→ 15′)	(2/3) 16, 64 (→ 15′)
Tip-toe	(3/3) 32–64 (→ 15′-30′) (1/3) 64 (60′)	(3/3) 64 (→ 15′) (1/3) 64 (30 → 60′)
Muscle tone	↑ (3/3) 16 (→ 24 h)	
Posture (loss of balance)	(3/3) 64 (→ 30′)	(1/3) 64 (→ 15′), (2/3) 64 (30′)
Forepaw treading	(1/3) 64 (→ 15′)	
Motor incoordination	(3/3) 64 (→ 30′), (1/3) 64 (60′)	(1, 3/3) 16, 64 (→ 30′)
Grip strength (loss of grasping)	(3/3) 64 (15′)	

Table 2 (continued)

Observation	Single-doses tested (mg/kg)	
	Buph (4–64)	NEH (1–64)
Autonomic effects		
Ptosis (palpebral closure)		(1/3) 32 (15′)
Respiration	↑ (3/3) 64 (→ 30′)	↑ (3/3) 32–64 (→ 30′)

(*)Neurobehavioural signs observed (first 15′) were transitory and recovered on a short time

$p = 0.0070$ and $F(2,17) = 6.459$; $p = 0.0082$, respectively). Comparing NEH-treated mice with those conditioned with an equal dose of Buph, CPP score from animals treated with low doses of NEH was near 1.3 times higher than that induced by Buph, whereas highest CPP scores were obtained in animals exposed to Buph doses of 16 mg/kg ($p = 0.0003$).

Effects of N-Ethylhexedrone and Buphedrone Exposure on Emotional Behaviour

During conditioning days, the novel cage behaviour paradigm was evaluated through the defecation index. The results of single (day 1) and repeated administration (day 4) are shown in Fig. 5a, b. Both synthetic cathinones reduced the mice defecation index compared with the control but with slight distinctions. Following single-dose (Fig. 5a), faecal reduction around 30% was observed in animals treated with 4 mg/kg doses of NEH (2.7 ± 1.2), while significant faecal reduction of near 82% was observed in mice exposed to NEH at 16 mg/kg dose (0.7 ± 0.3, $F(2,25) = 3.447$; $p = 0.0296$) and in mice treated with Buph at 4 mg/kg (0.7 ± 0.6, $F(2,25) = 8.886$; $p = 0.0012$). Faecal reductions close to 100% were observed in mice exposed to 16 mg/kg Buph. The same pattern of decline was obtained, for each drug, when animals were repeatedly administered during the 4 conditioning days, as the results obtained on day 4 show in Fig. 5b ($F(2,25) = 3.434$; $p = 0.0295$ for NEH and for Buph $F(2,25) = 10.88$; $p = 0.0004$).

Dosing ranges of 4 and 16 mg/kg of NEH and Buph were also used to compare the effects of repeated exposure with each SC on mice marble burying behaviour 24 h post-last dosing. The results obtained are shown in Fig. 5c. The average buried marbles for the control groups was 84.1 ± 2.9% ($n = 14$). Whereas Buph significantly inhibited marble burying to 66.4 ± 8.8% and 50.3 ± 4.7% ($F(2,25) = 11.95$; $p = 0.0002$) in a dose-dependent manner compared with control, mice exposed to 4 mg/kg doses of NEH had no significant behaviour change, but when exposed to doses of 16 mg/kg the number of buried marbles then decreased significantly (63.6 ± 7.6%, $F(2,25) = 8.973$; $p = 0.0012$). Approximately 25% and 40% inhibition of marble burying behaviour was observed at the highest dose tested of 16 mg/kg for NEH and Buph, respectively.

Withdrawal from Either N-Ethylhexedrone or Buphedrone Distinctively Affects Behaviour

The effect of repeated exposure to the same dosing ranges of NEH or Buph (4 and 16 mg/kg) on mice self-care and

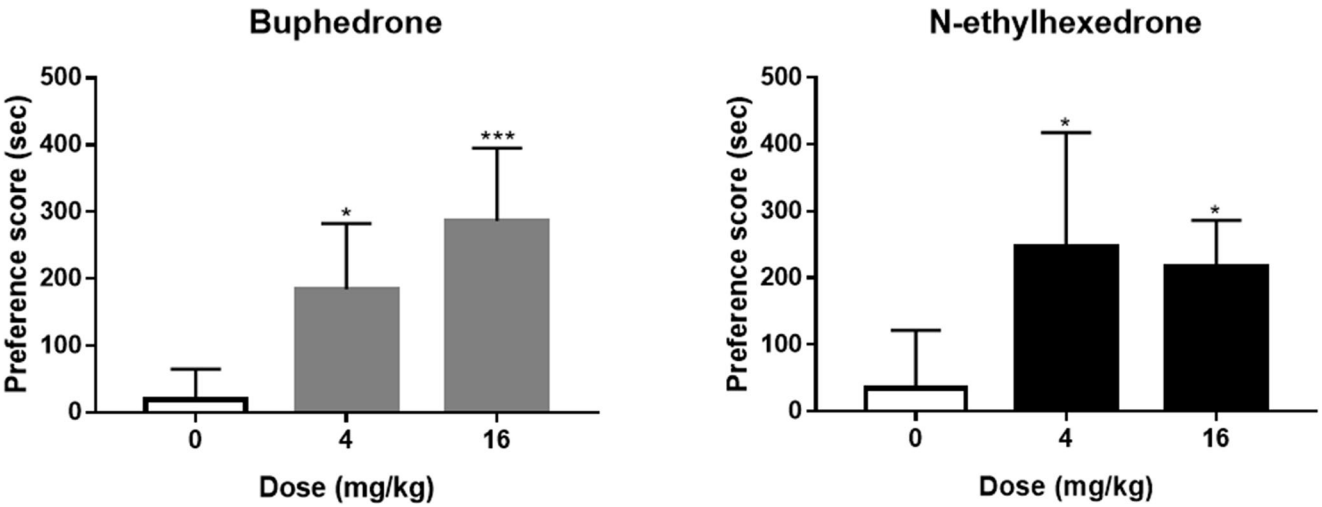


Fig. 4 Abuse potential of buphedrone and N-ethylhexedrone (4 and 16 mg/kg, i.p., $n = 7$/group) assessed by the conditioned place preference. Results are expressed as preference score and presented as mean ± SD; $^{*}p < 0.05$ and $^{***}p < 0.001$ vs. saline-treated animals (control); comparisons were made by one-way ANOVA followed by Bonferroni's test

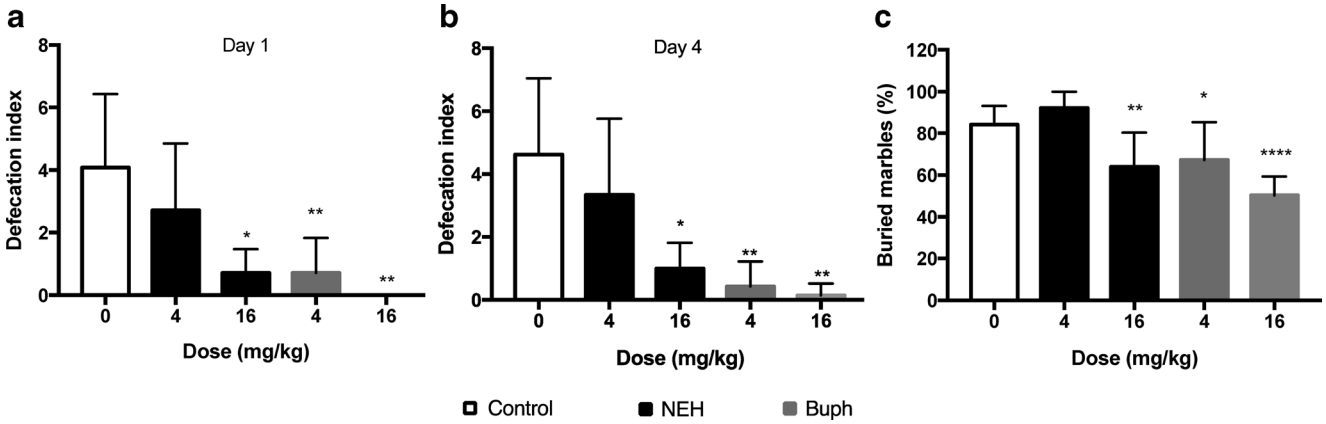


Fig. 5 Anxiolytic-like effect of N-ethylhexedrone (NEH) and buphedrone (Buph). Effect of single (**a**) and repeated (**b**) exposure to NEH and Buph (4 and 16 mg/kg, i.p., $n = 7$/group) on defecation index and on marble burying test (**c**) on day 1 post-last-repeated exposure. Results are expressed as mean ± SD; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{****}p < 0.001$ vs. saline-treated animals (control, $n = 14$); comparisons were made by one-way ANOVA followed by Bonferroni's test

motivational behaviour was evaluated by the time spent grooming in the splash test, 3 days post-last dosing. The average grooming time for the control groups was 16.0 ± 2.5% ($n = 14$). Repeated exposure to NEH significantly decreased time spent grooming 3 days post-last dosing in a dose-dependent manner compared with control to 6.3 ± 1.9% and 3.5 ± 2.4% ($F(2,25) = 7.116$; $p = 0.0036$), respectively, while mice exposed to Buph increased the grooming behaviour with significant alterations at doses of 16 mg/kg (34.2 ± 2.9%, $F(2,25) = 10.90$; $p = 0.0004$), as compared with controls ($p = 0.0003$) or to 4 mg/kg doses ($p = 0.0168$) (Fig. 6). Approximately 80% inhibition of grooming behaviour was observed at the highest dose tested of 16 mg/kg for NEH, whereas with Buph the increase was over 115% under the same conditions.

Effects of N-Ethylhexedrone and Buphedrone on Neuronal and Microglial Cell Viability

In order to define NPS cellular toxicity, we pursued our studies with human SH-SY5Y neuroblastoma and CHME3-microglial cell lines. This decision was performed considering that (1) primary cultures from mice are isolated from pups and as so show immature phenotypes; (2) translation of pathological mechanisms from animal models to humans represent low rate of success; (3) specific neuronal and microglia response differences towards toxic stimuli, such as NPS, are difficult to assess by brain histology that better represents the culminate of total cell dysfunctionalities; and (4) to minimize the number of animals that would be required to screen the different range of NPS concentrations.

We compared the cytotoxic effects of the selected concentrations (50–400 μM) of Buph and NEH, using the two referred human cell lines SH-SY5Y-neuron and CHME3-microglia, in comparison with the effect of staurosporine at 50 nM, known to induce apoptosis in either SH-SY5Y-neurons or mouse microglial cell lines and primary cultures (Prince and Oreland 1997; Soria et al. 2011). Methedrone was only used for comparative purposes based on its known interference with neuronal function in specific brain regions, namely at nucleus accumbens, striatum and hippocampus brain regions (Pail et al. 2015), together with known neurotoxic effects on SH-SY5Y neuroblastoma cells (mitochondrial dysfunction) (Soares et al. 2019). As indicated in Fig. 7a, all the tested NPS induced significant neuronal viability/function loss (evaluated by MTS test) at concentrations of 400 μM, but significant effects were early produced by Buph and NEH at 100 μM ($F(10,30) = 16.62$; $p < 0.0001$). We additionally determined the percentage of dying cells by specific

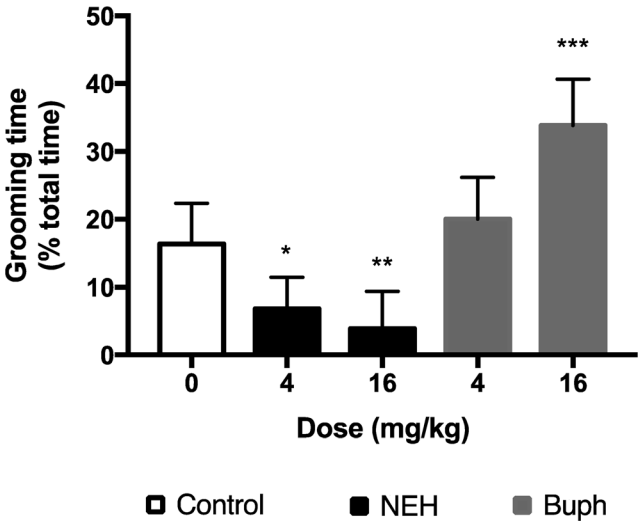
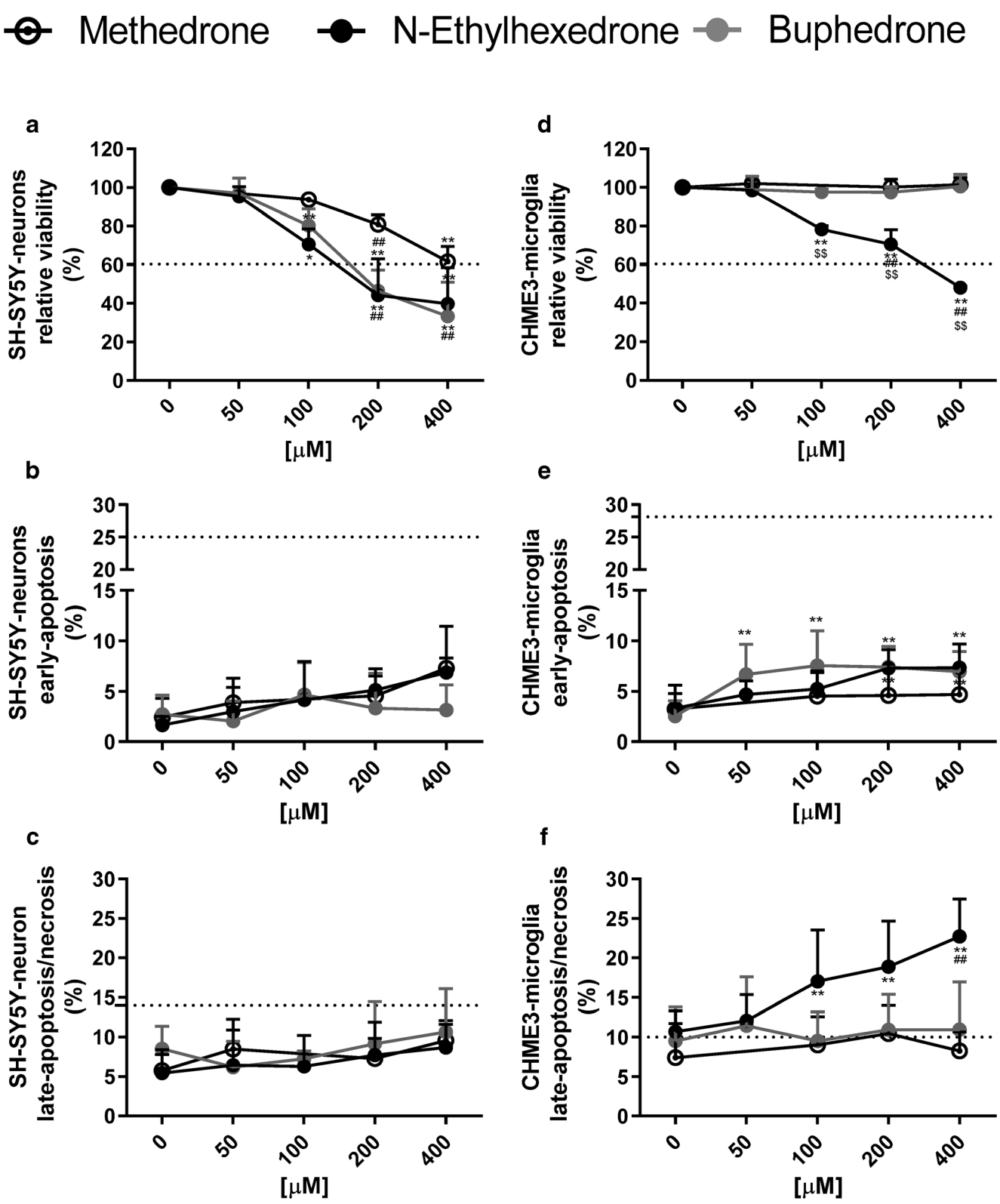


Fig. 6 Depressive-like effect of N-ethylhexedrone (NEH) but not buphedrone (Buph) on day 3 post-last-repeated exposure to NEH and Buph (4 and 16 mg/kg, i.p., $n = 7$ mice/group) observed on the Splash test. Results are expressed as percentage of test time and presented as mean ± SD; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. saline-treated animals (control, $n = 14$); comparisons were made by one-way ANOVA followed by Bonferroni's test



mechanisms, namely early and late apoptosis/necrosis by flow cytometry, but we did not find significant changes produced by none of the NPS tested in neurons (Fig. 7b, c). Regarding microglia, only NEH was able to produce deleterious microglia demise (MTS test, Fig. 7d), with significant effects that started at 100 μM ($F(11,32) = 53.74$; $p < 0.0001$), as those in neurons. Increased early apoptotic cells was observed for Buph at 50 μM $F(4,41) = 8.687$; $p < 0.0001$ and at 200 μM for

◀ **Fig. 7** New psychoactive substances (NPS) induce neuronal and microglial loss of cell viability. Human SH-SY5Y-neuronal (**a**–**c**) and CHME3-microglial (**d**–**f**) cell lines were treated with increasing concentrations of methedrone, buphedrone or N-ethylhexedrone for 24 h, as indicated in methods. MTS reduction test (**a**, **d**) was performed in order to assess relative cellular viability. The percentage of cells in early or late apoptosis/necrosis was determined by flow cytometry with phycoerythrin-conjugated annexin V (annexin V-PE) and 7-aminoactinomycin D (7-AAD). The two populations were distinguished as follows: early apoptotic cells (annexin V-PE positive and 7-AA negative, panels **b**, **e**) and late apoptotic/necrotic cells (annexin V-PE and 7-AA positive, panels **c**, **f**). All results are mean ± SD from five independent experiments performed in duplicate. Dashed line indicates the values obtained upon incubation with staurosporine at 100 nM, used as a positive control for apoptosis. Comparisons were made by one-way ANOVA followed by multiple comparisons Bonferroni post hoc correction. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. non-treated cells (control); $^{\#\#}p < 0.01$ vs. cells treated with methedrone at the same concentration; $^{\$\$}p < 0.01$ vs. cells treated with buphedrone at the same concentration

NEH ($F(4,39) = 10.69$; $p < 0.0001$), while late apoptotic cells were notorious only with NEH and for the concentration of 100 μM ($F(10,70) = 3.573$; $p = 0.0007$) or higher (Fig. 7e, f). Data evidence that Buph and NEH cause different cell death mechanisms and cell type susceptibilities. To note that despite the described neurotoxic effects for methedrone, the loss of neuronal viability was less evident than that for Buph and NEH. Indeed, methedrone effects on the overall viability of neurons by the MTS test were only noticed for the highest concentration used (400 μM), as recently indicated (Soares et al. 2019). Therefore, both Buph and NEH supplanted the neurotoxic properties of methedrone, reason why we decided to exclude this SC from the determinations to be undertaken.

Effects of N-Ethylhexedrone and Buphedrone on Neuronal and Microglial Lysosomal Activity

Having demonstrated that both Buph and NEH reduced neuronal viability and microglia alterations at 100 μM or greater, we decided to only use this one and the highest dose of 400 μM in the next assessments. Considering that lysosome dysfunction is implicated in neurodegenerative and microglia-mediated inflammatory processes (Tanaka et al. 2013; Usenovic and Krainc 2012), as well as in the regulation and function of autophagy during cell survival and death (Chazotte 2011; Das et al. 2012), we then assessed the effects produced by our tested SC in lysosome homeostasis. To do so, we incubated cells with LysoTracker red® probe that preferentially accumulates in the acidic vesicular compartments (Zhitomirsky et al. 2018). 3-Methyladenine (3-MA) was used as a negative control at 5 mM concentration to inhibit autophagy, as reported in either SH-SY5Y-neurons or mouse microglial cell lines and primary cultures (Jin et al. 2018; Sukumaran et al. 2015). As indicated in Fig. 8, NEH and Buph had similar effects in enhancing lysosomal activity in neurons, which was observed only upon incubation with 400 μM of each NPS ($F(6,20) = 5.873$; $p = 0.0012$). However, the effect of NEH was markedly enhanced over the one of Buph when tested in the human microglial cell line (Fig. 9). Actually, a significant increase of lysosomal stress was obtained with only 100 μM NEH, though both SC were effective in enhancing lysosomal activity at 400 μM ($F(6,21) = 16.57$; $p < 0.0001$). Data indicate a microglia increased lysosomal biogenesis proneness by NEH that may result in cell autophagic changes, which showed to be also determined by Buph but only at higher doses. This specific effect may be associated with an attempt of the cells to degrade specific metabolites, thus requiring autophagic clearance of cytoplasmic material by glial cells, such as microglia.

Effects of N-Ethylhexedrone and Buphedrone in Microglial Morphological Alterations

In order to assess whether increased lysosomal stress by NEH was associated to microglia activation, we next evaluated alterations on cell morphology, demonstrated to be associated with pro-inflammatory properties of adherent microglia. As depicted in Fig. 10, only the incubation with NEH was able to significantly disturb microglia shape. Indeed, no modifications were produced by Buph, even if considering the most elevated concentration tested. In contrast, NEH caused a reduction of cell perimeter for both 100 and 400 μM ($F(6,21) = 5.50$; $p = 0.0015$), and a decreased cell area, though only significant for 400 μM ($F(6,21) = 2.671$: $p = 0.0437$). Such morphological changes are linked to a switch of ramified cells to amoeboid morphologies with less, but thicker and shorter branches, described to be associated with increased microglia activation (Caldeira et al. 2014; Cunha et al. 2016).

Overall, the most marked effects from in vivo data for NEH vs. Buph, namely in retropulsion and circling behaviour, as well as in inducing depressive-like effects and reduced weight gain are in line with in vitro data showing that NEH increasingly produces loss of cell viability in both neurons and microglia. Our novel findings in microglia additionally show that NEH is more effective than Buph in causing lysosomal stress and morphological alterations compatible with cell activation and neuroinflammatory-associated events. Since such effects may concur to exacerbate neuronal damage, they may have contributed to the higher mice behaviour impairment observed by NEH.

Discussion

This study provides new data on distinct effects produced by two SC, Buph and NEH, emerging NPS in EU for which little is known in terms of their potential to produce changes in either behaviour or nerve cell function. For that, we performed an integrative study that combined both in vivo (animal

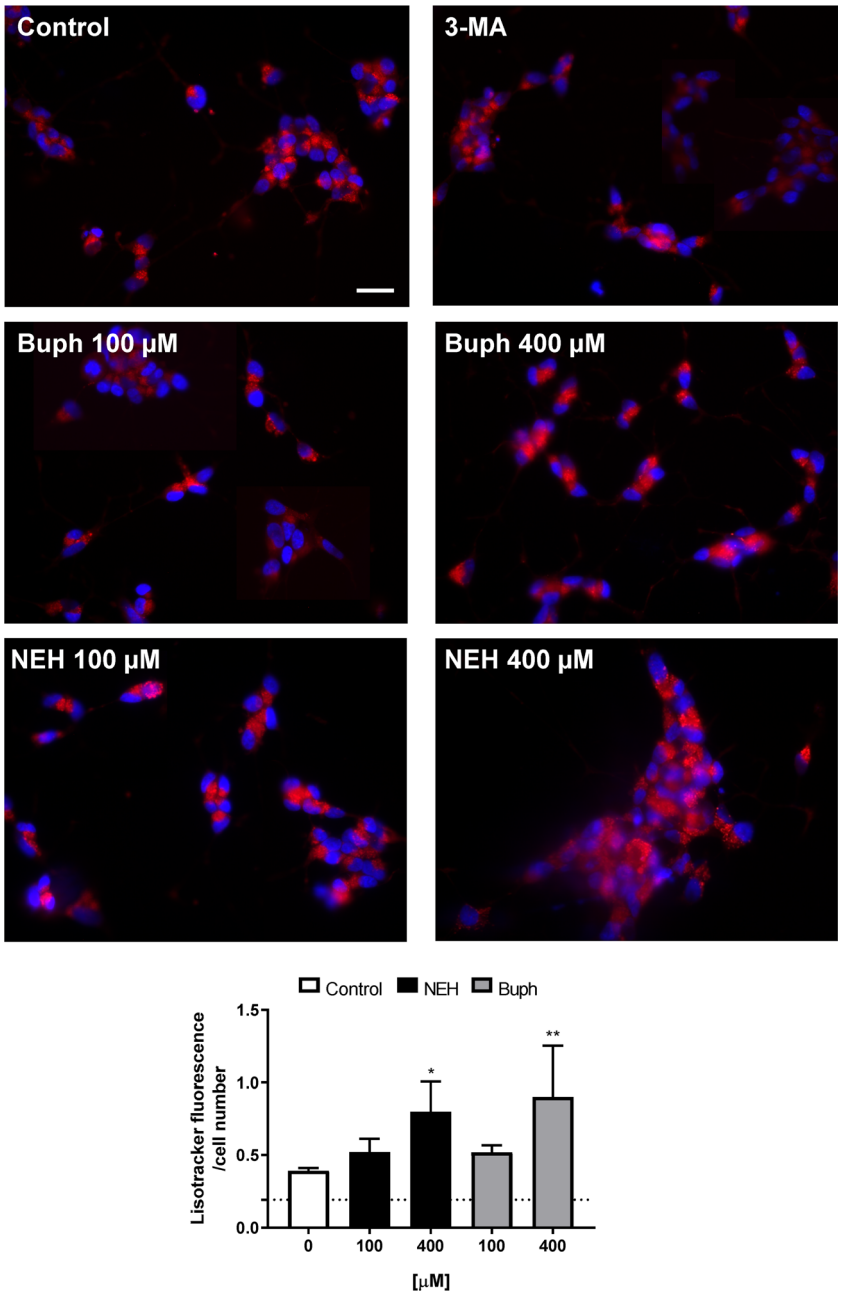


Fig. 8 N-ethylhexedrone (NEH) and buphedrone (Buph) promote lysosomal activity in SH-SY5Y-neurons. Human SH-SY5Y-neuronal cell line was treated with increasing concentrations of NEH or Buph for 24 h. After incubation period, lysosomes were labelled with the LysoTracker™ Red DND-99 fluorescent probe, as indicated in methods. Cell nuclei were stained with Hoechst 33258 dye (blue). Representative images of one experiment are shown. Images were quantified in ImageJ® software and normalized to the total number of cells. Scale bar represents 40 μm. Results are mean ± SD from five independent experiments. Dashed line indicates the values obtained upon incubation with 3-methyladenine (3-MA) at 5 mM, used as a positive control for autophagy inhibition. Comparisons were made by one-way ANOVA followed by multiple comparisons Bonferroni post hoc correction. *$p < 0.05$ and **$p < 0.01$ vs. non-treated cells (control)

models) and in vitro (human cellular models) data, in order to evaluate mice behavioural changes, as well as neuronal and microglial dysfunctionalities, upon exposure to each of these NPS.

For evaluation of behavioural effects in mice of these two SC, the tested range of 4 to 16 mg/kg doses was selected following the single-dose study by the primary observation (Irwin) test. As observed in mice treated with mephedrone (Martinez-Clemente et al. 2014), animals' single/repeatedly exposed to either NEH or Buph doses, equal or higher than 16 or 32 mg/kg (depending on the type of exposure), experienced a transient reduction on weight gain that was undetectable, at least from day 3 post-last exposure. It is important to mention that the human equivalent doses of here reported abuse potential and emotional behaviour doses inducing changes in mice are, respectively, 19 and 77 mg, using the animals' body surface area/weight dose conversion approach and assuming a human adult of 60 kg (US.FDA, 2005). Such doses are in the range of a light or heavy experience level in humans, respectively, for both SC, according to users reporting site Tripsit (Tripsit 2018a,Tripsit b).

In the Irwin test, the highest tested dose of NEH (128 mg/kg) was lethal, and as both drugs induced severe motor and CNS stimulation at doses of 32 mg/kg followed by neurotoxic signs at doses of 64 mg/kg, no higher doses for Buph were tested. Methylone, other NPS, also produces

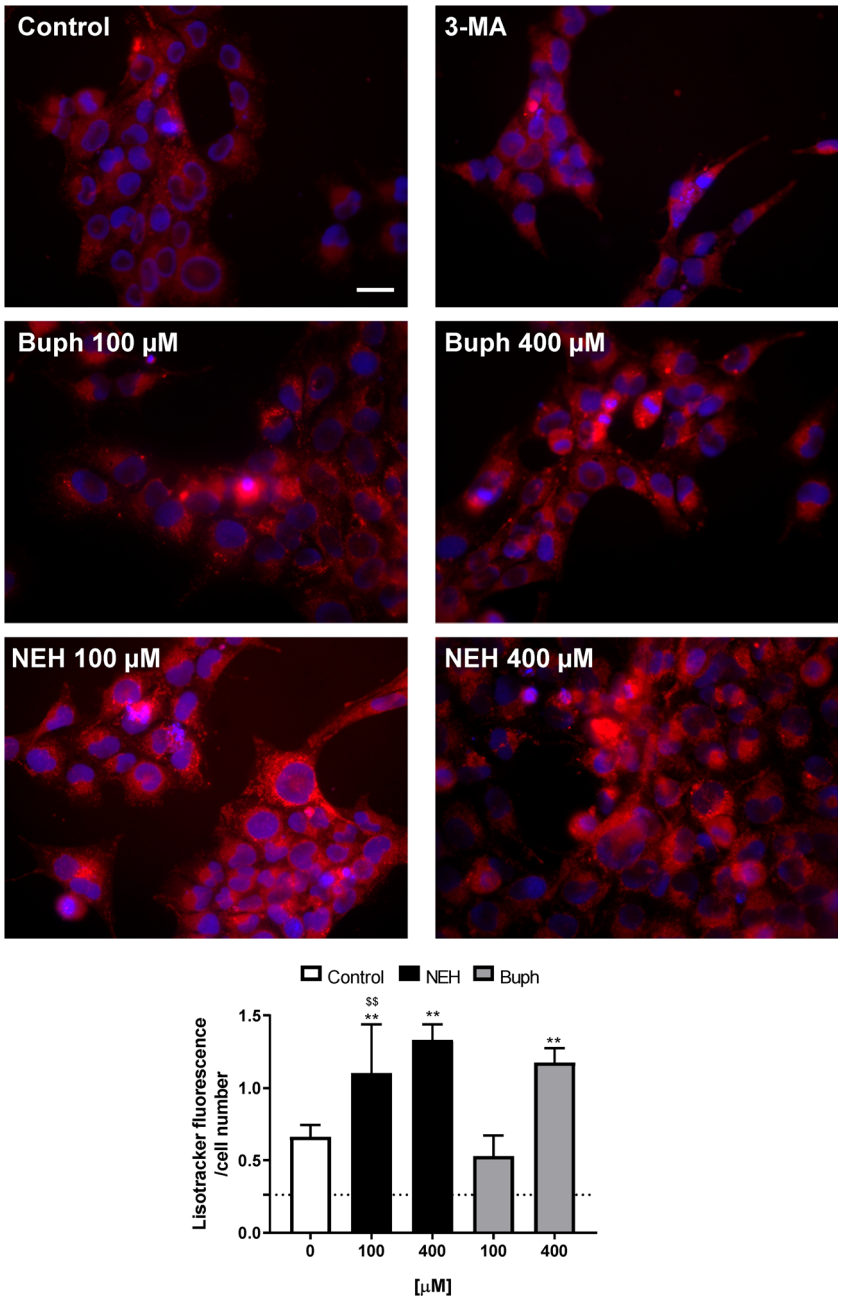


Fig. 9 N-ethylhexedrone (NEH) is more efficient in inducing lysosomal activity in CHME3-microglia than buphedrone (Buph). Human CHME3-microglial cell line was treated with increasing concentrations of NEH or Buph for 24 h. After incubation period, lysosomes were labelled with the LysoTracker™ Red DND-99 fluorescent probe, as indicated in methods. Cell nuclei were stained with Hoechst 33258 dye (blue). Representative images of one experiment are shown. Images were quantified in ImageJ® software and normalized to the total number of cells. Scale bar represents 40 µm. Results are mean ± SD from five independent experiments. Dashed line indicates the values obtained upon incubation with 3-methyladenine (3-MA) at 5 mM, used as a positive control for autophagy inhibition. Comparisons were made by one-way ANOVA followed by multiple comparisons Bonferroni post hoc correction. $^{**}p < 0.01$ vs. non-treated cells (control); $^{\$\$}p < 0.01$ vs. cells treated with Buph at the same concentration

convulsions at 56 mg/kg in mice (Marusich et al. 2012) and other studies reported designer cathinone-induced seizures at high doses (Berardelli et al. 1980). CNS stimulation including increased activity, excitation signs and stereotyped head moving, and sniffing were observed for both NEH and Buph, with a dose response effect within the tested dose range (4 to 64 mg/kg), and marked effects starting at 32 mg/kg. Remarkably, NEH had a bigger impact on stereotypy behaviour compared with Buph, inducing several other dose-dependent stereotypies, starting at 16 mg/kg, emphasizing circling effects. Mice exposed to 3-fluoromethcathinone, 4-fluoromethcathinone, methylenedioxypyrovalerone (MDPV), mephedrone, methedrone, methylone, α-PVP, α-PBP, α-PPP and 5-IT also showed stimulant effects, including hyperactivity and stereotyped head movements, sniffing and circling (Karlsson et al. 2014; Marusich et al. 2014; Marusich et al. 2012; Marusich et al. 2016). Cathinone has also been shown to increase stereotyped movements in rats, including those observed in this study, namely biting, licking, sniffing and head twitches (Zelger et al. 1980). Behaviour signs, such as jumping, biting and aggressiveness were observed, with short duration, mostly for Buph high-dose-treated mice while tremor and backward walking were associated to NEH. Specific behaviour effects as biting and retropulsion have

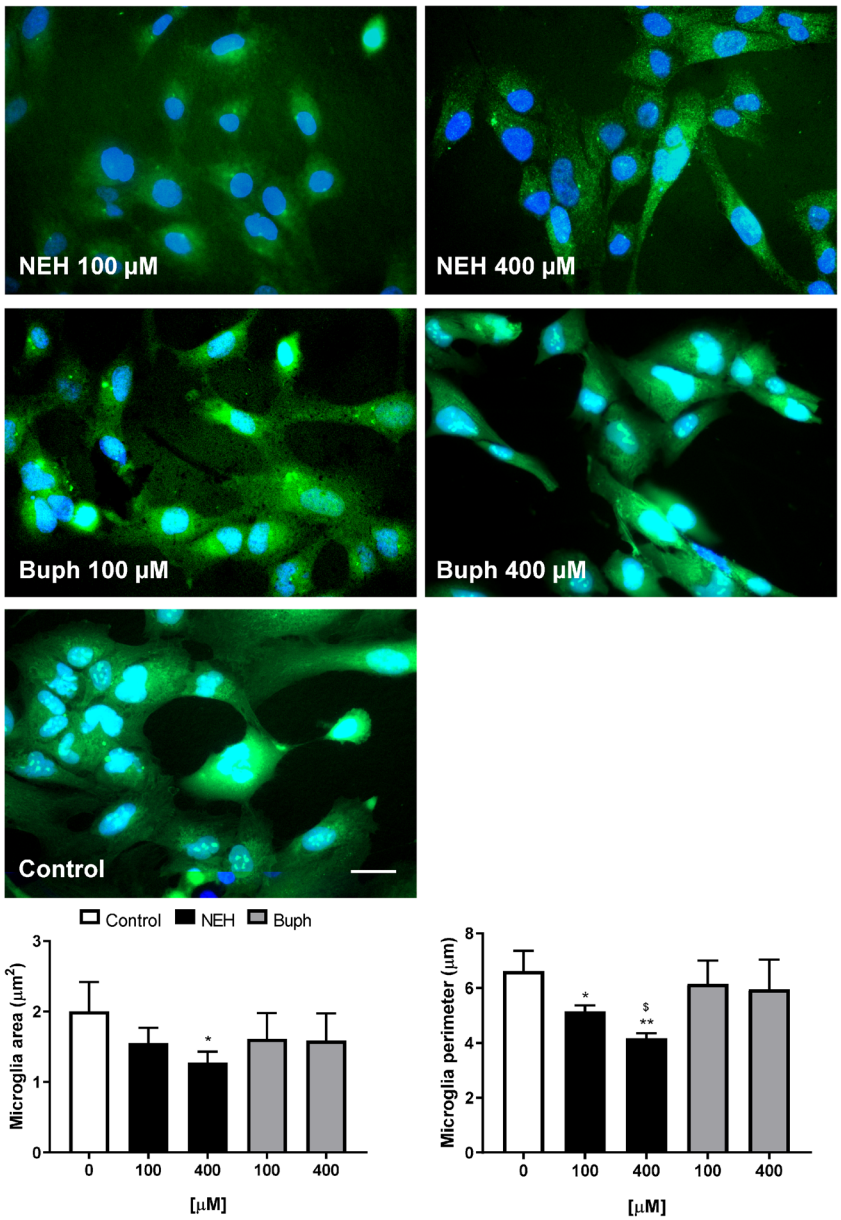


Fig. 10 N-ethylhexedrone (NEH) markedly decreases cell perimeter and area in CHME3-microglia, which is not observed upon buphedrone (Buph) treatment. Human CHME3-microglial cell line was treated with increasing concentrations of NEH or Buph for 24 h. In order to evaluate cell morphology, cells were fixed and immunofluorescence detected using TMEM-119 antibody, followed by a green-fluorescent-labelled secondary antibody, as indicated in methods. Cell nuclei were stained with Hoechst 33258 dye (blue). Cell area and perimeter were quantified by manually tracing using ImageJ® software. The average of at least 100 cells was considered for each condition. Representative images of one experiment are shown. Scale bar represents 40 μm. Results are mean ± SD from five independent experiments. Comparisons were made by one-way ANOVA followed by multiple comparisons Bonferroni post hoc correction. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. non-treated cells (control); $^{\$}p < 0.05$ vs. cells treated with Buph at the same concentration

been observed in mice exposed to MDPV, α-PPP and mephedrone, as well (Karlsson et al. 2014; Martinez-Clemente et al. 2014; Marusich et al. 2014). In vivo induced effects, such as excitation followed by the presence of stereotypies (at high doses), could be predictive of prodopaminergic or even proserotoninergic activities (Roux et al. 2005). Other behaviours observed for Buph and NEH like head twitches and, at high doses, backward walking, tremor and straub tail have been associated to serotoninergic hyperactivity too (Haberzettl et al. 2013). Furthermore, the presence of activity stimulation, clear stereotyped behaviour, straub tail and tip-toe gait (at high doses), as observed with Buph and NEH, suggest the likelihood presence of reinforcing, hence abuse potential, and even withdrawal effects in these drugs. Additionally, onset of such observed behaviours produced by Buph and NEH was very rapid being evident, for most of them, during the first minutes of the session. Activity stimulation produced by Buph and NEH started at 8 and 4 mg/kg doses, respectively, with short duration of action (30′ and 15′), having the longest duration of action for the 32 mg/kg doses. These induced effects completed fadeout 24 h after administration for all behaviours and doses tested in the Irwin. As reported (Marusich et al. 2012; Marusich et al., 2016a), drugs with quick onset and shorter duration of action are associated with greater abuse potential.

As expected, following the above described, when the abuse potential of NEH and of Buph was evaluated, the place preference shift observed after conditioning with each studied SC indicates that these drugs display rewarding effects, posing a risk of abuse at doses as low as 4 mg/kg. The suggested

abuse potential here observed for NEH and Buph has been described in Buph-treated mice at similar dose ranges (3 and 10 mg/kg) and for other SC (Glennon and Young 2016; Karlsson et al. 2014; Lisek et al. 2012; Marusich et al. 2016; Oh et al. 2018; Watterson et al. 2012). Studies demonstrated that mephedrone, methylone, MDPV and α-PVP induce similar or even higher abuse potential than amphetamine (Karlsson et al. 2014; Marusich et al. 2016; Oh et al. 2018). Evidences also suggest that addiction and rewarding effects following exposure to drugs of abuse, including MDMA, are correlated with increased levels of dopamine in the brain (Blum et al. 2012; Marona-Lewicka et al. 1996; Oh et al. 2018; Wise 2009).

It is known that new environments trigger a reaction of fear, anxiety and anguish. We evaluated the anxiety-induced behaviour of being in a novel environment (novel cage) during the sessions of the conditioning phase. The observed reduction of defecation index that revealed to be dose-dependent following single and repeated exposure for both NEH and Buph drugs was also observed with amphetamine (Moscardo et al. 2007) and may result from the effects of these SC on the autonomic nervous system. Neuronal control of defecation is associated to parasympathetic and sympathetic modulation. Hence, it is possible that Buph and NEH brain interaction, following either single or repeated exposure, results in sympathetic stimulation (noradrenergic action, NA) and consequent decreased defecation. On the other hand, the reduced defecation index, a validated parameter for measuring the emotional changes of being in a novel environment (Walsh and Cummins 1976), suggests a hyposensitivity to new environments and, therefore, an induced anxiolytic effect, possible through NA modulation. Amphetamine and several SC have been shown to increase noradrenaline release in addition to dopamine and serotonin (Baumann et al. 2014; Martinez-Clemente et al. 2014). These results added to the reduced burying behaviour observed one day after mice that had been exposed to NEH and Buph are in line with those burying effects reported for anxiolytic and antidepressant drugs (Kobayashi et al. 2008). They suggest an induced anxiolytic effect for Buph at 4 mg/kg (single/repeated) doses and for NEH at higher doses (16 mg/kg), which is sustained at least up to 1 day post-last-repeated dose. Grooming, one of the most frequently performed behavioural activities in rodents, was also evaluated. Given that rodent models of anxiety, as for example mice treated with anxiogenic drugs, display a longer duration of grooming behaviour (Kalueff et al. 2016), we would expect that our animals treated with NEH and Buph spent less time in grooming as they were presenting anxiolytic-like behaviour. Interestingly, Buph-treated animals displayed a longer duration of grooming behaviour than controls, contrasting with those treated with NEH. These effects on grooming behaviour were observed 3 days post-last dose, suggesting a withdrawn induced anxiety-like behaviour in mice repeatedly exposed to Buph. On the other hand, in the rodent model of depression (chronic unpredictable mild stress—CUMS), which mimics a variety of neurochemical and behavioural changes similar to those described for human depression, it is often seen deficits in self-grooming activity (d'Audiffret et al. 2010; Kalueff et al. 2016), hence suggesting a withdrawn depression-like behaviour in those mice exposed to NEH. This is not without precedent, as others have shown an increase in anxiety- and depression-like behaviours in rats withdrawn from chronic cocaine or MDPV (Philogene-Khalid et al. 2017).

In order to better distinguish the differential neurotoxicological effects of NEH and Buph we subsequently tested these NPS in human neuronal and microglial cell lines. We decided to use such approach in order to have models possibly more relevant to the human context and NPS abuse profile, as well as to better characterize each cell type individually for its response to specific NPS concentrations. We used the differentiated SH-SY5Y neuroblastoma human cell line that shows neuronal characteristics and a mature neuron-like phenotype based on neuronal markers (Kovalevich and Langford 2013). These neuronal cells were already used as a well-established model for amphetamine neurotoxicity research (den Hollander et al. 2015) and more recently to determine the cytotoxicity of several SC lacking the methylenedioxy ring, including Buph (Soares et al. 2019). Although it has been proposed that NPS, including SC, can induce neurotoxicity and alterations in neuron-microglia communication with consequent neuroinflammation that could explain the delayed response upon consumption (Blum et al. 2013), the precise mechanisms by which microglia can become activated upon exposure to NPS remains unknown. To increase our knowledge on the effects of the selected NPS as stressful compounds on microglia activation, we used the CHME3 microglial human cell line. This human microglia cell line was shown to retain properties of primary microglial cells in terms of phagocytic ability and production of inflammatory mediators upon stimulation (Janabi et al. 1995). Cells were exposed to either Buph or NEH, at concentrations that varied from 5 to 400 μM, using methedrone at similar doses for comparative purposes. Among the three tested drugs, NEH was as deleterious as Buph in causing neuronal demise, with lower effects being produced by methedrone. In fact, methedrone, despite previously indicated to be dangerous for recreational purposes based on the reported two fatal intoxications (Wikstrom et al. 2010), was recently indicated to induce neuronal toxicity in SH-SY5Y cells for higher concentrations than those observed in the present study (> 500 μM) (Soares et al. 2019). In the same study, Soares et al. (2019) also showed that Buph induced neuronal loss of viability. Here, we additionally demonstrated that NEH led to increased cytotoxicity towards microglial cells than Buph or methedrone, once it was the only NPS that significantly

induced microglia demise. Although Buph also revealed to enhance microglia apoptosis to values that do not surpass 10% of the cells, NEH caused increased late apoptosis reaching values of roughly 20%. Classical amphetamines were demonstrated to cause neuronal apoptosis (Angoa-Perez et al. 2017), but information on the role of SC to induce apoptosis in cellular models, as we here describe, is scarce. In previous studies, mephedrone was shown to elicit cytotoxicity against cortical neurons (Martinez-Clemente et al. 2014), pyrovalerone and its derivatives to reduce the viability of human neuroblastoma SHSY5Y cells (Wojcieszak et al. 2016), and 3-fluoromethcathinone (a mephedrone analog) to inhibit growth and cause cell arrest in HT22 immortalized mouse hippocampal cells (Siedlecka-Kroplewska et al. 2014). Though identification and analytical characterization of the newest cathinone NEH was lately reported (Liu et al. 2017), no clear data for their neurotoxic properties on cell death was so far reported. Recently, some SC were shown to cause autophagy and oxidative stress on neuronal cells (Matsunaga et al. 2017; Siedlecka-Kroplewska et al. 2014; Soares et al. 2019; Valente et al. 2014), but no reports exist on their potential effects on microglial cells. LysoTrack staining is currently used to label lysosomes (Chazotte 2011). In addition, mechanisms regulating autophagy were shown to contribute to cell survival and death (Das et al. 2012), turning its evaluation important when assessing pathological mechanisms, once its excessive activation may have deleterious effects in bioenergetics or in cell death mechanisms. In the present study, we demonstrate for the first time that both NEH and Buph promote neuronal lysosomal activation. This finding is in accordance with the cathinone-induced autophagy as mentioned above, but contrast with methamphetamine reported to downregulate lysosomal activity in SH-SY5Y neurons (Nara et al. 2012). Explanation for lysosomal stress by cathinones may derive from the neuronal attempt to maintain their synaptic plasticity mediated by lysosomal activity (Padamsey et al. 2017), or from the increased neuronal β-glucuronidase activity (involved in lysosomal pathway) after exposure to cathinone following the ingestion of *Catha edulis* leaves (Al-Mamary et al. 2014). Effects of NEH and Buph on lysosomal activation were higher in microglia than in neurons, mainly for the first NPS. Treatments with inflammatory agents were shown to acidify microglial lysosomes, making them more efficient for degradation processes (Majumdar et al. 2007). In this case, autophagic response resides on the recognition of the toxic cargo, thus targeting it to lysosome for degradation (Plaza-Zabala et al. 2017). If autophagy is long sustained, it may lead to detrimental flux dysregulations ending on cell death by apoptosis or necrosis. Nevertheless, upregulation of autophagy was also shown to promote the activation of microglia towards inflammation repair (Jin et al. 2018). If NEH and Buph increase of lysosomal activation relates with enhanced autophagy will require further studies.

When assessing morphological alterations caused by NEH and Buph on microglia, only the incubation with NEH led to a significant reduction in microglial perimeter and cell area that relate with a shift from a ramified to an amoeboid cell morphology (Caldeira et al. 2014; Cunha et al. 2016), thus supporting its ability to activate microglia. Indeed, such morphological changes based on soma size and roundness are widely utilized as markers of microglial activation (Davis et al. 2017; Heindl et al. 2018). Neuroinflammation was previously identified in human methamphetamine abusers by imaging (Blum et al. 2013), but absent in mephedrone exposure (Bowyer et al. 2008). Our study was innovative in exploring alterations caused by the SC NEH and Buph on human microglial cells better translating the effects that may be produced in drug users. Findings here obtained on microglia highlight NEH as having higher stressful properties than Buph in causing demise, lysosomal stress and cell morphological changes indicative of a dysfunctional and activated microglia. To the best of our knowledge, this is the first study reporting microglia activation to NEH per se, and not just as a consequence of neuronal demise.

The differences observed among NEH and Buph on the effects produced at equal doses do suggest non-equipotent doses. Both the duration of effects and the toxicity of these SC, following their absorption and distribution, depend not only on the pharmacological mechanism at the central nervous system but also on their elimination rate from the blood stream. There are no published reports on the pharmacokinetics of NEH (WHO 2019). Yet, user reports of short-lived psychoactive effects suggest that these two drugs are absorbed, distributed and eliminated relatively quickly (Erowid 2018). However, the greater lipophilicity of NEH compared with Buph (Log P 3.4 vs. 2.1, respectively, computed by XLogP3, PubChem) has been associated with longest plasma half-life and highest permeability.

In conclusion, in vivo data show that NEH and Buph induce a typical behaviour as reported for other SC in terms of excitation and stereotypies, but with intrinsic differences in several other fine-tuned behaviours: jumping, biting and aggressiveness for Buph; retropulsion and circling for NEH. Buph is more powerful than NEH in triggering features suggestive of anxiolytic behaviour. Importantly, either NEH or Buph displayed CPP shift in mice for doses as low as 4 mg/kg, supporting a potential risk of abuse for both drugs. Such property is here described for the first time for NEH, as far as authors' best knowledge and the World Health Organization report (WHO 2019). Additionally, withdrawn of NEH displayed depressive-like, but that of Buph produced an anxious-like behaviour in mice. Increased effects of NEH over those of Buph are also clear from in vitro studies relatively to microglia viability loss, late apoptosis, lysosomal activation and morphological changes, despite the equivalent results of these SC on neuronal cell demise and lysosomal stress.

Microglia preferential activation by NEH may then translate in increased neurodegeneration and toxicities in vivo. In sum, both Buph and NEH produce alterations either in nerve cell function or in mice behaviour, but the evidence of extended and multiple alterations observed for NEH and its specific microglia-induced dysfunctional properties points out that NEH represents a higher risk/safety concern than Buph.

Authors' Contribution AL, DB, CMS and RM were responsible for the study concept and design. CMS, SCH and AL performed the behaviour experiments, acquired and processed animal data. PF performed the synthesis of NPS. ARV, FB, PF and AF performed the cell culture maintenance, incubation with NPS and in vitro data acquisition and processing. CMS, ARV, DB and AL drafted the manuscript. All authors critically reviewed content and approved final version for publication.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

Al-Mamary H, El-Shaibany A, Al-Habori M, Al-Meeri A, Al-Zubairi AS et al (2014) Effect of *Catha edulis* on the activities of enzyme markers of carcinogenicity in chemically-induced hepatocellular carcinoma in rabbits. Int J Cancer Res 10:1–13. https://doi.org/10.3923/ijcr.2014.1.13

Angoa-Perez M, Anneken JH, Kuhn DM (2017) Neurotoxicology of synthetic cathinone analogs. Curr Top Behav Neurosci 32:209–230. https://doi.org/10.1007/7854_2016_21

Annau Z, Cuomo V (1988) Mechanisms of neurotoxicity and their relationship to behavioral changes. Toxicology 49:219–225. https://doi.org/10.1016/0300-483x(88)90002-9

Baumann MH, Solis E Jr, Watterson LR, Marusich JA, Fantegrossi WE et al (2014) Baths salts, spice, and related designer drugs: the science behind the headlines. J Neurosci 34:15150–15158. https://doi.org/10.1523/JNEUROSCI.3223-14.2014

Berardelli A, Capocaccia L, Pacitti C, Tancredi V, Quinteri F et al (1980) Behavioural and EEG effects induced by an amphetamine like substance (cathinone) in rats. Pharmacol Res Commun 12:959–964

Blum K, Chen AL, Giordano J, Borsten J, Chen TJ et al (2012) The addictive brain: all roads lead to dopamine. J Psychoactive Drugs 44:134–143. https://doi.org/10.1080/02791072.2012.685407

Blum K, Foster Olive M, Wang KK, Febo M, Borsten J et al (2013) Hypothesizing that designer drugs containing cathinones ("bath salts") have profound neuro-inflammatory effects and dangerous neurotoxic response following human consumption. Med Hypotheses 81:450–455. https://doi.org/10.1016/j.mehy.2013.06.007

Bowyer JF, Robinson B, Ali S, Schmued LC (2008) Neurotoxic-related changes in tyrosine hydroxylase, microglia, myelin, and the blood-brain barrier in the caudate-putamen from acute methamphetamine exposure. Synapse 62:193–204. https://doi.org/10.1002/syn.20478

Brites D, Vaz AR (2014) Microglia centered pathogenesis in ALS: insights in cell interconnectivity. Front Cell Neurosci 8:117. https://doi.org/10.3389/fncel.2014.00117

Caldeira C, Oliveira AF, Cunha C, Vaz AR, Falcão AS et al (2014) Microglia change from a reactive to an age-like phenotype with the time in culture. Front Cell Neurosci 8:152. https://doi.org/10.3389/fncel.2014.00152

Chazotte B (2011) Labeling lysosomes in live cells with LysoTracker. Cold Spring Harb Protoc 2011:pdb prot5571. https://doi.org/10.1101/pdb.prot5571

Cunha C, Gomes C, Vaz AR, Brites D (2016, 2016) Exploring new inflammatory biomarkers and pathways during LPS-induced M1 polarization. Mediators Inflamm:6986175. https://doi.org/10.1155/2016/6986175

d'Audiffret AC, Frisbee SJ, Stapleton PA, Goodwill AG, Isingrini E, Frisbee JC (2010) Depressive behavior and vascular dysfunction: a link between clinical depression and vascular disease? J Appl Physiol 108:1041–1051. https://doi.org/10.1152/japplphysiol.01440.2009

Das G, Shravage BV, Baehrecke EH (2012) Regulation and function of autophagy during cell survival and cell death. Cold Spring Harb Perspect Biol:4. https://doi.org/10.1101/cshperspect.a008813

Davis BM, Salinas-Navarro M, Cordeiro MF, Moons L, De Groef L (2017) Characterizing microglia activation: a spatial statistics approach to maximize information extraction. Sci Rep 7:1576. https://doi.org/10.1038/s41598-017-01747-8

Deacon RM (2006) Digging and marble burying in mice: simple methods for in vivo identification of biological impacts. Nat Protoc 1:122–124. https://doi.org/10.1038/nprot.2006.20

den Hollander B, Sundstrom M, Pelander A, Ojanpera I, Mervaala E et al (2014) Keto amphetamine toxicity-focus on the redox reactivity of the cathinone designer drug mephedrone. Toxicol Sci 141:120–131. https://doi.org/10.1093/toxsci/kfu108

den Hollander B, Sundstrom M, Pelander A, Siltanen A, Ojanpera I et al (2015) Mitochondrial respiratory dysfunction due to the conversion of substituted cathinones to methylbenzamides in SH-SY5Y cells. Sci Rep 5:14924. https://doi.org/10.1038/srep14924

EMCDDA (2017a) European drug report 2017: trends and developments. European Monitoring Centre for Drugs and Drug Addiction, Lisbon

EMCDDA (2017b) High-risk drug use and new psychoactive substances. Publications Office of the European Union, European Monitoring Centre for Drugs and Drug Addiction https://doi.org/10.2810/583405

EMCDDA (2017c) New drugs emerging at a slower pace - Drugnet Europe 98. European Monitoring Centre for Drugs and Drug Addiction, Lisbon

EMCDDA (2018a) European drug report 2018: trends and developments. European Monitoring Centre for Drugs and Drug Addiction, Lisbon. https://doi.org/10.2810/800331

EMCDDA (2018b) Fentanils and synthetic cannabinoids: driving greater complexity into the drug situation - — an update from the EU Early Warning System. European Monitoring Centre for Drugs and Drug Addiction, Lisbon

EMCDDA (2019) European drug report 2019: trends and developments. European Monitoring Centre for Drugs and Drug Addiction, Lisbon. https://doi.org/10.2810/191370

Erowid (2018) N-ethylhexedrone reports. https://erowid.org/experiences/subs/exp_NEthylhexedrone.shtml. Accessed 1 Sept 2019

Falcão AS, Carvalho LA, Lidónio G, Vaz AR, Lucas SD et al (2017) Dipeptidyl vinyl sulfone as a novel chemical tool to inhibit HMGB1/NLRP3-inflammasome and inflamma-miRs in Abeta-

mediated microglial inflammation. ACS Chem Neurosci 8:89–99. https://doi.org/10.1021/acschemneuro.6b00250

FDA (2017) Assessment of abuse potential of drugs: guidance for industry. https://www.fda.gov/media/116739/download. Accessed 1 Sept 2019

Fernandes A, Ribeiro AR, Monteiro M, Garcia G, Vaz AR et al (2018) Secretome from SH-SY5Y APPSwe cells trigger time-dependent CHME3 microglia activation phenotypes, ultimately leading to miR-21 exosome shuttling. Biochimie. https://doi.org/10.1016/j.biochi.2018.05.015

Glennon RA, Young R (2016) Neurobiology of 3,4-methylenedioxypyrovalerone (MDPV) and alpha-pyrrolidinovalerophenone (alpha-PVP). Brain Res Bull 126:111–126. https://doi.org/10.1016/j.brainresbull.2016.04.011

Haberzettl R, Bert B, Fink H, Fox MA (2013) Animal models of the serotonin syndrome: a systematic review. Behav Brain Res 256:328–345. https://doi.org/10.1016/j.bbr.2013.08.045

Heindl S, Gesierich B, Benakis C, Llovera G, Duering M, Liesz A (2018) Automated morphological analysis of microglia after stroke. Front Cell Neurosci 12:106. https://doi.org/10.3389/fncel.2018.00106

Herbert K, Karl Z, Gerhard L (1965) Patent DE1545591 - Verfahren zur Herstellung von α-Aminoketonen mit heterocyclischer Aminogruppe

Janabi N, Peudenier S, Heron B, Ng KH, Tardieu M (1995) Establishment of human microglial cell lines after transfection of primary cultures of embryonic microglial cells with the SV40 large T antigen. Neurosci Lett 195:105–108

Jin MM, Wang F, Qi D, Liu WW, Gu C, Mao CJ, Yang YP, Zhao Z, Hu LF, Liu CF (2018) A critical role of autophagy in regulating microglia polarization in neurodegeneration. Front Aging Neurosci 10:378. https://doi.org/10.3389/fnagi.2018.00378

Kalueff AV, Stewart AM, Song C, Berridge KC, Graybiel AM et al (2016) Neurobiology of rodent self-grooming and its value for translational neuroscience. Nat Rev Neurosci 17:45–59. https://doi.org/10.1038/nrn.2015.8

Karlsson L, Andersson M, Kronstrand R, Kugelberg FC (2014) Mephedrone, methylone and 3,4-methylenedioxypyrovalerone (MDPV) induce conditioned place preference in mice. Basic Clin Pharmacol Toxicol 115:411–416. https://doi.org/10.1111/bcpt.12253

Kobayashi T, Hayashi E, Shimamura M, Kinoshita M, Murphy NP (2008) Neurochemical responses to antidepressants in the prefrontal cortex of mice and their efficacy in preclinical models of anxiety-like and depression-like behavior: a comparative and correlational study. Psychopharmacology 197:567–580. https://doi.org/10.1007/s00213-008-1070-6

Kodamullil AT, Iyappan A, Karki R, Madan S, Younesi E et al (2017) Of mice and men: comparative analysis of neuro-inflammatory mechanisms in human and mouse using cause-and-effect models. J Alzheimers Dis 59:1045–1055. https://doi.org/10.3233/JAD-170255

Kovalevich J, Langford D (2013) Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology. Methods Mol Biol 1078:9–21. https://doi.org/10.1007/978-1-62703-640-5_2

Lisek R, Xu W, Yuvasheva E, Chiu YT, Reitz AB et al (2012) Mephedrone ('bath salt') elicits conditioned place preference and dopamine-sensitive motor activation. Drug Alcohol Depend 126:257–262. https://doi.org/10.1016/j.drugalcdep.2012.04.021

Liu C, Jia W, Li T, Hua Z, Qian Z (2017) Identification and analytical characterization of nine synthetic cathinone derivatives N-ethylhexedrone, 4-Cl-pentedrone, 4-Cl-alpha-EAPP, propylone, N-ethylnorpentylone, 6-MeO-bk-MDMA, alpha-PiHP, 4-Cl-alpha-PHP, and 4-F-alpha-PHP. Drug Test Anal 9:1162–1171. https://doi.org/10.1002/dta.2136

Majumdar A, Cruz D, Asamoah N, Buxbaum A, Sohar I et al (2007) Activation of microglia acidifies lysosomes and leads to degradation of Alzheimer amyloid fibrils. Mol Biol Cell 18:1490–1496. https://doi.org/10.1091/mbc.e06-10-0975

Marona-Lewicka D, Rhee GS, Sprague JE, Nichols DE (1996) Reinforcing effects of certain serotonin-releasing amphetamine derivatives. Pharmacol Biochem Behav 53:99–105

Martinez-Clemente J, Lopez-Arnau R, Abad S, Pubill D, Escubedo E et al (2014) Dose and time-dependent selective neurotoxicity induced by mephedrone in mice. PLoS One 9:e99002. https://doi.org/10.1371/journal.pone.0099002

Marusich JA, Grant KR, Blough BE, Wiley JL (2012) Effects of synthetic cathinones contained in "bath salts" on motor behavior and a functional observational battery in mice. Neurotoxicology 33:1305–1313. https://doi.org/10.1016/j.neuro.2012.08.003

Marusich JA, Antonazzo KR, Wiley JL, Blough BE, Partilla JS et al (2014) Pharmacology of novel synthetic stimulants structurally related to the "bath salts" constituent 3,4-methylenedioxypyrovalerone (MDPV). Neuropharmacology 87:206–213. https://doi.org/10.1016/j.neuropharm.2014.02.016

Marusich JA, Lefever TW, Blough BE, Thomas BF, Wiley JL (2016) Pharmacological effects of methamphetamine and alpha-PVP vapor and injection. Neurotoxicology 55:83–91. https://doi.org/10.1016/j.neuro.2016.05.015

Marusich JA, Antonazzo KR, Blough BE, Brandt SD, Kavanagh PV, Partilla JS, Baumann MH (2016a) The new psychoactive substances 5-(2-aminopropyl)indole (5-IT) and 6-(2-aminopropyl)indole (6-IT) interact with monoamine transporters in brain tissue. Neuropharmacology 101:68–75

Matsunaga T, Morikawa Y, Kamata K, Shibata A, Miyazono H et al (2017) alpha-Pyrrolidinononanophenone provokes apoptosis of neuronal cells through alterations in antioxidant properties. Toxicology 386:93–102. https://doi.org/10.1016/j.tox.2017.05.017

Moscardo E, Maurin A, Dorigatti R, Champeroux P, Richard S (2007) An optimised methodology for the neurobehavioural assessment in rodents. J Pharmacol Tox Met 56(2):239-255

Nara A, Aki T, Funakoshi T, Unuma K, Uemura K (2012) Hyperstimulation of macropinocytosis leads to lysosomal dysfunction during exposure to methamphetamine in SH-SY5Y cells. Brain Res 1466:1–14. https://doi.org/10.1016/j.brainres.2012.05.017

Oh JH, Hwang JY, Hong SI, Ma SX, Seo JY et al (2018) The new designer drug buphedrone produces rewarding properties via dopamine D1 receptor activation. Addict Biol 23:69–79. https://doi.org/10.1111/adb.12472

Padamsey Z, McGuinness L, Bardo SJ, Reinhart M, Tong R, Hedegaard A, Hart ML, Emptage NJ (2017) Activity-dependent exocytosis of lysosomes regulates the structural plasticity of dendritic spines. Neuron 93:132–146. https://doi.org/10.1016/j.neuron.2016.11.013

Pail PB, Costa KM, Leite CE, Campos MM (2015) Comparative pharmacological evaluation of the cathinone derivatives, mephedrone and methedrone, in mice. Neurotoxicology 50:71–80. https://doi.org/10.1016/j.neuro.2015.08.004

Philogene-Khalid HL, Hicks C, Reitz AB, Liu-Chen LY, Rawls SM (2017) Synthetic cathinones and stereochemistry: S enantiomer of mephedrone reduces anxiety- and depressant-like effects in cocaine- or MDPV-abstinent rats. Drug Alcohol Depend 178:119–125. https://doi.org/10.1016/j.drugalcdep.2017.04.024

Plaza-Zabala A, Sierra-Torre V, Sierra A (2017) Autophagy and microglia: novel partners in neurodegeneration and aging. Int J Mol Sci 18. https://doi.org/10.3390/ijms18030598

Prince JA, Oreland L (1997) Staurosporine differentiated human SH-SY5Y neuroblastoma cultures exhibit transient apoptosis and trophic factor independence. Brain Res Bull 43:515–523. https://doi.org/10.1016/s0361-9230(97)00328-6

Roux S, Sable E, Porsolt RD (2005) Primary observation (Irwin) test in rodents for assessing acute toxicity of a test agent and its effects on behavior and physiological function. Curr Protoc Pharmacol

Chapter 10:Unit 10 10. https://doi.org/10.1002/0471141755.ph1010s27

Schifano F, Napoletano F, Chiappini S, Guirguis A, Corkery JM et al (2019) New/emerging psychoactive substances and associated psychopathological consequences. Psycholog Med:1–13. https://doi.org/10.1017/S0033291719001727

Siedlecka-Kroplewska K, Szczerba A, Lipinska A, Slebioda T, Kmiec Z (2014) 3-Fluoromethcathinone, a structural analog of mephedrone, inhibits growth and induces cell cycle arrest in HT22 mouse hippocampal cells. J Physiol Pharmacol 65:241–246

Smolinsky AN, Bergner CL, LaPorte JL, Kalueff AV (2009) Analysis of grooming behavior and its utility in studying animal stress, anxiety, and depression. In: Gould T (ed) Mood and anxiety related phenotypes in mice. Neuromethods, vol 42. Humana Press, Totowa

Soares J, Costa VM, Gaspar H, Santos S, de Lourdes BM et al (2019) Structure-cytotoxicity relationship profile of 13 synthetic cathinones in differentiated human SH-SY5Y neuronal cells. Neurotoxicology. https://doi.org/10.1016/j.neuro.2019.08.009

Soria JA, Arroyo DS, Gaviglio EA, Rodriguez-Galan MC, Wang JM et al (2011) Interleukin 4 induces the apoptosis of mouse microglial cells by a caspase-dependent mechanism. Neurobiol Dis 43:616–624. https://doi.org/10.1016/j.nbd.2011.05.010

Spiller HA, Ryan ML, Weston RG, Jansen J (2011) Clinical experience with and analytical confirmation of "bath salts" and "legal highs" (synthetic cathinones) in the United States. Clin Toxicol 49:499–505. https://doi.org/10.3109/15563650.2011.590812

Sukumaran P, Sun Y, Vyas M, Singh BB (2015) TRPC1-mediated Ca(2)(+) entry is essential for the regulation of hypoxia and nutrient depletion-dependent autophagy. Cell Death Dis 6:e1674. https://doi.org/10.1038/cddis.2015.7

Tanaka Y, Matsuwaki T, Yamanouchi K, Nishihara M (2013) Increased lysosomal biogenesis in activated microglia and exacerbated neuronal damage after traumatic brain injury in progranulin-deficient mice. Neuroscience 250:8–19. https://doi.org/10.1016/j.neuroscience.2013.06.049

Tripsit (2018a) Buphedrone. Factsheets 2018 - http://drugs.tripsit.me/buphedrone. Accessed 1 Sept 2019

Tripsit (2018b) Hexen. Factsheets 2018 - http://drugs.tripsit.me/hexen. Accessed 1 Sept 2019

Ugolini F, Lana D, Nardiello P, Nosi D, Pantano D et al (2018) Different patterns of neurodegeneration and glia activation in CA1 and CA3 hippocampal regions of TgCRND8 mice. Front Aging Neurosci 10:372. https://doi.org/10.3389/fnagi.2018.00372

US.FDA (2005) Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers. Food and Drug Administration Center for Drug Evaluation and Research, U.S. Department of Health and Human Services. https://www.fda.gov/media/72309/download

Usenovic M, Krainc D (2012) Lysosomal dysfunction in neurodegeneration: the role of ATP13A2/PARK9. Autophagy 8:987–988. https://doi.org/10.4161/auto.20256

Valente MJ, Guedes de Pinho P, de Lourdes Bastos M, Carvalho F, Carvalho M (2014) Khat and synthetic cathinones: a review. Arch Toxicol 88:15–45. https://doi.org/10.1007/s00204-013-1163-9

Walsh RN, Cummins RA (1976) The open-field test: a critical review. Psychol Bull 83:482–504

Watterson LR, Hood L, Sewalia K, Tomek SE, Yahn S et al (2012) The reinforcing and rewarding effects of methylone, a synthetic cathinone commonly found in "Bath Salts". J Addict Res Ther (Suppl 9). https://doi.org/10.4172/2155-6105.S9-002

WHO (2019) Critical review report: N-ethylhexedrone. 42nd ECDD (2019): N-ethylhexedrone. https://www.who.int/medicines/access/controlled-substances/Final_N-ethylhexedrone.pdf?ua=1. Accessed 1 March 2020

Wikstrom M, Thelander G, Nystrom I, Kronstrand R (2010) Two fatal intoxications with the new designer drug methedrone (4-methoxymethcathinone). J Anal Toxicol 34:594–598

Wise RA (2009) Roles for nigrostriatal–not just mesocorticolimbic–dopamine in reward and addiction. Trends Neurosci 32:517–524. https://doi.org/10.1016/j.tins.2009.06.004

Wojcieszak J, Andrzejczak D, Woldan-Tambor A, Zawilska JB (2016) Cytotoxic activity of pyrovalerone derivatives, an emerging group of psychostimulant designer cathinones. Neurotoxicity Res 30:239–250. https://doi.org/10.1007/s12640-016-9640-6

Zancajo VM, Brito J, Carrasco MP, Bronze MR, Moreira R et al (2014) Analytical profiles of "legal highs" containing cathinones available in the area of Lisbon. Portugal Forensic Sci Int 244:102–110. https://doi.org/10.1016/j.forsciint.2014.08.010

Zelger JL, Schorno HX, Carlini EA (1980) Behavioural effects of cathinone, an amine obtained from Catha edulis Forsk.: comparisons with amphetamine, norpseudoephedrine, apomorphine and nomifensine. Bull Narc 32:67–81

Zhitomirsky B, Farber H, Assaraf YG (2018) LysoTracker and MitoTracker Red are transport substrates of P-glycoprotein: implications for anticancer drug design evading multidrug resistance. J Cell Mol Med 22:2131–2141. https://doi.org/10.1111/jcmm.13485

Zuba D, Adamowicz P, Byrska B (2013) Detection of buphedrone in biological and non-biological material–two case reports. Forensic Sci Int 227:15–20. https://doi.org/10.1016/j.forsciint.2012.08.034