

## Role of amino terminal substitutions in the pharmacological, rewarding and psychostimulant profiles of novel synthetic cathinones

L. Duart-Castells<sup>a</sup>, N. Nadal-Gratacós<sup>b</sup>, M. Muralter<sup>a</sup>, B. Puster<sup>a</sup>, X. Berzosa<sup>b</sup>,  
R. Estrada-Tejedor<sup>b</sup>, M. Niello<sup>c</sup>, S. Bhat<sup>c</sup>, D. Pubill<sup>a</sup>, J. Camarasa<sup>a</sup>, H.H. Sitte<sup>c,d</sup>,  
E. Escubedo<sup>a,\*</sup>, R. López-Arnau<sup>a</sup>

<sup>a</sup> Department of Pharmacology, Toxicology and Therapeutic Chemistry, Pharmacology Section and Institute of Biomedicine (IBUB), Faculty of Pharmacy, University of Barcelona, Barcelona, Spain

<sup>b</sup> Pharmaceutical Chemistry Group (GQF), IQS School of Engineering, Universitat Ramon Llull, Barcelona, Spain

<sup>c</sup> Medical University Vienna, Center for Physiology and Pharmacology, Institute of Pharmacology, Vienna, Austria, A-1090

<sup>d</sup> Center for Addiction Research and Science, Medical University Vienna, Waehringerstrasse 13 A, 1090, Vienna, Austria

### ARTICLE INFO

#### Keywords:

New psychoactive substances  
Synthetic cathinones  
Psychostimulant  
Reward  
Dopamine  
Dopamine transporter

### ABSTRACT

The emergence of new synthetic cathinones continues to be a matter of public health concern. In fact, they are quickly replaced by new structurally related alternatives. The main goal of the present study was to characterize the pharmacological profile, the psychostimulant and rewarding properties of novel cathinones (pentedrone, N-ethyl-pentadron,  $\alpha$ -PVP, N,N-diethyl-pentadron and  $\alpha$ -PpVP) which only differs in their amino terminal substitution.

Rat synaptosomes were used for [<sup>3</sup>H]dopamine uptake experiments. HEK293 transfected cells (hDAT, hSERT, hOCT; human dopamine, serotonin and organic cation transporter) were also used for [<sup>3</sup>H]monoamine uptake and transporter binding assays. Molecular docking was used to investigate the effect of the amino substitutions on the biological activity. Hyperlocomotion and conditioned place preference paradigm were used in order to study the psychostimulant and rewarding effects in mice.

All compounds tested are potent inhibitors of DAT with very low affinity for SERT, hOCT-2 and -3, and their potency for inhibiting DAT increased when the amino-substituent expanded from a methyl to either an ethyl-, a pyrrolidine- or a piperidine-ring. Regarding the *in vivo* results, all the compounds induced an increase in locomotor activity and possess rewarding properties. Results also showed a significant correlation between predicted binding affinities by molecular docking and affinity constants ( $K_i$ ) for hDAT as well as the cLogP of their amino-substituent with their hDAT/hSERT ratios.

Our study demonstrates the role of the amino-substituent in the pharmacological profile of novel synthetic cathinones as well as their potency inhibiting DA uptake and ability to induce psychostimulant and rewarding effects in mice.

### 1. Introduction

The emergence of New Psychoactive Substances (NPS) in the illicit drug market, including synthetic cathinones, continues to be a matter of public health concern. Their consumption is associated with acute intoxications and even some related fatalities have been reported. As of 2018, 119 countries and territories have reported 890 NPS to the United Nations Office on Drugs and Crime, with more than 300 identified in the

United States of America (USA). Although some of them are under law restrictions, others are easily available through online shops or through the *darknet* (European Monitoring Centre for Drugs and Drug Addiction, 2019; United Nations Office on Drugs and Crime (UNODC), 2013). When one of these NPS falls under legislative control, the drug market responds by producing different structurally related alternatives, through minor chemical modifications. For instance, when “first-generation cathinones” (i.e., methylone, mephedrone and 3,4-methylenedioxypropylvalerone (MDPV)) were classified by the Drug

\* Corresponding author. Department of Pharmacology, Toxicology and Therapeutic Chemistry, Faculty of Pharmacy, University of Barcelona. Avda. Joan XXIII 27-31. Barcelona, 08028, Spain.

E-mail address: [eescubedo@ub.edu](mailto:eescubedo@ub.edu) (E. Escubedo).

<https://doi.org/10.1016/j.neuropharm.2021.108475>

Received 13 October 2020; Received in revised form 18 January 2021; Accepted 23 January 2021

Available online 30 January 2021

0028-3908/© 2021 The Authors.

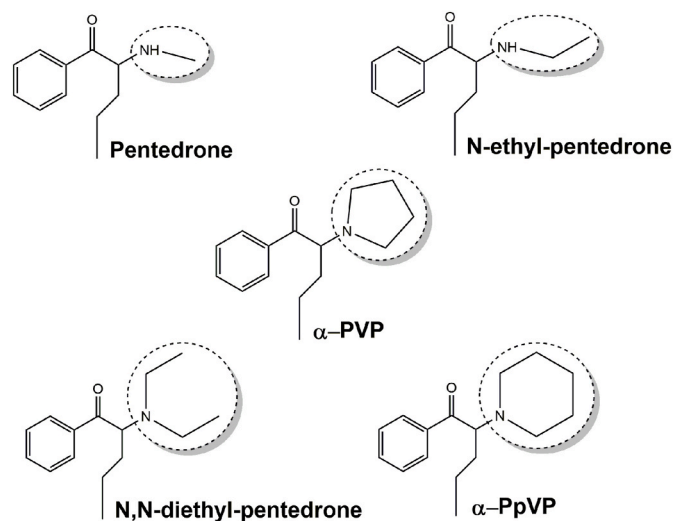
Published by Elsevier Ltd.

This is an open access article under the CC BY-NC-ND license

(<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

### Abbreviations

1-Methyl-4-phenylpyridinium	MPP <sup>+</sup>
$\alpha$ -diethylaminovalerophenone	N,N-diethyl-pentedrone
$\alpha$ -ethylaminovalerophenone	N-ethyl-pentedrone
$\alpha$ -piperidinevalerophenone	$\alpha$ -PpVP
$\alpha$ -pyrrolidinovalerophenone	$\alpha$ -PVP
Calculated molar refractivity	CMR
Calculated partition coefficient	CLogP
Conditioned place preference	CPP
Dopamine	DA
Dopamine transporter	DAT
Horizontal locomotor activity	HLA
Methylenedioxypropylvalerone	MDPV
Organic cation transporter	OCT
Quantitative Structure-Activity Relationship	QSAR
Serotonin	5-HT
Serotonin transporter	SERT
Structure-Activity Relationship	SAR



**Fig. 1.** Chemical structure of the aminovalerophenone derivatives. Compounds share a similar chemical structure, only differing by their amino-substituent: methylamino- (pentedrone), ethylamino- (N-ethyl-pentedrone), diethylamino- (N,N-diethyl-pentedrone), pyrrolidine ring ( $\alpha$ -PVP) or piperidine ring ( $\alpha$ -PpVP).

Enforcement Administration (DEA) as Schedule I compounds (Drug Enforcement Administration Department of Justice, 2011), a “second-generation” emerged, which includes  $\alpha$ -pyrrolidinovalerophenone ( $\alpha$ -PVP) and pentedrone (Drug Enforcement Administration, Department of Justice, 2014). The simple removal of the 3,4-methylenedioxy group from MDPV led to the  $\alpha$ -PVP structure, which at that time was not scheduled, and largely replaced MDPV on the drug market. Toxicity and abuse potential have been demonstrated for several synthetic cathinones regardless their mode of action (Baumann et al., 2018; Luethi et al., 2017; Rickli et al., 2015; Simmler et al., 2013, 2014; Zhou et al., 2019). However, scientific knowledge of many other synthetic cathinones that may appear or have recently appeared in the illicit market is still limited and thus, some of them have not yet been scheduled by the concerned authorities.

It has been demonstrated that  $\alpha$ -PVP acts as a potent blocker of the dopamine (DA) transporters (DAT) (Marusich et al., 2014; Meltzer et al., 2006; Rickli et al., 2015). Preclinical studies have also described  $\alpha$ -PVP to produce long-lasting increases in locomotor activity, to induce conditioned place preference and to fully substitute for discriminative stimulus effects of both cocaine and methamphetamine (Gatch et al., 2015b; Marusich et al., 2014). Moreover,  $\alpha$ -PVP has also been shown to facilitate intracranial self-stimulation and maintain self-administration in rats (Huskinson et al., 2017; Watterson et al., 2014). Pentedrone is another “second-generation” cathinone closely related to  $\alpha$ -PVP, only differing in its amino-group substituent (see Fig. 1). Similar to  $\alpha$ -PVP, pentedrone, as well as many other synthetic cathinones, blocks DAT and exhibits psychostimulant, rewarding and reinforcing properties as widely reported by other authors (Gatch et al., 2015a; Hwang et al., 2017; Javadi-Paydar et al., 2018; Simmler et al., 2014). Numerous structure-activity relationship (SAR) studies on synthetic cathinones have focused on the relationship between the molecular properties of different substituents and their pharmacological and toxicological profile (Eshleman et al., 2017; Kolanos et al., 2015; Niello et al., 2019; Walther et al., 2019) (for review see also (Baumann et al., 2018; Glennon and Dukat, 2016)). Saha and colleagues demonstrated how modifications at both,  $\alpha$ -carbon alkyl chain and the N-group of methcathinone, generate a “hybrid compound” that behaves as a blocker at DAT and as a releaser at the serotonin transporter (SERT) (Saha et al., 2015, 2019).

Thus, we suggest that some novel synthetic cathinones may induce potent psychostimulant and rewarding effects due to their structural similarities with other well-known synthetic relatives, such as  $\alpha$ -PVP and pentedrone (Gatch et al., 2015a; Hwang et al., 2017; Javadi-Paydar

et al., 2018; Simmler et al., 2014). This hypothesis also extends to the fact that, changes in the amino terminal group may also affect to their potency and selectivity as it occurs when changing other molecular substituents in other synthetic cathinones (Eshleman et al., 2017; Kolanos et al., 2015; Niello et al., 2019; Saha et al., 2015, 2019; Walther et al., 2019).

To explore the possible role of the amino-terminal group of synthetic cathinones, we have studied a set of five  $\alpha$ -aminovalerophenone derivatives: pentedrone,  $\alpha$ -ethylaminovalerophenone (N-ethyl-pentedrone),  $\alpha$ -diethylaminovalerophenone (N,N-diethyl-pentedrone),  $\alpha$ -PVP and  $\alpha$ -piperidinevalerophenone ( $\alpha$ -PpVP) (See Fig. 1). This may shine light on how these structural modifications may shape their activity at monoamine transporters together with their psychostimulant and rewarding effects. Importantly, the present work also highlights pharmacological and behavioural effects of N-ethyl-pentedrone, a novel NPS currently available ([www.erowid.com](http://www.erowid.com)) and identified in fatal cases and seizures (Majchrzak et al., 2018; Zaami et al., 2018) with no legal ramifications to prevent its distribution for consumption. Moreover, we provide insights into other novel synthetic cathinones which may appear as next generation NPS in the near future, and following also specific structural modifications in the amino-terminal group, increasing or reducing its steric bulk, lipophilicity and volume, with the aim of finding a relationship between these structural physicochemical parameters and the effects of these substances.

Accordingly, the aims of the present study were i) to characterize the *in vitro* pharmacology of five  $\alpha$ -aminovalerophenone derivatives; ii) to study the interaction mechanism of these compounds at the molecular level by means of molecular docking; iii) to assess their potential psychostimulant and rewarding effects at different doses; iv) to establish a structure-activity relationship (SAR) between the different amino-substituents and their pharmacological profile. These were achieved by monoamine uptake and binding experiments, together with molecular docking to investigate the molecular aspects of  $\alpha$ -aminovalerophenone derivatives. Combined with behavioural experiments in mice, which provided insight regarding their potential psychostimulant and rewarding effects, our study allowed to establish the SAR between the different amino-substituents and their pharmacological profile. Altogether, this study aims to provide a molecular explanation and behavioural evidence about the psychostimulant and rewarding effects induced by novel synthetic cathinones.

## 2. Materials and methods

### 2.1. Subjects

Male Swiss CD-1 mice (Charles River, Lyon, France) weighing 25–30 g (8 weeks-old) were randomly assigned to an experimental group and used for the behavioural experiments. For the synaptosomal preparation, male Sprague-Dawley rats (Janvier, Le Genest, France) weighing 225–250 g (2–3 months-old) were used. The animals were housed in polycarbonate cages with wood-derived bedding, in temperature-controlled conditions ( $22 \pm 1$  °C) under a 12 h light/dark cycle and had free access to food (standard laboratory diet, Panlab SL, Barcelona, Spain) and drinking water. All the behavioural experiments were performed during mice light cycle and a different batch of animals were used in HLA and CPP experiments in order to avoid any pre-exposure effect. All animal care and experimental protocols in this study complied with the guidelines of the European Community Council (2010/63/EU) and were approved by the Animal Ethics Committee of the University of Barcelona under the supervision of the Autonomous Government of Catalonia. Efforts were made to minimize suffering and reduce the number of used animals. All studies involving animals are reported in accordance with the ARRIVE guidelines (McGrath and Lilley, 2015).

### 2.2. Drugs and materials

$\alpha$ -Aminovalerophenone derivatives were synthesized in racemic form as hydrochloride salts and identified as described in Supplementary information section (A). Solutions for injection were freshly prepared daily in isotonic saline solution (0.9% NaCl, pH 7.4). [ $^3$ H]DA, [ $^3$ H]serotonin ([ $^3$ H]5-HT), [ $^3$ H]imipramine and [ $^3$ H]WIN35,428 were purchased from PerkinElmer Inc. (Boston, MA, USA). [ $^3$ H]1-Methyl-4-phenylpyridinium ([ $^3$ H]MPP<sup>+</sup>), was supplied by American Radio-labelled Chemicals (St. Louis, USA). Cocaine was generously provided by the Spanish National Institute of Toxicology. Pargyline, HEPES sodium and ascorbic acid as well as cell culture media (Dulbecco's Modified Eagle's medium (DMEM) high-glucose) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell culture dishes and 96-well plates were obtained from Sarstedt (Nuembrecht, Germany). All other reagents were of analytical grade and purchased from several commercial sources.

### 2.3. [ $^3$ H]DA uptake experiments in rat synaptosomes

#### 2.3.1. Rat synaptosome preparation

Rat synaptosome suspensions were prepared as described (Pubill et al., 2005), with minor modifications. Briefly, in each uptake inhibition experiment for each compound, two rats (independent experimental unit) were decapitated under isoflurane anesthesia and the striatum was dissected out, homogenized ( $\times 20$  vol; 5 mM Tris-HCl and 320 mM sucrose in Milli-Q water) and centrifuged at  $1000 \times g$ , 4 °C for 10 min. After discarding the pellet, the supernatant was centrifuged at  $13,000 \times g$  for 30 min at 4 °C. Then the supernatant was discarded and the pellet was diluted in HEPES-buffered solution (composition in mM: 140 NaCl, 5.37 KCl, 1.26 CaCl<sub>2</sub>, 0.44 KH<sub>2</sub>PO<sub>4</sub>, 0.49 MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.41 MgSO<sub>4</sub>·7H<sub>2</sub>O, 4.17 NaHCO<sub>3</sub>, 0.34 Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 5.5 glucose and 20 HEPES-Na) containing pargyline (20 mM) and ascorbic acid (1 mM). In order to reduce the number of animals used in this study, rats were used instead of mice for obtaining striatal synaptosomes.

#### 2.3.2. Plasmalemmal [ $^3$ H]DA uptake

In order to obtain a first evidence of the direct blockade (competitive inhibition) of [ $^3$ H]DA uptake in the presence of different  $\alpha$ -aminovalerophenone derivatives, synaptosomes from rat striatum were prepared as described above. Competitive blockade of [ $^3$ H]DA uptake was performed as described by López-Arnau et al. (2012), with some

modifications. Briefly, reaction tubes were composed of 0.125 ml of pentedrone, N-ethyl-pentedrone, N,N-diethyl-pentedrone,  $\alpha$ -PVP or  $\alpha$ -PpVP at different concentrations in HEPES-buffered solution containing pargyline and ascorbic acid. Moreover, 0.025 ml of [ $^3$ H]DA was also added (final concentration 5 nM). The reaction tubes as well as the synaptosomal suspension were warmed for 10 min at 37 °C before the addition of 0.1 ml of synaptosome suspension, after which incubation was carried out for a further 5 min. Then, the uptake reaction was terminated by rapid vacuum filtration through Whatman GF/B glass fibre filters (Whatman Intl Ltd., Maidstone, UK) pre-soaked in 0.5% polyethyleneimine. Tubes and filters were washed rapidly three times with 4 mL ice-cold 50 mM Tris-HCl. The radioactivity trapped on the filters was measured by liquid scintillation spectrometry. Non-specific uptake was determined at 4 °C in parallel samples, which also contained cocaine (300  $\mu$ M) due to its DAT blocking and uptake inhibitor properties. This non-specific uptake provides the non-specific [ $^3$ H]DA uptake through other mechanism ways different to DAT which has to be subtracted from the total uptake values. So, the specific uptake was calculated as follows: Total uptake (radioactivity in the presence of the synthetic cathinone) minus non-specific uptake (radioactivity in the presence of cocaine). All determinations were performed per duplicate (technical replicates). Moreover, each uptake inhibition assay for each compound was obtained from three independent experimental units (N = 3). No [ $^3$ H]5-HT uptake assays in rat synaptosomes were performed due to the results obtained in inhibition assays in HEK293 cells thus reducing the number of rats used.

### 2.4. Uptake inhibition and transporter binding assays in HEK293 cells

#### 2.4.1. Cell culture and membrane preparation

Human embryonic kidney (HEK293) cells were used for the uptake and binding experiments. The generation and maintenance of stable, monoclonal cell lines expressing the human isoforms of DAT, SERT and the organic cation transporter 2 or 3 (OCT-2, OCT-3) was carried out as described previously (Mayer et al., 2016a, 2016b). HEK293 were maintained in DMEM supplemented with heat-inactivated 10% FBS, 100 U/ml penicillin and 100  $\mu$ g/100 ml streptomycin, and cultured to a subconfluent state in a humidified atmosphere (5% CO<sub>2</sub>, 37 °C). Geneticin (G418; 50  $\mu$ g/ml) was added to maintain the selection process.

For the uptake inhibition assays, HEK293 cells (passages 10–13) expressing different monoamine transporters were seeded at a density of 0.36 million cells per well onto poly-D-lysine (PDL) coated 96-well plates, 24 h prior to the experiment.

For membrane preparations, stably transfected HEK293 cells (hDAT and hSERT) were harvested from 15-cm dishes 80–90% confluent. Briefly, cells were washed twice with ice-cold phosphate buffered saline (PBS), mechanically detached from the dish with a plastic scraper in the same ice-cold PBS and pelleted by centrifugation ( $400 \times g$  for 10 min at 4 °C). The resulting pellet was resuspended in hypotonic HME buffer (20 mM HEPES NaOH, 2 mM MgCl<sub>2</sub>, 1 mM EDTA; pH 7.4), followed by two freeze-thaw cycles in liquid nitrogen and homogenization through sonication at 4 °C. Thereafter, membranes were collected by centrifugation ( $40,000 \times g$  for 30 min at 4 °C) and resuspended in an appropriate volume of HME buffer. The membrane preparations were kept at  $-80$  °C until use. Protein concentration was determined using the Bio-Rad Protein Reagent (Bio Rad Laboratories, Hercules, CA).

#### 2.4.2. Uptake inhibition assays

Before starting the uptake inhibition experiments, the media was removed from the cell culture 96-well plates and replaced with 200  $\mu$ l per well of Krebs-HEPES-Buffer (KHB; 10 mM HEPES, 120 mM NaCl, 3 mM KCl, 2 mM CaCl<sub>2</sub> · 2 H<sub>2</sub>O, 2 mM MgCl<sub>2</sub> · 6 H<sub>2</sub>O supplemented with 20 mM D-glucose; pH 7.3). Afterwards, cells were incubated with different concentrations of the test drugs diluted in KHB at a final volume of 50  $\mu$ l per well for 5 or 10 min, depending on the uptake experiment performed, to ensure equilibrated conditions (preincubation). The

preincubation time was 5 min in case of the uptake-1 inhibition assays (hDAT and hSERT) and 10 min for the uptake-2 assays (OCT-2, OCT-3). On preincubation, the tritiated substrates were added: 0.02  $\mu\text{M}$  [ $^3\text{H}$ ]MPP + for hDAT, 0.1  $\mu\text{M}$  [ $^3\text{H}$ ]5-HT for hSERT and 0.05  $\mu\text{M}$  [ $^3\text{H}$ ]MPP + for hOCT-2 and hOCT-3. The uptake incubation times were 3 min for hDAT, 1 min for hSERT and 10 min for uptake-2 experiments. The uptake was terminated by removing the tritiated substrate, washing the cells with ice-cold KHB and lysing them with sodium dodecyl sulfate (SDS) 1%. The lysate was added to scintillation fluid and the released radioactivity was quantified with a beta-scintillation counter (PerkinElmer, Waltham, MA, USA). Non-specific uptake was determined in parallel samples containing cocaine 100  $\mu\text{M}$  for hDAT, paroxetine 30  $\mu\text{M}$  for hSERT and decynium-22 (D22) 100  $\mu\text{M}$  for hOCT-2 and hOCT-3. The non-specific uptake value was <10% of total uptake and was subtracted from the data to yield specific uptake, as described above. The uptake in absence of the test compounds was normalized to 100% and uptake in the presence of different concentrations of drugs was expressed as a percentage thereof. All determinations were performed per triplicate. All experiments were performed three times ( $N = 3$ ).

#### 2.4.3. Transporter binding assays

Membrane preparations expressing the transporters hDAT and hSERT were incubated with radiolabelled selective ligands at concentrations equal or close to  $K_d$ , and ligand displacement by the tested drugs at different concentrations was measured. The drugs were diluted in binding buffer (120 mM NaCl, 3 mM KCl, 2 mM  $\text{MgCl}_2$ , 10  $\mu\text{M}$   $\text{ZnCl}_2$  and 20 mM Tris pH 7.4 for hDAT, and 120 mM NaCl, 3 mM KCl, 2 mM  $\text{MgCl}_2$ , 1 mM EDTA and 20 mM Tris pH 7.4 for hSERT) and tested at increasing concentrations (ranged from 0.1 nM–300  $\mu\text{M}$ ) in duplicate. The binding reactions were performed in tubes containing 25  $\mu\text{l}$  of the radioligand: [ $^3\text{H}$ ]WIN35,428,  $K_d = 12$  nM;  $B_{\text{max}} = 6.75$  pmol/mg (Sucic et al., 2010) (hDAT assay, final concentration 10 nM) or [ $^3\text{H}$ ]imipramine,  $K_d = 4.5$  nM;  $B_{\text{max}} = 15$  pmol/mg (Sucic et al., 2010) (hSERT assay, final concentration 3 nM) diluted in the corresponding reaction buffer, 5  $\mu\text{g}$  of membranes and 100  $\mu\text{l}$  of the tested drug dilution. Non-specific binding was determined in the presence of cocaine 100  $\mu\text{M}$  (for hDAT radioligand) and paroxetine 3  $\mu\text{M}$  (for hSERT radioligand) since high concentrations of these two compounds are able to fully displace [ $^3\text{H}$ ]WIN35,428 and [ $^3\text{H}$ ]imipramine binding, respectively. This non-specific binding of [ $^3\text{H}$ ]WIN35,428 or [ $^3\text{H}$ ]imipramine provides the binding to other components such as membrane lipids or the microfiber filters, which has also to be subtracted from the total binding values. Incubation was performed for 1 h at 20 °C. The binding reactions were terminated by rapid filtration of the membranes through GF/C glass microfiber filters pre-soaked with 0.5% polyethyleneimine and rapid washing with ice-cold wash buffer (120 mM NaCl, 2 mM  $\text{MgCl}_2$ , 10 mM Tris and 100  $\mu\text{M}$   $\text{ZnCl}_2$  for hDAT, and 120 mM NaCl, 2 mM  $\text{MgCl}_2$  and 10 mM Tris, for hSERT). Afterwards, scintillation cocktail was added to the vials containing the filters, and the trapped radioactivity was quantified by liquid scintillation counting. Specific binding of each compound to the transporter was defined as the difference between total binding (binding buffer alone) and non-specific binding. All determinations were performed per triplicate. All experiments were performed three times ( $N = 3$ ).

#### 2.5. Docking of $\alpha$ -aminovaleerophenone derivatives

All computational procedures were conducted using MOE 2019.01 software (Chemical Computing Group, Montreal, Canada). Structural model of hDAT was obtained by applying homology modeling on hDAT amino acid sequence (Uniprot ID: Q01959) and considering the crystal structure of *Drosophila* DAT (dDAT) complexed with methamphetamine as template (Protein Data Bank, 4XP6) (Wang et al., 2015). Although hDAT and dDAT proteins have moderate sequence similarity (56%), calculated as the pairwise percentage identity between sequences using MOE software, the active site is highly conserved (>80%). The obtained

hDAT three-dimensional model (RMSD = 0.191 Å) was finally prepared by applying the QuickPrep protocol available in MOE. The drugs were built as (S)-enantiomers in protonated form and they were docked into the hDAT model using the GBVI/WSA  $\Delta G$  score function was used to quantify the free energy of binding of the 100 resulting conformations for each molecule.

#### 2.6. Horizontal locomotor activity (HLA)

During the habituation phase (2 consecutive days), mice received an intraperitoneal (i.p.) saline injection and placed into a black Plexiglass open field arena (25 × 25 × 40 cm) under low-light conditions and white noise for 60 min. On the test day, horizontal locomotor activity (HLA) was measured as described by Duart-Castells et al. (2019), with minor modifications. Briefly, the animals were given their corresponding i.p. injection (saline 5 ml/kg, pentedrone 3, 10 or 30 mg  $\text{kg}^{-1}$ , N-ethyl-pentedrone 1, 3 or 10 mg  $\text{kg}^{-1}$ , N,N-diethyl-pentedrone 3.5, 12.5 or 35 mg  $\text{kg}^{-1}$ ,  $\alpha$ -PVP 1, 3 or 10 mg  $\text{kg}^{-1}$  or  $\alpha$ -PpVP 7.5, 25 or 75 mg  $\text{kg}^{-1}$ ) and immediately placed in the open field arena. HLA was video-monitored for 60 min using a tracking software (Smart 3.0 Panlab, Barcelona, Spain) and their total travelled distance (in cm) was measured. All HLA experiments were performed between 8:00 a.m. and 3:00 p.m. On one hand, pentedrone and  $\alpha$ -PVP doses were chosen following the results reported by Hwang et al. (2017) and Wojcieszak et al. (2018). On the other hand, there is no data available about the doses of N-ethyl-pentedrone, N,N-diethyl-pentedrone and  $\alpha$ -PpVP used in animal research. Therefore, these doses were chosen in pilot experiments and according to the psychostimulant effect induced by pentedrone or  $\alpha$ -PVP in our experiments at the medium doses tested in order to have, at least, one dose of N-ethyl-pentedrone, N,N-diethyl-pentedrone and  $\alpha$ -PpVP equally effective (similar distance travelled after injection). Moreover, all the highest doses used were always 10-fold higher than the lowest dose tested, a protocol also used by the same authors when injecting pentedrone or  $\alpha$ -PVP (Hwang et al., 2017; Wojcieszak et al., 2018).

#### 2.7. Conditioned place preference (CPP)

The potential of the five  $\alpha$ -aminovaleerophenone derivatives to induce rewarding effects was determined using a place conditioning paradigm (unbiased), as described by Duart-Castells et al. (2019), with minor modifications. The apparatus consisted of three distinct compartments with differences in visual and tactile cues (two compartments communicated by a central corridor). CPP was performed in three different phases: preconditioning, conditioning and post-conditioning test. During the preconditioning phase (Day 0), mice were placed in the middle of the corridor and had free access to all the apparatus compartments for 15 min. The mean time spent in each compartment was video-monitored and recorded (Smart 3.0, Panlab, Barcelona, Spain).

During the conditioning phase (Day 1–4, sessions 1–8) mice received the corresponding i.p. injection using the same doses used previously in the HLA experiments (saline, pentedrone, N-ethyl-pentedrone, N,N-diethyl-pentedrone,  $\alpha$ -PVP, or  $\alpha$ -PpVP) and immediately placed into one of the two conditioning compartments for 20 min (sessions 1, 3, 5 and 7). On the alternate sessions (2, 4, 6 and 8), mice were given a saline injection and placed in the other compartment for 20 min. Two conditioning sessions per day were performed, separated by a 5-h period. Control groups received a saline injection in every session. Sessions were counterbalanced as much as possible between compartments. Animals that spent >70% of the total session time in one compartment during the preconditioning phase were excluded due to the high bias.

Finally, the post-conditioning test was conducted as the preconditioning phase. A preference score was calculated as the difference between the time spent in the drug-paired compartment in the post-conditioning test minus the time spent in the same compartment in the preconditioning phase.

## 2.8. Data acquisition and statistical analysis

Data from biochemical analyses were normalized with 100% defined as the mean of the technical replicates in the control group. Competition curves were plotted and fitted by nonlinear regression. Data were best fitted to a sigmoidal dose-response curve and an IC<sub>50</sub> or EC<sub>50</sub> value was obtained. Transporter ratios were calculated as (1/DAT IC<sub>50</sub>: 1/SERT IC<sub>50</sub>), with higher values indicating greater selectivity for DAT.  $K_i$  (affinity) values were calculated using the Cheng-Prusoff equation:  $K_i = EC_{50}/(1 + [radioligand\ concentration/K_d])$  (Yung-Chi and Prusoff, 1973). One-way or two-way ANOVA of repeated measures, and subsequent post hoc test (Tukey-Kramer) which was conducted only if F was significant, was used to determine overall  $\alpha$ -aminovalesterophenone derivatives effects on HLA and CPP. The  $\alpha$  error probability was set at 0.05 ( $p < 0.05$ ). The exact group size for the individual experiments is shown in the corresponding figure legends. Pearson correlation analyses were also performed when needed. All analysis were carried out using GraphPad Prism (GraphPad software, San Diego, CA, USA). Molecular and physicochemical descriptors were calculated for the different amino-terminal group using ChemBioOffice Ultra and Data Warrior software. Lipophilicity descriptors included calculated partition coefficient (CLogP). Molecular surface and steric bulk were also investigated using Total Surface Area (TSA) and calculated molar refractivity (CMR), respectively (Hevener et al., 2008).

## 3. Results

### 3.1. Monoamine uptake inhibition

[<sup>3</sup>H]DA uptake inhibition by  $\alpha$ -aminovalesterophenone derivatives was assessed in rat brain synaptosomes in order to obtain a first evidence of the direct blockade of DA uptake, but more extensively in transfected HEK293 cells.

#### 3.1.1. [<sup>3</sup>H]DA uptake inhibition in rat brain synaptosomes

DA uptake inhibition profile of the tested compounds are depicted in Fig. S1 and their respective IC<sub>50</sub> values for [<sup>3</sup>H]DA uptake inhibition at DAT are compiled in Table 1. Data showed that  $\alpha$ -PVP appeared to be > 5-fold more potent inhibiting DA uptake than both N-ethylpentedrone and  $\alpha$ -PpVP. Moreover,  $\alpha$ -PVP was also >10- and >20-fold more potent

**Table 1**

Affinity and potency of substituted cathinones and standard compounds at monoamine transporters. Monoamine uptake-1 and uptake-2 inhibition and Transporter binding affinities: values are  $K_i$  given as  $\mu\text{M}$  (mean  $\pm$  SEM).

Compound	Monoamine uptake inhibition						Transporter binding affinities	
	Rat brain synaptosomes		Transfected HEK293 cells			Transfected HEK293 cells		
	Uptake-1 [ <sup>3</sup> H]DA	Uptake-1 [ <sup>3</sup> H]MPP <sup>+</sup> uptake at hDAT	Uptake-1 [ <sup>3</sup> H]5-HT uptake at hSERT	hDAT/hSERT ratio	Uptake-2 [ <sup>3</sup> H]MPP <sup>+</sup> uptake at hOCT-2	Uptake-2 [ <sup>3</sup> H]MPP <sup>+</sup> uptake at hOCT-3	hDAT	hSERT
Pentedrone	1.74 $\pm$ 0.37	0.22 $\pm$ 0.03	78 $\pm$ 5.5	363	27.0 $\pm$ 1.8	>1000	0.15 $\pm$ 0.02	11.4 $\pm$ 0.7
N-ethyl-pentedrone	0.82 $\pm$ 0.07	0.08 $\pm$ 0.02	78.5 $\pm$ 0.2	1029	57.6 $\pm$ 6.6	>1000	0.04 $\pm$ 0.01	17.1 $\pm$ 0.5
N,N-diethyl-pentedrone	3.54 $\pm$ 0.22	0.45 $\pm$ 0.07	>100	>2000	47.8 $\pm$ 0.7	>1000	0.4 $\pm$ 0.03	75.9 $\pm$ 21.2
$\alpha$ -PVP	0.14 $\pm$ 0.02	0.03 $\pm$ 0.01	>100	>2000	17.5 $\pm$ 2.5	>1000	0.01 $\pm$ 0.002	52.2 $\pm$ 2.9
$\alpha$ -PpVP	1.06 $\pm$ 0.08	0.06 $\pm$ 0.01	>100	>2000	12.9 $\pm$ 0.4	>1000	0.08 $\pm$ 0.02	>100
Cocaine <sup>a</sup>	N.A.	1.19 $\pm$ 0.31	7.94 $\pm$ 1.25	6.68	N.A.	N.A.	0.22 $\pm$ 0.01	N.A.
Imipramine <sup>a</sup>	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	0.01 $\pm$ 0.004

hDAT/hSERT ratio = 1/DAT IC<sub>50</sub>: 1/SERT IC<sub>50</sub>.

N.A., not assessed; <sup>a</sup> Control compounds.

than pentedrone and N,N-diethyl-pentedrone, respectively.

#### 3.1.2. Monoamine uptake inhibition in transfected HEK293 cells

The corresponding IC<sub>50</sub> values and hDAT/hSERT inhibition ratios are presented in Table 1 and concentration-response curves are depicted in Fig. S2. While assessing uptake-1 assays, all tested drugs displayed low IC<sub>50</sub> values for hDAT and negligible activity at hSERT. Regarding hDAT inhibition,  $\alpha$ -PVP was 2-fold more potent than both N-ethylpentedrone and  $\alpha$ -PpVP. Moreover,  $\alpha$ -PVP also appeared to be > 5-, >10- and 40-fold more potent than pentedrone, N,N-diethyl-pentedrone and cocaine, respectively. So, the order of potency inhibiting DA uptake appears to be  $\alpha$ -PVP  $\geq$  N-ethylpentedrone  $\geq$   $\alpha$ -PpVP > pentedrone > N,N-diethyl-pentedrone. On the other hand, cocaine appeared to be > 50- and >100-fold more potent than pentedrone and N-ethylpentedrone at inhibiting 5-HT uptake. Additionally, it is important to point out the very low inhibitory potencies of N,N-diethyl-pentedrone,  $\alpha$ -PVP and  $\alpha$ -PpVP when studying [<sup>3</sup>H]5-HT uptake inhibition at hSERT. However, an estimation of the IC<sub>50</sub> values, as well as hDAT/hSERT ratios, were calculated in order to perform the corresponding correlation analysis. Accordingly, all compounds appeared to be > 100-fold more potent blocking capacity at hDAT over hSERT.

Molecular and physicochemical descriptors were also calculated for the different amino-substituents, correlating some of these QSAR parameters, such as CLogP, total surface area, volume and CMR, with the corresponding logIC<sub>50</sub> value and loghDAT/hSERT ratio. As shown in Table 2, hSERT, but not hDAT IC<sub>50</sub> values, significantly correlated with CLogP, total surface area, volume and CMR of the amino-substituents. In addition, the hDAT/hSERT ratios also correlated with CLogP.

With regards to uptake-2 inhibition experiments (Table 1; Fig. S2),  $\alpha$ -aminovalesterophenone derivatives blocked hOCT-2 within the IC<sub>50</sub> value range of 10–60  $\mu\text{M}$ , with no considerable effect on hOCT-3.

### 3.2. Transporter binding affinities

The binding affinities of the  $\alpha$ -aminovalesterophenone derivatives for hDAT and hSERT were assessed by their ability to displace the corresponding radioligand binding to membranes prepared from HEK293 cells expressing these transporters. Their affinity constants ( $K_i$ ) are summarized in Table 1. All drugs exhibited higher binding affinity for hDAT in the medium-low nanomolar range (<400 nM), when compared

**Table 2**

Correlation analysis between molecular or physicochemical descriptors and logIC<sub>50</sub> or log hDAT/hSERT ratio values of monoamine uptake in transfected HEK293 cells.

	Log IC <sub>50</sub> hDAT		Log IC <sub>50</sub> hSERT		Log hDAT/hSERT ratio	
	r <sup>2</sup>	p	r <sup>2</sup>	p	r <sup>2</sup>	p
CLogP	0.082	P > 0.05	<b>0.917</b>	<b>P &lt; 0.05</b>	<b>0.806</b>	<b>P &lt; 0.05</b>
Total Surface Area (Å <sup>2</sup> )	0.013	P > 0.05	<b>0.922</b>	<b>P &lt; 0.05</b>	0.639	P > 0.05
Volume (Å <sup>3</sup> )	0.024	P > 0.05	<b>0.925</b>	<b>P &lt; 0.05</b>	0.677	P > 0.05
CMR	0.045	P > 0.05	<b>0.926</b>	<b>P &lt; 0.05</b>	0.734	P > 0.05

CLogP: partition coefficient.

CMR: calculated molar refractivity.

to hSERT (>10000 nM). Therefore, all the compounds seem to be hDAT-selective. For instance, N-ethyl-pentadron, α-PVP and α-PpVP were more potent than cocaine in binding to hDAT. Conversely, all drugs presented substantially lower affinity to hSERT, with pentadron showing the highest affinity (*K<sub>i</sub>*). α-PVP and the compound with the bulkier amino-substituent (α-PpVP) displayed poor binding affinity to hSERT. These results are in accordance with uptake-1 experiments in which we demonstrated a progressively lower potency at hSERT when increasing the volume, steric bulk or total surface area of the amino-substituent.

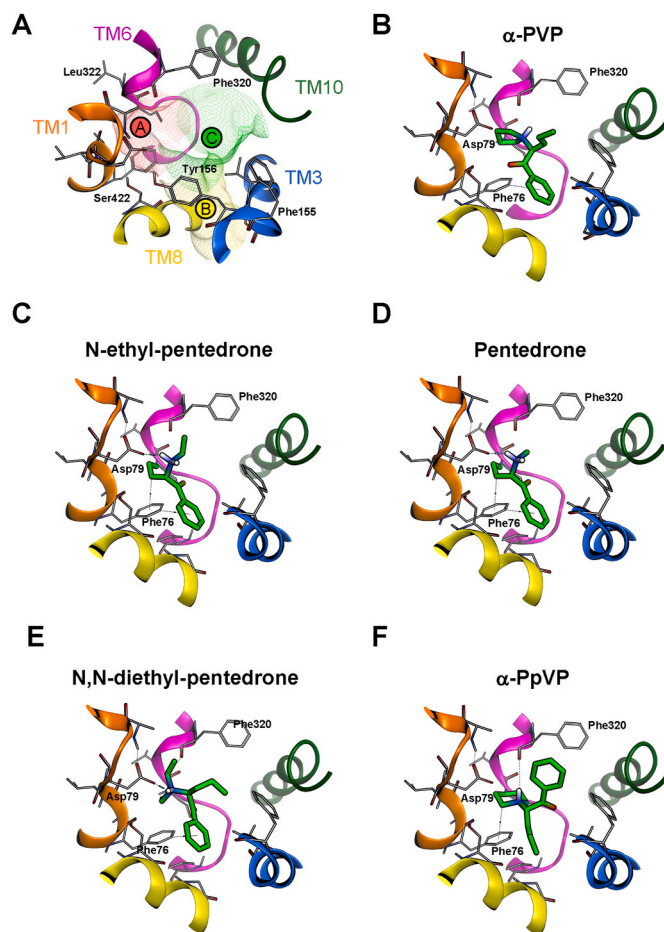
### 3.3. Molecular docking with α-aminovaleophenone derivatives

The binding pocket can be divided into three subsites (A-C), (Fig. 2A) (Andersen et al., 2010; Cheng et al., 2015). As expected, the phenyl ring was mainly located in subsite B (Saha et al., 2015), which corresponds to an amphiphilic site where the phenyl group can interact with Phe76 of TM1 helix through π-interaction. However, results suggested a more stable configuration for α-PpVP (Fig. 2F), in which the phenyl group was oriented towards the hydrophobic subsite C. Interestingly, the pyrrolidine group of α-PVP was subtly directed to TM6, renouncing to interact with Asp79, amino-acid that interacts with methamphetamine indeed (Fig. 2B). This is in contrast to N-ethyl-pentadron (Fig. 2C), pentadron (Fig. 2D) and N,N-diethyl-pentadron (Fig. 2E), which showed similar binding mechanism establishing an hydrogen bond between the amino group and Asp79. The addition of an extra carbon atom in pentadron seemed not to affect the binding mechanism (Fig. 2C and D) but the high hydrophobicity of N,N-diethyl-pentadron contrasted with the high polarity of subsite A and modified its interaction mechanism (Fig. 2E). Nevertheless, the major change was observed in α-PpVP where the phenyl and propyl groups exchange their orientations in comparison to α-PVP. It may be due to the steric hindrance found by the piperidine substructure when attempting to fit subsite A and the hydrophobic complementarity obtained when the phenyl group is located within subsite C (Fig. 2F). Predicted binding affinities (approximated as docking score value) agreed with *K<sub>i</sub>* experimental data ( $R^2_{SCORE-\log_{10}(K_i)} = 0.93$ ) and following the same order (Spearman correlation coefficient,  $\rho = 1$ ).

### 3.4. Effect of α-aminovaleophenone derivatives on HLA

One-way ANOVA of HLA results revealed a significant effect of the variable *Dose* for all the drugs tested (Pentadron:  $F_{(3,36)} = 22.01$ ;  $p < 0.001$ ; N-ethyl-pentadron:  $F_{(3,36)} = 98.42$ ;  $p < 0.001$ ; N,N-diethyl-pentadron:  $F_{(3,36)} = 52.89$ ;  $p < 0.001$ ; α-PVP:  $F_{(3,40)} = 72.09$ ;  $p < 0.001$ ; α-PpVP:  $F_{(3,36)} = 12.01$ ;  $p < 0.001$ ). Overall, all compounds increased HLA in a dose-dependent manner in mice.

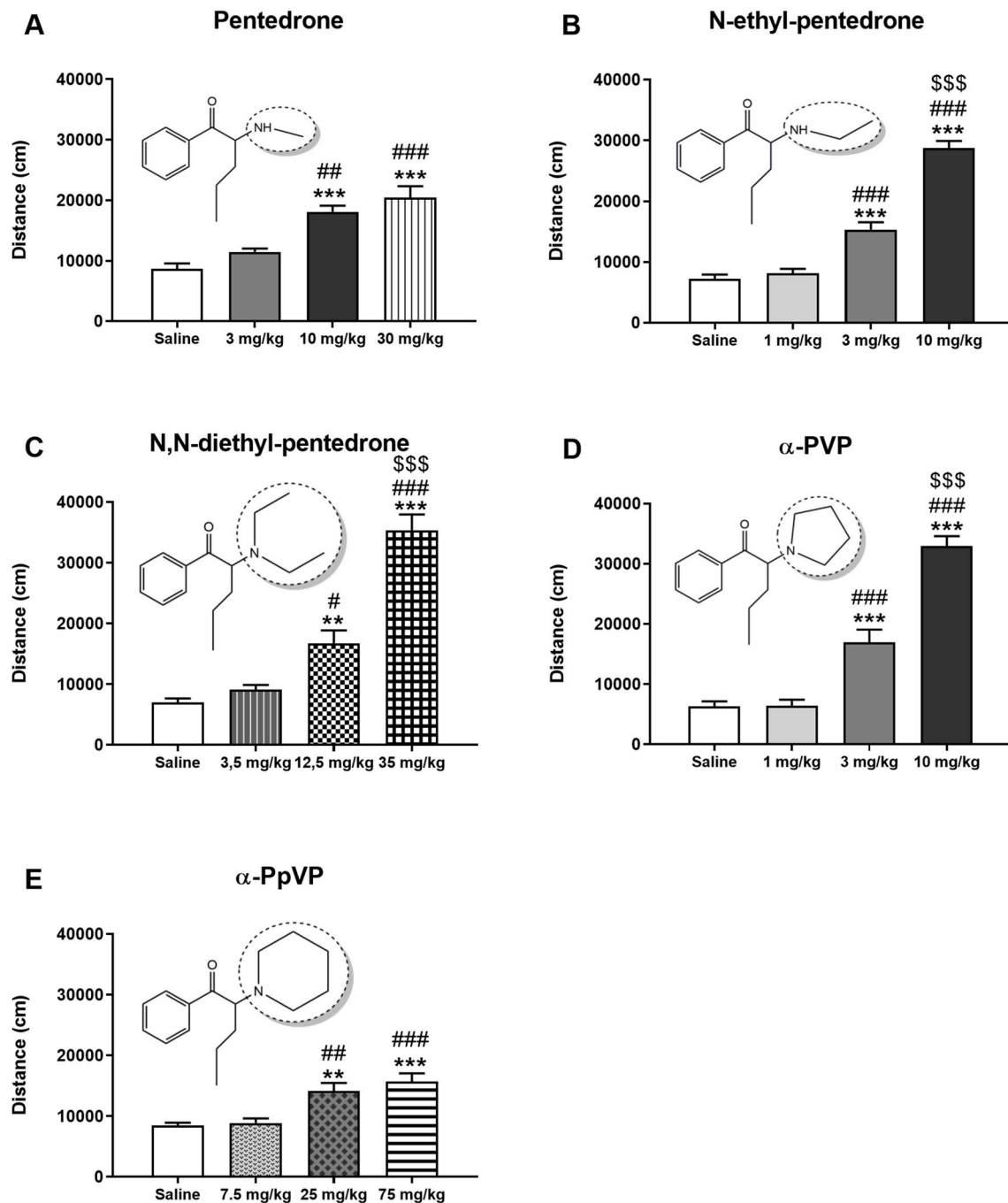
For pentadron and α-PpVP, subsequent post-hoc Tukey-Kramer test



**Fig. 2.** Molecular representation of hDAT binding site identifying the three different subsites A to C by colored surfaces (A). Binding mechanism predicted by molecular docking for α-PVP (B), N-ethyl-pentadron (C), pentadron (D), N,N-diethyl-pentadron (E) and α-PpVP (F). IN COLOR

demonstrated a significant increase in HLA after 10 and 30 mg kg<sup>-1</sup> (pentadron) and 25 and 75 mg kg<sup>-1</sup> (α-PpVP) injections, respectively, compared to the saline group. As for N-ethyl-pentadron (3 and 10 mg kg<sup>-1</sup>), N,N-diethyl-pentadron (12.5 and 35 mg kg<sup>-1</sup>) and α-PVP (3 and 10 mg kg<sup>-1</sup>), a similar increase in the distance travelled was observed when compared with the control group with the highest dose tested showing significant difference in locomotion when compared to the second highest concentration (Fig. 3).

HLA profiles are depicted in Fig. 4. Two-way ANOVA of repeated measures of the results yielded the following results: Pentadron (*Time*:  $F_{(11, 396)} = 21.46$ ,  $p < 0.001$ ; *Dose*:  $F_{(3,36)} = 15.20$ ,  $p < 0.001$ ; *Interaction*:  $F_{(33,396)} = 2.721$ ,  $p < 0.001$ ); N-ethyl-pentadron (*Time*:  $F_{(11,396)} = 26.88$ ,  $p < 0.001$ ; *Dose*:  $F_{(3,36)} = 98.11$ ,  $p < 0.001$ ; *Interaction*:  $F_{(33,396)} = 3.591$ ,  $p < 0.001$ ); N,N-diethyl-pentadron (*Time*:  $F_{(11,396)} = 20.01$ ,  $p < 0.001$ ; *Dose*:  $F_{(3,36)} = 52.89$ ,  $p < 0.001$ ; *Interaction*:  $F_{(33,396)} = 11.63$ ,  $p < 0.001$ ); α-PVP (*Time*:  $F_{(11,440)} = 30$ ,  $p < 0.001$ ; *Dose*:  $F_{(3,40)} = 67.53$ ,  $p < 0.001$ ; *Interaction*:  $F_{(33,440)} = 3.701$ ,  $p < 0.001$ ); α-PpVP (*Time*:  $F_{(11,396)} = 83.30$ ,  $p < 0.001$ ; *Dose*:  $F_{(3,36)} = 9.947$ ,  $p < 0.001$ ; *Interaction*:  $F_{(33,396)} = 8.543$ ,  $p < 0.001$ ). HLA profile revealed a rapid onset effect (5 min) for all the compounds at the medium and highest dose tested whereas no significant effect was observed at the lowest doses tested at any time point. Moreover, at the medium doses tested, the increase in locomotor activity ended before a 60 min period for all the compounds studied. Specifically, the psychostimulant effect induced by pentadron, N,N-diethyl-pentadron and α-PpVP at the medium dose tested lasted for 50, 25 and 15 min, respectively. On the other hand, the increase in the locomotor activity induced by both N-ethyl-pentadron



**Fig. 3.** Effects of aminovalerophenone derivatives on cumulative locomotor activity in CD-1 mice. Bars represent mean  $\pm$  SEM of the distance travelled in 60 min. Panel A: saline, pentedrone 3 mg kg<sup>-1</sup>, 10 mg kg<sup>-1</sup> and 30 mg kg<sup>-1</sup> groups, N = 10/group. Panel B: saline, N-ethyl-pentedrone 1, 3, and 10 mg kg<sup>-1</sup>, N = 10/group. Panel C: saline, N,N-diethyl-pentedrone 3.5, 12.5, 35 mg kg<sup>-1</sup>, N = 10/group. Panel D: saline,  $\alpha$ -PVP 1, 3, 10 mg kg<sup>-1</sup>, N = 11/group. Panel E: saline,  $\alpha$ -PpVP 7.5, 25, 75 mg kg<sup>-1</sup>, N = 10/group. \*\*P < 0.01 and \*\*\*P < 0.001 vs saline; #P < 0.05, ##P < 0.01 and ###P < 0.001 vs the lower drug-dose; \$\$\$P < 0.001 vs the medium drug-dose.

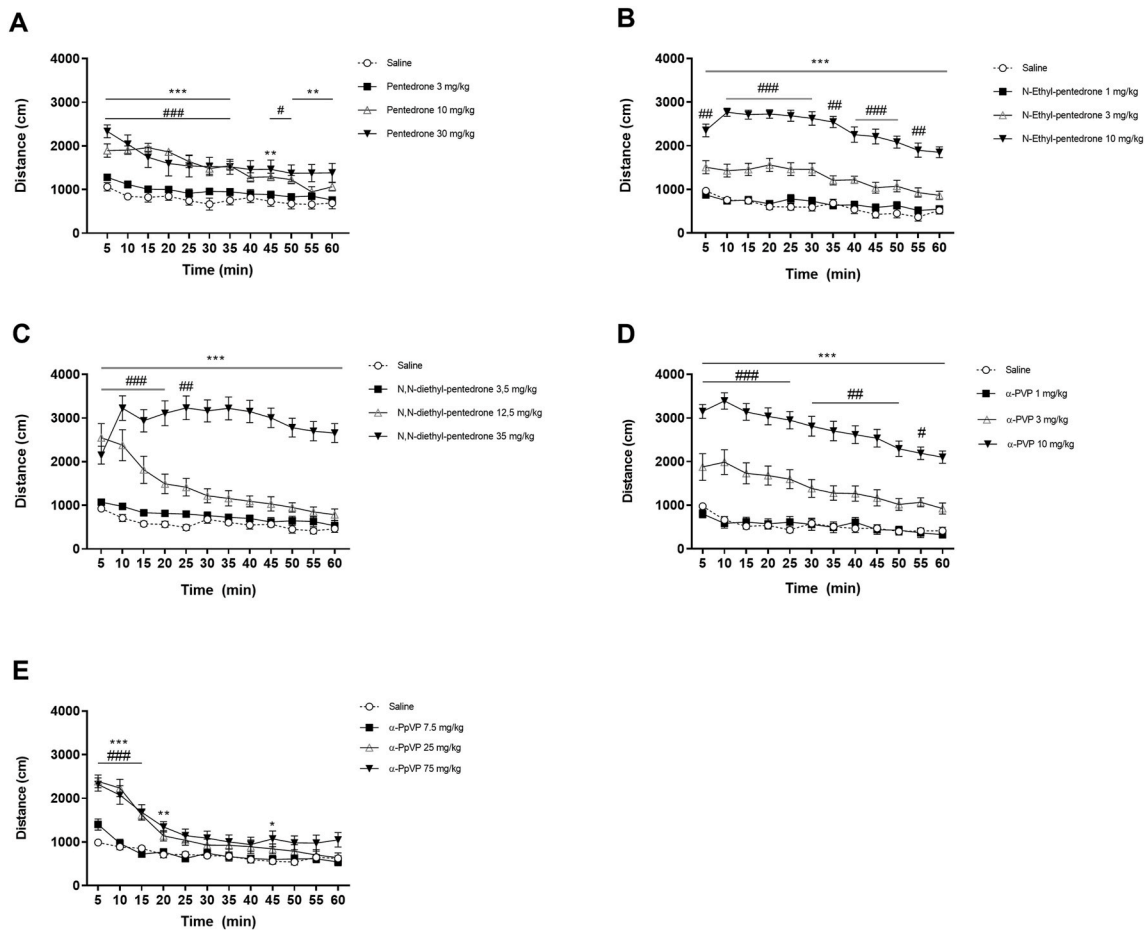
and  $\alpha$ -PVP at a dose of 3 mg kg<sup>-1</sup> lasted for 55 min. However, at the highest doses tested for pentedrone, N-ethyl-pentedrone, N,N-diethyl-pentedrone and  $\alpha$ -PVP, the psychostimulant effect lasted for more than 60 min.

When analysing the locomotor activity induced by the medium doses tested of each  $\alpha$ -aminovalerophenone derivative, one-way ANOVA yielded no significant effect of the variable *Compound*, which means that the medium doses used in this study were equally effective. Therefore,  $\alpha$ -PVP and N-ethyl-pentedrone elicited similar locomotor response at a dose of 3 mg kg<sup>-1</sup>, while pentedrone and N,N-diethyl-pentedrone

induced such effect at doses of 10 and 12.5 mg kg<sup>-1</sup>, respectively and  $\alpha$ -PpVP produced it at 25 mg kg<sup>-1</sup>. Consequently, the rank order of potency according to the doses used in this study for eliciting hyperlocomotion was  $\alpha$ -PVP = N-ethyl-pentedrone > pentedrone > N,N-diethyl-pentedrone >  $\alpha$ -PpVP.

### 3.5. Effect of $\alpha$ -aminovalerophenone derivatives on CPP

The CPP paradigm was used to study the rewarding effect of five  $\alpha$ -aminovalerophenone derivatives. The percentages of time spent in



**Fig. 4.** Time course profile of HLA induced by pentedrone (Panel A), N-ethyl-pentedrone (Panel B), N,N-diethyl-pentedrone (Panel C),  $\alpha$ -PVP (Panel D) and  $\alpha$ -PpVP (Panel E) in CD-1 mice. Each time point represents mean  $\pm$  SEM of the distance travelled displayed in 5 min blocks. Only comparisons versus the corresponding saline group are shown for clarity purposes. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  highest dose tested vs. saline; # $P < 0.05$ , ## $P < 0.01$  and ### $P < 0.001$  medium dose tested vs. saline.

both compartments during the preconditioning phase of all five experiments performed were around 50% (with no statistical differences found between compartments), indicating a total lack of preference for either compartment. Seven animals were withdrawn from the experiments due to an initial bias for one of the compartments (>70% of the total session time). Fig. 5 shows the effects of  $\alpha$ -aminovalerophenone derivatives on CPP paradigm. On the test day, one-way ANOVA revealed a significant effect of Dose for all the compounds tested (Pentedrone:  $F(3,51) = 8.819$ ;  $p < 0.001$ ; N-ethyl-pentedrone:  $F(3,49) = 5.044$ ;  $p < 0.01$ ; N,N-diethyl-pentedrone:  $F(3,49) = 2.802$ ;  $p < 0.05$ ;  $\alpha$ -PVP:  $F(3,52) = 4.555$ ,  $p < 0.01$ ,  $\alpha$ -PpVP: ( $F_{3,52} = 4.179$ ;  $p = 0.01$ ).

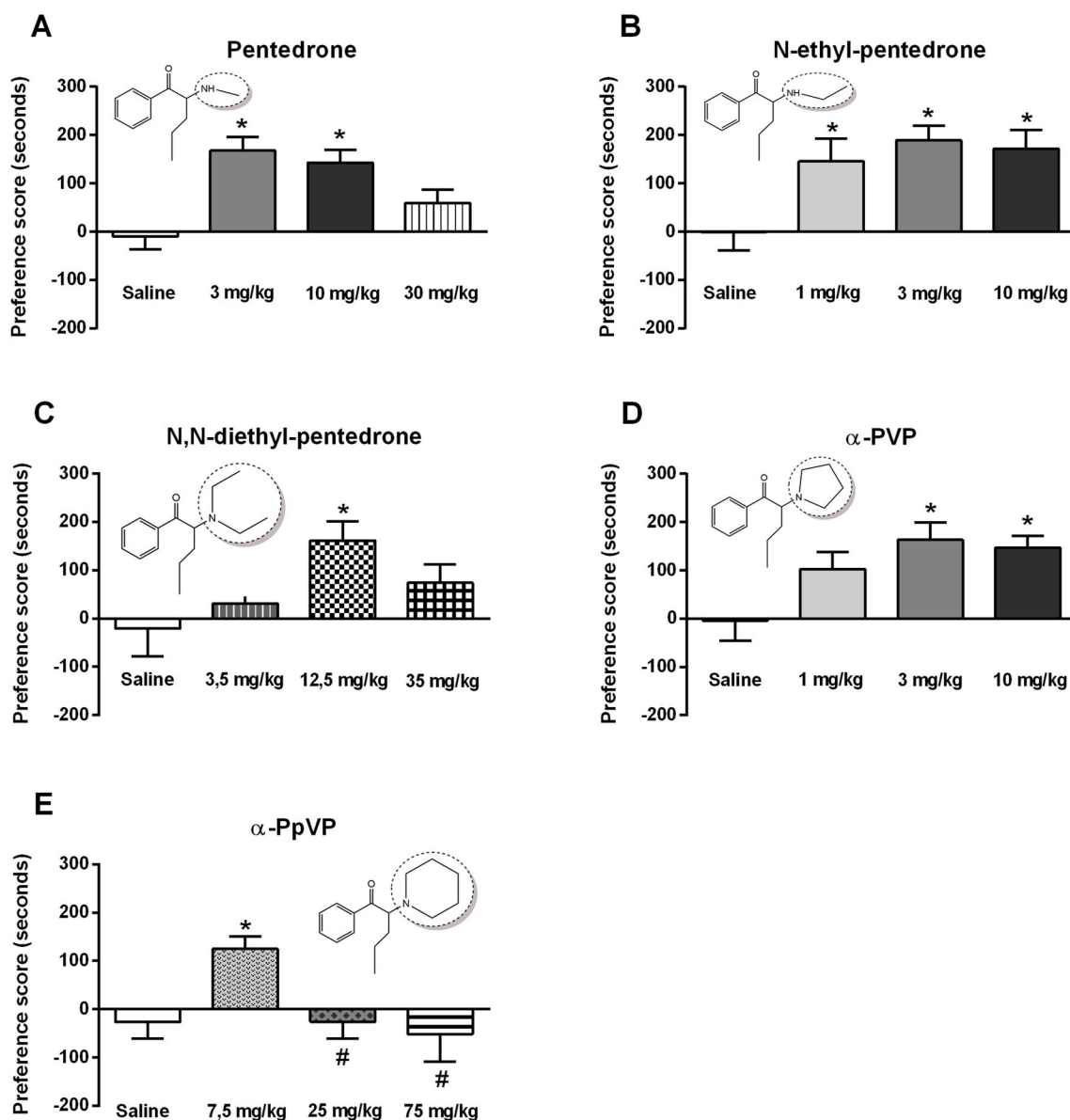
Particularly, animals conditioned with pentedrone and  $\alpha$ -PVP at 3 and 10  $\text{mg kg}^{-1}$  showed a significant increase in the preference score compared with the saline-treated animals (See Fig. 5A and D). Moreover, a significant increase in the preference score after N-ethyl-pentedrone repeated administrations was observed for all doses tested (1, 3 and 10  $\text{mg kg}^{-1}$ ) compared to the control group (See Fig. 5B). Post hoc analysis also confirmed a significant increase in the preference score compared to the control group, after N,N-diethyl-pentedrone administrations, but only at a dose of 12.5  $\text{mg kg}^{-1}$  (See Fig. 5C). Finally, after conditioning with  $\alpha$ -PpVP, post hoc analysis revealed an increase in the preference score only at the lowest dose tested (7.5  $\text{mg kg}^{-1}$ ) while a significant reduction of the preference score was obtained at the medium and highest dose tested (25 and 75  $\text{mg kg}^{-1}$ ) in comparison to the lowest dose tested (7.5  $\text{mg kg}^{-1}$ ) (See Fig. 5E).

#### 4. Discussion

The necessity of scheduling NPS under the Controlled Substances Act (CSA) is supported by studies that investigate their potential for abuse and provide scientific evidence of its pharmacological and toxicological profiles *in vitro* and *in vivo* (for review see (Bonson et al., 2019)). Thus, the main goal of the present study was to characterize the pharmacological profile and the role of the amino substituent, as well as the psychostimulant and rewarding properties of five different  $\alpha$ -aminovalerophenone derivatives, which structurally differ only in their amino substituent (Fig. 1).

A first evidence of the direct blockade of DA uptake by five different  $\alpha$ -aminovalerophenone derivatives was obtained in rat brain synaptosomes. Thereafter, pharmacological profiling of the five drugs from synaptosomal preparations was further confirmed by heterologous assays. The expression of cloned transporters in heterologous systems enabled us to investigate direct interactions of the synthetic cathinones to a single human transporter type. In this case, uptake inhibition in HEK293 cells demonstrated that all five compounds tested also potently inhibit DA uptake, relative to cocaine, but with low effect on 5-HT uptake inhibition at the concentrations tested. However, the absolute  $\text{IC}_{50}$  values obtained from rat brain synaptosomes differ when compared to those obtained from HEK293 cells. In fact, frequent discrepancies between both preparations in absolute potency estimations have been reported (Baumann et al., 2014; Mayer et al., 2016b; Saha et al., 2015; Sandtner et al., 2016). However, the absolute  $\text{IC}_{50}$  values of  $\alpha$ -PVP and pentedrone for DA and 5-HT uptake in HEK293 cells obtained in the





**Fig. 5.** Effects of aminovalerophenone derivatives on conditioned place preference (CPP) test in CD-1 mice. Bars represent mean  $\pm$  SEM of the preference score (difference between the time spent in the drug-paired compartment on the test day and the pre-conditioning day). Panel A: saline and pentedrone 3 and 10 mg kg<sup>-1</sup>, N = 14/group, pentedrone 30 mg kg<sup>-1</sup>, N = 13/group. Panel B: saline and N-ethyl-penedrone 1 mg kg<sup>-1</sup>, N = 14/group, N-ethyl-penedrone 3 mg kg<sup>-1</sup>, N = 13/group, N-ethyl-penedrone 10 mg kg<sup>-1</sup>, N = 12/group. Panel C: saline, N,N-diethyl-pentedrone 35 mg kg<sup>-1</sup>, N = 14/group, N,N-diethyl-pentedrone 3.5 mg kg<sup>-1</sup>, N = 12/group, N,N-diethyl-pentedrone 12.5 mg kg<sup>-1</sup>, N = 13/group. Panel D: saline,  $\alpha$ -PVP 1, 3, 10 mg kg<sup>-1</sup>, N = 14/group. Panel E: saline,  $\alpha$ -PpVP 7.5, 25, 75 mg kg<sup>-1</sup>, N = 14/group. \*P < 0.05, \*\*P < 0.01 and \*\*\*P < 0.001 vs saline; #P < 0.05 vs the lower drug-dose.

present study are very similar to those reported by other authors (Eshleman et al., 2017). Moreover, we corroborated that these compounds may act as DAT blockers (cocaine-like compounds) since a good correlation between DA uptake inhibition (IC<sub>50</sub> values) and binding assays (K<sub>i</sub> values) were observed (Eshleman et al., 2013).

Drug selectivity for DAT vs SERT may play a role in the abuse potential of some psychostimulant drugs such as synthetic cathinones and amphetamine derivatives targeting monoamine transporters (Baumann et al., 2018; Negus and Banks, 2017; Simmler and Liechti, 2017; Stevens Negus and Miller, 2014). Thus, the high hDAT/hSERT ratios observed in the present study may indicate a high abuse potential of these substances and, therefore, a threat to public health.

SAR and QSAR studies attempt to explain how a functional group of a molecule influences its action at its target (Glennon and Dukat, 2016). Kolanos and colleagues performed a SAR study with “deconstructed” MDPV analogues (Kolanos et al., 2013). They conclude that a tertiary

amine is the major contributor to the potent effect of MDPV as a DAT blocker, compared to analogues with a secondary and a primary amine. These results agree with our molecular docking results in which the geometry defined by the amine induces a differential binding mechanism. Similarly, the shortening of the pyrrolidine ring of MDPV or the expansion of the  $\alpha$ -PVP pyrrolidine ring to a piperidine ring resulted in a progressive decrease in the DAT potency (Glennon and Young, 2016; Kolanos et al., 2015). The five compounds tested in the present study only differ in their amino-substituent, including secondary and tertiary amine analogues. The *in vitro* data extend previous findings and reveal that the potency of these compounds to block hDAT reuptake increases when the amino group expands from a methyl to an ethyl but decreases from a pyrrolidine to a diethyl and piperidine ring. However, the fact that the N,N-diethyl-pentedrone showed a low affinity to hDAT suggests that carrying a primary, secondary or tertiary amine may not be the sole contributing factor to the activity at these transporters. Moreover, a

significant correlation was observed between predicted binding affinities (docking analysis) and  $K_i$  values for hDAT. Additionally, a positive correlation was also observed between hDAT/hSERT ratio and the CLogP; the higher the lipophilicity of the substituent, the higher is its selectivity for hDAT vs hSERT. In parallel, it seems that the potency in inhibiting 5-HT uptake improves with decreasing bulk, surface and lipophilicity of the amino-substituent. Although these SAR on hSERT inhibition is extremely useful, we must point out that these studies must be expanded and corroborated with other synthetic cathinones that have a higher affinity for SERT.

OCT-2 and OCT-3 are low-affinity/high-capacity transporters (Engel and Wang, 2005; Koepsell, 2020) that play a role in neurotransmitter uptake in the brain (Jonker and Schinkel, 2004; Koepsell et al., 1999; Koepsell and Endou, 2004). There is evidence both for and against a direct interaction of psychostimulants such as D-Amphetamine and MDMA to significantly inhibit OCTs (Amphoux et al., 2006; Mayer et al., 2018). In fact, amphetamine is known to promote non-exocytosis release of substrates in the presence of cocaine in an OCT-3-dependent manner (Sitte and Freissmuth, 2015). Our findings demonstrated that all the five compounds tested inhibit hOCT-2 function with similar potencies. By contrast, and like cocaine (Amphoux et al., 2006), all the drugs tested do not produce any effect on hOCT-3 function.

To assess the psychostimulant and rewarding properties of the five compounds, we used a motor performance and CPP test, respectively. A dose-response effect in locomotor activity was observed, confirming previous reports on synthetic cathinones (Gatch et al., 2015a, 2015b; Giannotti et al., 2017; Hwang et al., 2017; Javadi-Paydar et al., 2018; Marusich et al., 2014, 2016; Wojcieszak et al., 2018), as well as a psychostimulant effect similar to cocaine (López-Arnau et al., 2017; Duart-Castells et al., 2020). Particularly,  $\alpha$ -PVP and N-ethyl-pentedrone appear to be equally effective for inducing hyperlocomotion at all the doses tested. However, pentedrone seems to be less effective than  $\alpha$ -PVP and N-ethyl-pentedrone since at the doses of 3 and 10 mg kg<sup>-1</sup> pentedrone induced a reduced hyperlocomotion in comparison to  $\alpha$ -PVP and N-ethyl-pentedrone. Moreover, likely due to an increase of other aspects of the animal behaviour, a ceiling effect was observed at the highest dose tested of pentedrone and  $\alpha$ -PVP. In fact, it has been recently demonstrated by our research group that an increase of stereotyped behaviour after repeated injections of MDPV, a structural related synthetic cathinone, directly affects HLA (Lopez-Arnau et al., 2019). Moreover, further research also focused on the effects of these compounds on other aspects of the animals' emotional status (rearing, depressive-, anxiolytic- or anxiogenic-like symptoms, impulsivity, etc ...) is needed. As expected, the substances that needed lower doses to produce the same behavioural effects (N-ethyl-pentedrone and  $\alpha$ -PVP) are those with highest hDAT affinities and potencies. However, despite being one of the substances with the higher affinity for hDAT, a higher dose of  $\alpha$ -PVP was required to induce the same locomotor effect when compared to the other compounds. At this point, we can only hypothesize due to the lack of pharmacokinetic studies (Saeidnia et al., 2015) that some pharmacokinetic parameters such as absorption and/or crossing blood-brain-barrier may be involved in such difference between *in vitro* and *in vivo* results obtained with by  $\alpha$ -PVP. However, other possibilities should be kept in mind. For instance,  $\alpha$ -PVP could act as an atypical DAT inhibitor due to the different binding mode observed of this compound, which may trap the dopamine transporter protein in an atypical conformation. Some researchers (Hong et al., 2018; Tomlinson et al., 2019) have investigated the effects of bantzopine analogues, potent and selective probes for the DAT. In general, they have shown that these compounds, do not demonstrate efficacious locomotor stimulation in mice, do not fully substitute for a cocaine discriminative stimulus and are not appreciably self-administered in rats or nonhuman primates. These compounds are generally more potent than cocaine as dopamine uptake inhibitors *in vitro*, although their actions *in vivo* are not consistent with this action. As such, they have described this class of compounds as atypical dopamine uptake inhibitors. Molecular pharmacology has revealed differences in

binding domains between typical and atypical ligands that correlated with their distinct behavioural profiles (Reith et al., 2015). Alternatively, an off-target effect could also contribute to the different behavioural profile of  $\alpha$ -PVP. Moreover, DAT inhibition may not be the only factor in determining the behavioural effects induced by  $\alpha$ -PVP, N-ethyl-pentedrone, pentedrone and N,N-diethyl-pentedrone. In fact, we cannot also rule out an interaction of these compounds with other receptors that may play an important role on their psychostimulant effects, as it was observed for mephedrone in previous studies reported by our research group, in which, we demonstrated that the psychostimulant effect induced by mephedrone was partially mediated by a 5-HT<sub>2A</sub> receptor interaction (López-Arnau et al., 2012).

Finally, our results also demonstrated that all the compounds tested induced rewarding effects similar to cocaine (López-Arnau et al., 2017; Duart-Castells et al., 2020) and are consistent with previous studies about the behavioural effects of synthetic cathinones (Gatch et al., 2015b; Hwang et al., 2017). Particularly, the lowest tested dose of  $\alpha$ -PVP, pentedrone and N-ethyl-pentedrone induced place-conditioning despite not producing hyperlocomotion, while the other two doses of N-ethyl-pentedrone induced both effects. In contrast, the other two doses tested of  $\alpha$ -PVP produced an increase in the locomotor activity but do not exert rewarding properties. This fact might be related to the unpleasant and/or deleterious effects that animals may suffer at such high doses.

## 5. Conclusions

In summary, all of the  $\alpha$ -aminovalerophenone compounds studied act as potent DA uptake inhibitors. Increasing the length of the amino group from a methyl to an ethyl group decreased hDAT IC<sub>50</sub>, while changing a pyrrolidine to a diethyl and piperidine ring increased the IC<sub>50</sub> at hDAT. A positive correlation between the hDAT/hSERT ratio and the CLogP of the amino-substituent exists, pointing to a high abuse liability with increased lipophilicity of the amino-substituent. Finally, our study also demonstrate that N-ethyl-pentedrone, N,N-diethyl-pentedrone and  $\alpha$ -PVP are able to induce psychostimulant and rewarding effects in mice, suggesting their abuse liability. However, not all these findings might apply to females since only male mice were used in this study. Accordingly, further research is needed in order to identify any gender differences in the psychostimulant and rewarding properties of novel synthetic cathinones, as it has already been observed for some first and second generation of synthetic cathinones (King et al., 2015; Nelson et al., 2019).

## CRedit authorship contribution statement

**L. Duart-Castells:** performed *in vitro* and *in vivo* experiments, Synthesis and chemical characterization of compounds was carried, contributed to analysis and interpretation of data, Writing - original draftwrote the manuscript. **N. Nadal-Gratacós:** conducted computational docking Formal analysis. **M. Muralter:** performed *in vitro* and *in vivo* experiments, Synthesis and chemical characterization of compounds was carried. **B. Puster:** performed *in vitro* and *in vivo* experiments, Synthesis and chemical characterization of compounds was carried. **X. Berzosa:** conducted computational docking analysis. **R. Estrada-Tejedor:** conducted computational docking analysis. **M. Niello:** performed *in vitro* and *in vivo* experiments. **S. Bhat:** performed *in vitro* and *in vivo* experiments, Synthesis and chemical characterization of compounds was carried. **D. Pubill:** Writing - review & editing. **J. Camarasa:** Writing - review & editing. **H.H. Sitte:** conceived and designed the research study, contributed to analysis. **E. Escubedo:** conceived and designed the research study, contributed to analysis. **R. López-Arnau:** conceived and designed the research study, performed *in vitro* and *in vivo* experiments, Synthesis and chemical characterization of compounds was carried, contributed to analysis.

## Declaration of competing interest

HHS has received honoraria for lectures and consulting from AbbVie, Amgen, AstraZeneca, Astropharma, Bano Healthcare, Chiesi, FOPI, Gebro, IIR, Janssen-Cilag, Lundbeck, MSD, Novartis, Pfizer, Roche, Sanofi-Aventis, Shire, Vertex (past 5 years). All other authors declare no conflicts of interest.

## Acknowledgements

This study was supported by Ministerio de Economía y

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.neuropharm.2021.108475>.



## Role of funding source

None of the organizations that have funded this research had no further role in study design, analysis and interpretation of data, in the writing of the manuscript, and in the decision to submit the paper for publication.

## References

- Amphoux, A., Vialou, V., Drescher, E., Brüsch, M., Mannoury La Cour, C., Rochat, C., Millan, M.J., Giros, B., Bönisch, H., Gautron, S., 2006. Differential pharmacological in vitro properties of organic cation transporters and regional distribution in rat brain. *Neuropharmacology* 50, 941–952. <https://doi.org/10.1016/j.neuropharm.2006.01.005>.
- Andersen, J., Olsen, L., Hansen, K.B., Taboureau, O., Jørgensen, F.S., Jørgensen, A.M., Bang-Andersen, B., Egebjerg, J., Strömgaard, K., Kristensen, A.S., 2010. Mutational mapping and modeling of the binding site for (S)-citalopram in the human serotonin transporter. *J. Biol. Chem.* 285, 2051–2063. <https://doi.org/10.1074/jbc.M109.072587>.
- Baumann, M.H., Bulling, S., Benaderet, T.S., Saha, K., Ayestas, M.A., Partilla, J.S., Ali, S. F., Stockner, T., Rothman, R.B., Sandtner, W., Sitte, H.H., 2014. Evidence for a role of transporter-mediated currents in the depletion of brain serotonin induced by serotonin transporter substrates. *Neuropsychopharmacology* 39, 1355–1365. <https://doi.org/10.1038/npp.2013.331>.
- Baumann, M.H., Walters, H.M., Niello, M., Sitte, H.H., 2018. Neuropharmacology of synthetic cathinones. In: *Handbook of Experimental Pharmacology*. Springer New York LLC, pp. 113–142. [https://doi.org/10.1007/164\\_2018\\_178](https://doi.org/10.1007/164_2018_178).
- Bonson, K.R., Dalton, T., Chiapperino, D., 2019. Scheduling synthetic cathinone substances under the controlled substances act. *Psychopharmacology (Berlin)*. <https://doi.org/10.1007/s00213-018-5129-8>.
- Cheng, M.H., Block, E., Hu, F., Cobanoglu, M.C., Sorkin, A., Bahar, I., 2015. Insights into the modulation of dopamine transporter function by amphetamine, orphenadrine, and cocaine binding. *Front. Neurol.* 6, 134. <https://doi.org/10.3389/fneur.2015.00134>.
- Drug Enforcement Administration, Department of Justice, 2011. Schedules of controlled substances: temporary placement of three synthetic cathinones in Schedule I. *Final Order. Fed. Regist.* 76, 65371–65375.
- Duart-Castells, L., López-Arnau, R., Vizcaíno, S., Camarasa, J., Pubill, D., Escubedo, E., 2019. 7,8-Dihydroxyflavone blocks the development of behavioural sensitization to MDPV, but not to cocaine: differential role of the BDNF-TrkB pathway. *Biochem. Pharmacol.* 163, 84–93. <https://doi.org/10.1016/j.bcp.2019.02.004>.
- Drug Enforcement Administration, Department of Justice, 2014. Schedules of controlled substances: temporary placement of 10 synthetic cathinones into Schedule I. *Final order. Fed. Regist.* 79 (45), 12938–12943.
- Duart-Castells, L., Blanco-Gandía, M.C., Ferrer-Pérez, C., Puster, B., Pubill, D., Miñarro, J., Escubedo, E., Rodríguez-Arias, M., 2020. Cross-reinstatement between 3,4-methylenedioxypyrovalerone (MDPV) and cocaine using conditioned place preference. *Prog. in Neuro-Psych. and Biol. Psych.* 100 <https://doi.org/10.1016/j.pnpbp.2020.109876>, 109876.
- Engel, K., Wang, J., 2005. Interaction of organic cations with a newly identified plasma membrane monoamine transporter. *Mol. Pharmacol.* 68, 1397–1407. <https://doi.org/10.1124/mol.105.016832>.
- Competitividad (grant number SAF2016-75347-R), Ministerio de Ciencia e Innovación (PID2019-109390RB-I00) and Plan Nacional sobre Drogas (2020I051). LDC received FPU grants from the Ministerio de Economía y Competitividad (15/02492). JC, DP and EE belong to 2017SGR979 from Generalitat de Catalunya. This work was also supported by Austrian Science Foundation (FWF) F35–B06 and W1232 (HHS) and the Vienna Science and Technology Fund (WWTF) CS15-033 (HHS). We thank Marion Holy and Kathrin Jäntschi for excellent technical assistance. NNG has also been awarded by Col·legi de Farmacèutics de Barcelona for its contribution in the synthesis section.
- Eshleman, A.J., Wolfrum, K.M., Reed, J.F., Kim, S.O., Swanson, T., Johnson, R.A., Janowsky, A., 2017. Structure-activity relationships of substituted cathinones, with transporter binding, uptake, and release. *J. Pharmacol. Exp. Therapeut.* 360, 33–47. <https://doi.org/10.1124/jpet.116.236349>.
- European Monitoring Centre for Drugs and Drug Addiction, 2019. *European Drug Report*. European Union Publications Office.
- Gatch, M.B., Dolan, S.B., Forster, M.J., 2015a. Comparative behavioural pharmacology of three pyrrolidine-containing synthetic cathinone derivatives. *J. Pharmacol. Exp. Therapeut.* 354, 103–110. <https://doi.org/10.1124/jpet.115.223586>.
- Gatch, M.B., Rutledge, M.A., Forster, M.J., 2015b. Discriminative and locomotor effects of five synthetic cathinones in rats and mice. *Psychopharmacology (Berlin)* 232, 1197–1205. <https://doi.org/10.1007/s00213-014-3755-3>.
- Giannotti, G., Canazza, I., Caffino, L., Bilel, S., Ossato, A., Fumagalli, F., Marti, M., 2017. The cathinones MDPV and  $\alpha$ -PVP elicit different behavioural and molecular effects following acute exposure. *Neurotox. Res.* 32, 594–602. <https://doi.org/10.1007/s12640-017-9769-y>.
- Glennon, R.A., Dukat, M., 2016. Structure-activity relationships of synthetic cathinones. In: *Current Topics in Behavioural Neurosciences*. Springer Verlag, pp. 19–47. [https://doi.org/10.1007/7854\\_2016\\_41](https://doi.org/10.1007/7854_2016_41).
- Glennon, R.A., 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>.
- Hevener, K.E., Ball, D.M., Buolamwini, J.K., Lee, R.E., 2008. Quantitative structure-activity relationship studies on nitrofuranyl anti-tubercular agents. *Bioorg. Med. Chem.* 16, 8042–8053. <https://doi.org/10.1016/j.bmc.2008.07.070>.
- Hong, W.C., Wasko, M.J., Wilkinson, D.S., Hiranita, T., Li, L., Hayashi, S., Snell, D.B., Madura, J.D., Surratt, C.K., Katz, J.L., 2018. Dopamine transporter dynamics of N-substituted benzotropine analogs with atypical behavioural effects. *J. Pharmacol. Exp. Therapeut.* 366, 527–540. <https://doi.org/10.1124/jpet.118.250498>.
- Huskinson, S.L., Naylor, J.E., Townsend, E.A., Rowlett, J.K., Blough, B.E., Freeman, K.B., 2017. Self-administration and behavioural economics of second-generation synthetic cathinones in male rats. *Psychopharmacology (Berlin)* 234, 589–598. <https://doi.org/10.1007/s00213-016-4492-6>.
- Hwang, J.-Y., Kim, J.-S., Oh, J.-H., Hong, S.-I., Ma, S.-X., Jung, Y.-H., Ko, Y.-H., Lee, S.-Y., Kim, H.-C., Jang, C.-G., 2017. The new stimulant designer compound pentredone exhibits rewarding properties and affects dopaminergic activity. *Addiction Biol.* 22, 117–128. <https://doi.org/10.1111/adb.12299>.
- Javadi-Paydar, M., Nguyen, J.D., Vandewater, S.A., Dickerson, T.J., Taffe, M.A., 2018. Locomotor and reinforcing effects of pentredone, pentylone and methylone in rats. *Neuropharmacology* 134, 57–64. <https://doi.org/10.1016/j.neuropharm.2017.09.002>.
- Jonker, J.W., Schinkel, A.H., 2004. Pharmacological and physiological functions of the polyspecific organic cation transporters: OCT1, 2, and 3 (SLC22A1-3). *J. Pharmacol. Exp. Therapeut.* <https://doi.org/10.1124/jpet.103.053298>.
- King, H.E., Wakeford, A., Taylor, W., Wetzell, B., Rice, K.C., Riley, A.L., 2015. Sex differences in 3,4-methylenedioxypyrovalerone (MDPV)-induced taste avoidance

- and place preferences. *Pharmacol. Biochem. Behav.* 137, 16–22. <https://doi.org/10.1016/j.pbb.2015.07.013>.
- Koepsell, H., 2020. Organic cation transporters in health and disease. *Pharmacol. Rev.* 72, 253–319. <https://doi.org/10.1124/pr.118.015578>.
- Koepsell, H., Endou, H., 2004. The SLC22 drug transporter family. *Pflugers Arch. Eur. J. Physiol.* <https://doi.org/10.1007/s00424-003-1089-9>.
- Koepsell, H., Gorboulev, V., Arndt, P., 1999. Molecular pharmacology of organic cation transporters in kidney. *J. Membr. Biol.* <https://doi.org/10.1007/s002329900475>.
- Kolanos, R., Sakloth, F., Jain, A.D., Partilla, J.S., Baumann, M.H., Glennon, R.A., 2015. Structural modification of the designer stimulant  $\alpha$ -pyrrolidinovalephorone ( $\alpha$ -PVP) influences potency at dopamine transporters. *ACS Chem. Neurosci.* 6, 1726–1731. <https://doi.org/10.1021/acschemneuro.5b00160>.
- Kolanos, R., Solis, E., Sakloth, F., De Felice, L.J., Glennon, R.A., 2013. “Deconstruction” of the abused synthetic cathinone methylenedioxypyrovalerone (MDPV) and an examination of effects at the human dopamine transporter. *ACS Chem. Neurosci.* 4, 1524–1529. <https://doi.org/10.1021/cn4001236>.
- Lopez-Arnau, R., Duart-Castells, L., Aster, B., Camarasa, J., Escubedo, E., Pubill, D., 2019. Effects of MDPV on dopamine transporter regulation in male rats. Comparison with cocaine. *Psychopharmacology (Berlin)* 236, 925–938. <https://doi.org/10.1007/s00213-018-5052-z>.
- López-Arnau, R., Luján, M.A., Duart-Castells, L., Pubill, D., Camarasa, J., Valverde, O., Escubedo, E., 2017. Exposure of adolescent mice to 3,4-methylenedioxypyrovalerone increases the psychostimulant, rewarding and reinforcing effects of cocaine in adulthood. *Br. J. Pharmacol.* 174, 1161–1173. <https://doi.org/10.1111/bph.13771>.
- López-Arnau, R., Martínez-Clemente, J., Pubill, D., Escubedo, E., Camarasa, J., 2012. Comparative neuropharmacology of three psychostimulant cathinone derivatives: butylone, mephedrone and methylone. *Br. J. Pharmacol.* 167, 407–420. <https://doi.org/10.1111/j.1476-5381.2012.01998.x>.
- Luethi, D., Liechti, M.E., Krähenbühl, S., 2017. Mechanisms of hepatocellular toxicity associated with new psychoactive synthetic cathinones. *Toxicology* 387, 57–66. <https://doi.org/10.1016/j.tox.2017.06.004>.
- Majchrzak, M., Celiński, R., Kuś, P., Kowalska, T., Sajewicz, M., 2018. The newest cathinone derivatives as designer drugs: an analytical and toxicological review. *Forensic Toxicol.* <https://doi.org/10.1007/s11419-017-0385-6>.
- Marusich, J.A., Antonazzo, K.R., Wiley, J.L., Blough, B.E., Partilla, J.S., Baumann, M.H., 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, J.A., Lefever, T.W., Blough, B.E., Thomas, B.F., Wiley, J.L., 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>.
- Mayer, F.P., Luf, A., Nagy, C., Holy, M., Schmid, R., Freissmuth, M., Sitte, H.H., 2016a. Application of a combined approach to identify new psychoactive street drugs and decipher their mechanisms at monoamine transporters. *Curr. Top. Behav. Neurosci.* 32, 333–350. [https://doi.org/10.1007/7854\\_2016\\_63](https://doi.org/10.1007/7854_2016_63).
- Mayer, F.P., Schmid, D., Owens, W.A., Gould, G.G., Apuschkin, M., Kudlacek, O., Salzer, I., Boehm, S., Chiba, P., Williams, P.H., Wu, H.-H., Gether, U., Koek, W., Daws, L.C., Sitte, H.H., 2018. An unsuspected role for organic cation transporter 3 in the actions of amphetamine. *Neuropsychopharmacology* 43, 2408–2417. <https://doi.org/10.1038/s41386-018-0053-5>.
- Mayer, F.P., Wimmer, L., Dillon-Carter, O., Partilla, J.S., Burchardt, N.V., Mihovilovic, M.D., Baumann, M.H., Sitte, H.H., 2016b. Phase I metabolites of mephedrone display biological activity as substrates at monoamine transporters. *Br. J. Pharmacol.* 173, 2657–2668. <https://doi.org/10.1111/bph.13547>.
- McGrath, J.C., Lilley, E., 2015. Implementing guidelines on reporting research using animals (ARRIVE etc.): new requirements for publication in BJP. *Br. J. Pharmacol.* <https://doi.org/10.1111/bph.12955>.
- Meltzer, P.C., Butler, D., Deschamps, J.R., Madras, B.K., 2006. 1-(4-Methylphenyl)-2-pyrrolidin-1-yl-pentan-1-one (Pyrovalerone) analogues: a promising class of monoamine uptake inhibitors. *J. Med. Chem.* 49, 1420–1432. <https://doi.org/10.1021/jm050797a>.
- Negus, S.S., Banks, M.L., 2017. Decoding the structure of abuse potential for new psychoactive substances: structure-activity relationships for abuse-related effects of 4-substituted methcathinone analogs. *Curr. Top. Behav. Neurosci.* 32, 119–131. [https://doi.org/10.1007/7854\\_2016\\_18](https://doi.org/10.1007/7854_2016_18).
- Nelson, K.H., Manke, H.N., Imanalieva, A., Rice, K.C., Riley, A.L., 2019. Sex differences in  $\alpha$ -pyrrolidinopentophenone ( $\alpha$ -PVP)-induced taste avoidance, place preference, hyperthermia and locomotor activity in rats. *Pharmacol. Biochem. Behav.* 185. <https://doi.org/10.1016/j.pbb.2019.172762>.
- Niello, M., Cintulova, D., Hellsberg, E., Jantsch, K., Holy, M., Ayatollahi, L.H., Cozzi, N. V., Freissmuth, M., Sandtner, W., Ecker, G.F., Mihovilovic, M.D., Sitte, H.H., 2019. para-Trifluoromethyl-methcathinone is an allosteric modulator of the serotonin transporter. *Neuropharmacology*. <https://doi.org/10.1016/j.neuropharm.2019.04.021>.
- Pubill, D., Chipana, C., Camins, A., Pallàs, M., Camarasa, J., Escubedo, E., 2005. Free radical production induced by methamphetamine in rat striatal synaptosomes. *Toxicol. Appl. Pharmacol.* 204, 57–68. <https://doi.org/10.1016/j.taap.2004.08.008>.
- Reith, M.E.A., Blough, B.E., Hong, W.C., Jones, K.T., Schmitt, K.C., Baumann, M.H., Partilla, J.S., Rothman, R.B., Katz, J.L., 2015. Behavioural, biological, and chemical perspectives on atypical agents targeting the dopamine transporter. *Drug Alcohol Depend.* <https://doi.org/10.1016/j.drugalcdep.2014.12.005>.
- Rickli, A., Hoener, M.C., Liechti, M.E., 2015. Monoamine transporter and receptor interaction profiles of novel psychoactive substances: para-halogenated amphetamines and pyrovalerone cathinones. *Eur. Neuropsychopharmacol.* 25, 365–376. <https://doi.org/10.1016/j.euroneuro.2014.12.012>.
- Saeidnia, S., Azadeh, Manayi, Abdollahi, M., 2015. From in vitro experiments to in vivo and clinical studies; pros and cons. *Curr. Drug Discovery Technologies* 12, 4. <https://doi.org/10.2174/1570163813666160114093140>.
- Saha, K., Li, Y., Holy, M., Lehner, K.R., Bukhari, M.O., Partilla, J.S., Sandtner, W., Sitte, H.H., Baumann, M.H., 2019. The synthetic cathinones, butylone and pentylone, are stimulants that act as dopamine transporter blockers but 5-HT transporter substrates. *Psychopharmacology (Berlin)* 236, 953–962. <https://doi.org/10.1007/s00213-018-5075-5>.
- Saha, K., Partilla, J.S., Lehner, K.R., Seddik, A., Stockner, T., Holy, M., Sandtner, W., Ecker, G.F., Sitte, H.H., Baumann, M.H., 2015. “Second-generation” mephedrone analogs, 4-MEC and 4-MePPP, differentially affect monoamine transporter function. *Neuropsychopharmacology* 40, 1321–1331. <https://doi.org/10.1038/npp.2014.325>.
- Sandtner, W., Stockner, T., Hasenhuettl, P.S., Partilla, J.S., Seddik, A., Zhang, Y.-W., Cao, J., Holy, M., Steinkellner, T., Rudnick, G., Baumann, M.H., Ecker, G.F., Newman, A.H., Sitte, H.H., 2016. Binding mode selection determines the action of ecstasy homologs at monoamine transporters. *Mol. Pharmacol.* 89, 165–175. <https://doi.org/10.1124/mol.115.101394>.
- Simmler, L.D., Buser, T.A., Donzelli, M., Schramm, Y., Dieu, L.H., Huwyler, J., Chaboz, S., Hoener, M.C., Liechti, M.E., 2013. Pharmacological characterization of designer cathinones in vitro. *Br. J. Pharmacol.* 168, 458–470. <https://doi.org/10.1111/j.1476-5381.2012.02145.x>.
- Simmler, L.D., Liechti, M.E., 2017. Interactions of cathinone NPS with human transporters and receptors in transfected cells. In: *Current Topics in Behavioural Neurosciences*. Springer Verlag, pp. 49–72. [https://doi.org/10.1007/7854\\_2016\\_20](https://doi.org/10.1007/7854_2016_20).
- Simmler, L.D., Rickli, A., Hoener, M.C., Liechti, M.E., 2014. Monoamine transporter and receptor interaction profiles of a new series of designer cathinones. *Neuropharmacology* 79, 152–160. <https://doi.org/10.1016/j.neuropharm.2013.11.008>.
- Sitte, H.H., Freissmuth, M., 2015. Amphetamines, new psychoactive drugs and the monoamine transporter cycle. *Trends Pharmacol. Sci.* <https://doi.org/10.1016/j.tips.2014.11.006>.
- Stevens Negus, S., Miller, L.L., 2014. Intracranial self-stimulation to evaluate abuse potential of drugs. *Pharmacol. Rev.* 66, 869–917. <https://doi.org/10.1124/pr.112.007419>.
- Sucic, S., Dallinger, S., Zdrzil, B., Weissensteiner, R., Jørgensen, T.N., Holy, M., Kudlacek, O., Seidel, S., Hwan Cha, J., Gether, U., Newman, A.H., Ecker, G.F., Freissmuth, M., Sitte, H.H., 2010. The N terminus of monoamine transporters is a lever required for the action of amphetamines. *J. Biol. Chem.* 285, 10924–10938. <https://doi.org/10.1074/jbc.M109.083154>.
- Tomlinson, M.J., Krout, D., Pramod, A.B., Lever, J.R., Newman, A.H., Henry, L.K., Vaughan, R.A., 2019. Identification of the benzotropine analog [125 I]GA II 34 binding site on the human dopamine transporter. *Neurochem. Int.* 123, 34–45. <https://doi.org/10.1016/j.neuint.2018.08.008>.
- United Nations Office on Drugs and Crime (UNODC), 2013. UNODC early warning advisory (EWA) on new psychoactive substances (NPS) [WWW Document]. URL. <https://www.unodc.org/LSS/Home/NPS> (Accessed 3 February 2021).
- Walther, D., Shalabi, A.R., Baumann, M.H., Glennon, R.A., 2019. Systematic structure-activity studies on selected 2-, 3-, and 4-monosubstituted synthetic methcathinone analogs as monoamine transporter releasing agents. *ACS Chem. Neurosci.* 10, 740–745. <https://doi.org/10.1021/acschemneuro.8b00524>.
- Wang, K.H., Penmatsa, A., Gouaux, E., 2015. Neurotransmitter and psychostimulant recognition by the dopamine transporter. *Nature* 521, 322–327. <https://doi.org/10.1038/nature14431>.
- Watterson, L.R., Burrows, B.T., Hernandez, R.D., Moore, K.N., Grabenauer, M., Marusich, J.A., Olive, M.F., 2014. Effects of  $\alpha$ -pyrrolidinopentophenone and 4-methyl-N-ethylcathinone, two synthetic cathinones commonly found in second-generation “bath salts,” on intracranial self-stimulation thresholds in rats. *Int. J. Neuropsychopharmacol.* 18. <https://doi.org/10.1093/ijnp/ppy014>.
- Wojcieszak, J., Andrzejczak, D., Wojtas, A., Golembiowska, K., Zawilska, J.B., 2018. Effects of the new generation  $\alpha$ -pyrrolidinophenones on spontaneous locomotor activities in mice, and on extracellular dopamine and serotonin levels in the mouse striatum. *Forensic Toxicol.* 36, 334–350. <https://doi.org/10.1007/s11419-018-0409-x>.
- Yung-Chi, C., Prusoff, W.H., 1973. Relationship between the inhibition constant (KI) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem. Pharmacol.* 22, 3099–3108. [https://doi.org/10.1016/0006-2952\(73\)90196-2](https://doi.org/10.1016/0006-2952(73)90196-2).
- Zaami, S., Giorgetti, R., Pichini, S., Pantano, F., Marinelli, E., Busardò, F.P., 2018. Synthetic cathinones related fatalities: an update. *Eur. Rev. Med. Pharmacol. Sci.* 22, 268–274. <https://doi.org/10.26355/eurrev-201801-14129>.
- Zhou, X., Luethi, D., Sanvee, G.M., Bouitbir, J., Liechti, M.E., Krähenbühl, S., 2019. Molecular toxicological mechanisms of synthetic cathinones on c2c12 myoblasts. *Int. J. Mol. Sci.* 20. <https://doi.org/10.3390/ijms20071561>.