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

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Abstract

Background and Purpose: 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 synthetic cathinones differing in its amino terminal substitution. Experimental Approach: Rat brain synaptosomes were used for [3H]dopamine uptake experiments. HEK293 transfected cells (hDAT, hSERT, hOCT; human dopamine, serotonin and organic cation transporter, respectively) were also used for [3H]monoamine uptake and transporter binding assays. Molecular docking allowed to investigate the effect of the amino substitutions on the biological activity. Locomotor activity experiments and conditioned place preference paradigm were used in order to study the psychostimulant and rewarding effects in mice. Key Results: All compounds tested are potent inhibitors of DAT with very low affinity for SERT, hOCT-2 and -3, and their potency 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 (Ki) for hDAT. Conclusions and Implications: Our study demonstrated the role of the amino-substituent in the pharmacological profile of novel synthetic cathinones and provides the first evidence that some of them are potent DAT inhibitors and able to induce psychostimulant and rewarding effects in mice.

Keywords

New psychoactive substances, synthetic cathinones, psychostimulant, reward, dopamine, dopamine transporter.

Bullet point summary

What is already known:

- α-PVP and pentedrone act as potent DAT blockers and exhibit psychostimulant and rewarding properties.
- SAR studies on synthetic cathinones have shown to correlate molecular properties with their pharmacological profile.

What this study adds:

- The role of the amino-substituent in the pharmacological profile of novel synthetic cathinones.
- The first evidence that N-ethyl-pentedrone, N,N-diethyl-pentedrone and α-PpVP induce psychostimulant and rewarding effects.

Clinical significance:

The high abuse potential of these novel synthetic cathinones, therefore a threat to public health.

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 several deaths and acute intoxications. 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*(United Nations Office on Drugs and Crime (UNODC); European Monitoring Centre for Drugs and Drug, 2019). 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-methylenedioxypyrovalerone (MDPV)) were classified by the Drug Enforcement Administration (DEA) as Schedule I compounds (Drug Enforcement Administration, Department of Justice, 2011), a "second-generation" emerged, which includes α-pyrrolidinovalerophenone (α-PVP) and pentedrone (Drug Enforcement Administration, Department of Justice, 2014). The simple removal of the 3,4-methylendioxy group from MDPV led to the α-PVP structure, which at that time was not scheduled, and largely replaced MDPV on the drug market. However, since the scientific evidence regarding their mode of action, toxicity and/or abuse potential are still missing, many other synthetic cathinones have not yet been scheduled by the concerning authorities.

It has been demonstrated that α-PVP acts as a potent blocker of the dopamine (DA) transporters (DAT) (Meltzer et al., 2006; Marusich et al., 2014). Preclinical studies have also described α-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 (Marusich et al., 2014; Gatch et al., 2015b). Moreover, α-PVP has also been shown to facilitate intracranial self-stimulation and maintain self-administration in rats (Watterson et al., 2014; Huskinson et al., 2017). Pentedrone is another "second-generation" cathinone closely related to α-PVP, only differing in its amino-group substituent (see Figure 1A). Similar to α-PVP, pentedrone also blocks DAT and exhibits psychostimulant, rewarding and reinforcing properties (Simmler et al., 2014; Gatch et al., 2015a; Hwang et al., 2017; Javadi-Paydar et al., 2018). Numerous structure-activity relationship (SAR) studies on synthetic cathinones have shown to correlate molecular properties of different substituents with their pharmacological and toxicological profile (Kolanos et al., 2015; Eshleman et al., 2017; Niello et al., 2019; Walther et al., 2019) (for review see also (Glennon and Dukat, 2016; Baumann et al., 2018)). Saha and colleagues demonstrated how modifications at both, α-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).

In this study, we explore different structural modifications in the amino-terminal group of synthetic cathinones by describing a set of five α-aminovalerophenone derivatives: pentedrone, α-ethylaminovalerophenone (N-ethyl-pentedrone), α-diethylaminovalerophenone (N,N-diethyl-pentedrone), α-PVP and α-piperidinevalerophenone (α-PpVP) (See Figure 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) 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.

Accordingly, the aims of the present study were i) to characterize the*in vitro* pharmacology of five α-

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 α-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 and behavioural explanation for abuse liability associated with 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. The CD1 mouse strain was selected for its optimal sensitivity to the psychostimulant effects of cocaine (McKerchar et al., 2005). For the synaptosomal preparation, male Sprague-Dawley rats (Janvier, Le Genest, France) weighing 225–250 g (2-3 months-old) were used. This rat strain has been used widely to obtain pure rat brain synaptosomes (Kolanos et al., 2015; Gannon et al., 2018; Lopez-Arnau et al., 2019; Saha et al., 2019). The animals were housed in temperature-controlled conditions (22 ± 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 animal care and experimental protocols in this study complied with the guidelines of the European Community Council (2010/62/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 and BJP guidelines for reporting experiments involving animals (McGrath and Lilley, 2015).

2.2. Drugs and materials

α-Aminovalerophenone derivatives were synthetized in racemic form as hydrochloride salts as described below in section 2.2.1. *Συντηεσις οφ α-αμινοαλεροπηενονε δεριατιες* . Solutions for injection were freshly prepared daily in isotonic saline solution (0.9% NaCl, pH 7.4). [^{3}H]DA, [^{3}H]5-HT, [^{3}H]imipramine and [^{3}H]WIN35,428 were purchased from Perkin Elmer Inc. (Boston, MA, USA). [^{3}H]1-Methyl-4-phenylpyridinium ([^{3}H]MPP$^+$), was supplied by American Radiolabeled 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.

2.2.1. Συντηεσις οφ α-αμινοαλεροπηενονε

The synthesis and characterization of the five cathinones was carried out through three steps, following the procedure formerly described (Meltzer et al., 2006). First of all, the ketone intermediate was prepared by alkylation of the nitrile compound (Step 1), followed by acidic hydrolysis. The n-BuMgCl was added in a dropwise manner to a solution of benzonitrile in toluene in anhydrous conditions. After three hours at room temperature, the reaction was complete. A solution of H_2SO_4 (4%) was added into the reaction mixture previously cooled. The organic layer was extracted with Et_2O, dried ($MgSO_4$), filtered and reduced in vacuo to an oil. The α-bromination reaction was carried out by adding bromine in a dropwise manner to a solution of the ketone intermediate in Et_2O in presence of $AlCl_3$ in catalytic amounts (Step 2). The excess of bromine is neutralized with a solution of $Na_2S_2O_3$. The organic layer is separated, dried (MgSO4), filtrated and reduced in vacuo to an oil. The α-bromoketone was dissolved in Et_2O, and the corresponding amine (methylamine, ethylamine, diethylamine, pyrrolidine and piperidine), was added all at once (Step 3). After 24-48 h the reactions were completed. The reaction mixtures were extracted with HCl 1N and then back-extracted into Et_2O by basification to pH 10 with NaOH 1 M. The organic layers were dried (MgSO4),

filtered and reduced in vacuo to an oil. Each product was dissolved in EtOH and a mixture of Et_2O with HCl (3M) in CPME was added in a dropwise manner in order to obtain the hydrochloride salt. Solids were collected by filtration. The identification of the five compounds was assessed by proton nuclear magnetic resonance (^{1}H NMR) ($CDCl_3$) yielding the following results:

α-μετηψλαμινοαλεροπηενονε ηψδροχηλοριδε : δ 10.55 (s, 1H), 9.16 (s, 1H), 7.96 (dd, J = 8.4, 1.2 Hz, 2H), 7.70 – 7.64 (m, 1H), 7.53 (t, J = 7.8 Hz, 2H), 4.94 (s, 1H), 2.81 (s, 3H), 2.30 – 2.16 (m, 1H), 2.16 – 2.02 (m, 1H), 1.61 – 1.46 (m, 1H), 1.45 – 1.29 (m, 1H), 0.89 (t, J = 7.3 Hz, 3H); *α -ετηψλαμινοαλεροπηενονε ηψδροχηλοριδε* : d 7.99 – 7.98 (d, J = 7.4 Hz, 2H), 7.72 – 7.68 (t, J = 7.4 Hz, 1H), 7.58 – 7.54 (t,J = 7.6 Hz, 2H), 6.99 (s, 0H), 4.97 (s, 1H), 3.24 (s, 1H), 3.04 (s, 1H), 2.33 – 2.29 (d, J = 12.4 Hz, 1H), 2.21 – 2.14 (m, 1H), 1.56 – 1.53 (t, J = 7.2 Hz, 3H), 1.48 – 1.32 (m, 2H), 0.90 – 0.86 (t, J = 7.2 Hz, 3H); *α -διετηψλαμινοαλεροπηενονε ηψδροχηλοριδε* : 8.18 - 8.10 (m, 2H), 7.75 (ddt, J = 7.9, 7.0, 1.2 Hz, 1H), 7.66 - 7.57 (m, 2H), 5.34 (dd, J = 6.3, 4.9 Hz, 1H), 3.48 (dq, J = 14.6, 7.3 Hz, 1H), 3.36 - 3.25 (m, 3H), 3.11 (dq,J = 14.3, 7.3 Hz, 1H), 2.06 - 1.92 (m, 2H), 1.41 (t, J = 7.3 Hz, 3H), 1.33 (t, J = 7.3 Hz, 3H), 1.30 - 1.12 (m, 3H), 0.86 (t, J = 7.3 Hz, 3H); *α - πψρρολιδινοπεντιοπηενονε ηψδροχηλοριδε:* d : 12.42 (s, 1H), 8.02-7.96 (m, 2H), 7.72-7.67 (m, 1H), 7.58-7.54 (m, 2H), 5.25 (dt, J = 8.0, 5.1 Hz, 1H), 3.82 (m, 2H), 3.62 (m, 1H), 2.95 (dq, J = 10.5, 7.7 Hz, 1H), 2.03 (m, 2H), 2.19 (m, 3H), 1.35 (m, 1H), 1.47 (m, 1H), 0.89 (t, J = 7.3 Hz, 3H); *α - N-πιπεριδψλαλεροπηενονε ηψδροχηλοριδε* : δ : 12.40 (ς, 1H), 8.02 – 7.97 (μ, 2H), 7.73 – 7.67 (δδτ, Θ = 7.4 Hζ, 2H), 7.58 – 7.52 (μ, 1H), 5.03 (δτ, Θ = 10.3, 3.8 Hζ, 1H), 3.67 (δ, Θ = 12.0 Hζ, 1H), 3.51 – 3.45 (μ, 1H), 3.42 – 3.37 (δ, Θ = 21.1 Hζ, 1H), 2.75 – 2.64 (μ, 1H), 2.53 – 2.45 (μ, 1H), 2.41 – 2.33 (μ, 1H), 2.30 – 2.17 (μ, 1H), 2.03 – 1.97 (μ, 2H), 1.96 – 1.87 (μ, 2H), 1.75 – 1.63 (δ, Θ = 12.5 Hζ, 1H), 1.47 – 1.30 (μ, 2H), 0.91 (τ, 3H).

ῆεμιςαλ πυριτψ οφ τηε οβταινεδ χομπουνδ ωας αλσο ασσεσσεδ βψ τηιν λαψερ χηροματογραπηψ ^{1}H NMP, ^{13} NMP ανδ μασς σπεχτρομετρψ. Αλλ αναλψτιςαλ δατα ωερε χονσιστεντ ωιτη τηε ασσιγνεδ στρυχτυρε ωιτη οερ 98% πυριτψ φορ τηε χατηινονε δεριατιε.

2.3. [^{3}H]ΔA υπτακε εξπεριμεντς ιν ρατ σψναπτοσομες

2.3.1. Ρατ σψναπτοσομε πρεπαρατιον

Ρατ σψναπτοσομε συσπενσιονς ωερε πρεπαρεδ ας δεσχριβεδ, ωιτη μινορ μοδιφιχατιονς (Πυβιλλ ετ αλ., 2005). Βριεφλψ, ιν εαχη εξπεριμεντ τωο ρατς ωερε δεχαπιτατεδ υνδερ ισοφλυορανε ανεστηεσια ανδ τηε στριατυμ ωας δισσεχτεδ ουτ, ηομογενιζεδ (ξ20 ολυμες· 5 μM Τρισ-Η"λ ανδ 320 μM συχροσε ιν Μιλλι-Χ ωατερ) ανδ χεντριφυγεδ ατ 1,000ξγ, 4°C for 10 min. After discarding the pellet, the supernatant was centrifuged at 13.000 x g for 30 minutes 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 $CaCl_2$, 0.44 KH_2PO_4, 0.49 $MgCl_2.6H_2O$, 0.41 $MgSO_4.7H_2O$, 4.17 $NaHCO_3$, 0.34 Na_2HPO_4. $7H_2O$, 5.5 glucose and 20 HEPES-Na) containing pargyline (20 mM) and ascorbic acid (1 mM).

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 α-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, α-PVP or α-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) presoaked 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 4degC in parallel samples containing cocaine (300 μM). All determinations were performed per duplicate.

All experiments were performed three times (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., 2016b, 2016a). HEK293 were maintained in DMEM supplemented with heat-inactivated 10% FBS, 100 U/ml penicillin and 100 µg/ 100 ml streptomycin, and cultured to a subconfluential state in a humidified atmosphere (5% CO2, 37^{o}C). Geneticin (G418; 50 µg/ml) was added to maintain the selection process.

For the uptake inhibition assays, HEK293 cells 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, 24h 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 x g for 10 minutes at 4^{o}C). The resulting pellet was resuspended in hypotonic HME buffer (20 mM HEPES NaOH, 2 mM $MgCl_2$, 1 mM EDTA; pH 7.4), followed by two freeze-thaw cycles in liquid nitrogen and homogenization through sonication at 4^{o}C. Thereafter, membranes were collected by centrifugation (40.000 x g for 30 min at 4^{o}C) and resuspended in an appropriate volume of HME buffer. The membrane preparations were kept at -80^{o}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 µl per well of Krebs-HEPES-Buffer (KHB; 10 mM HEPES, 120 mM NaCl, 3 mM KCl, 2 mM $CaCl_2 \cdot 2\ H_2O$, 2mM $MgCl_2 \cdot 6\ H_2O$ 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 µl per well for some time 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 µM [^{3}H]MPP+ for hDAT, 0,1 µM [^{3}H]5-HT for hSERT and 0,05 µM [^{3}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 (Perkin Elmer, Waltham, MA, USA). Non-specific uptake was determined in parallel samples containing cocaine 100 µM for hDAT, paroxetine 30 µM for hSERT and decynium-22 (D22) 100 µM for hOCT-2 andhOCT-3. The non-specific uptake value was <10% of total uptake and was subtracted from the data to yield specific uptake. 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 $MgCl_2$, 10 µM $ZnCl_2$ and 20 mM Tris pH 7,4 for hDAT, and 120 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA and 20 mM Tris pH 7,4 for hSERT) and tested at increasing concentrations (ranged

from 0,1 nM – 300 μM) in duplicate. The binding reactions were performed in tubes containing 25 μl of the radioligand: [^{3}H]WIN35,428 (hDAT assay, final concentration 10 nM) or [^{3}H]imipramine (hSERT assay, final concentration 3 nM) diluted in the corresponding reaction buffer, 5 μg of membranes and 100 μl of the tested drug dilution. Non-specific binding was determined in the presence of cocaine 100 μM (for hDAT radioligand) and paroxetine 3 μM (for hSERT radioligand). Incubation was performed for 1 hour at 20 ºC. 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 nM NaCl, 2 mM $MgCl_2$, 10 mM Tris and 100 μM $ZnCl_2$ for hDAT, and 120 nM NaCl, 2 mM $MgCl_2$ 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. Horizontal locomotor activity (HLA)

During the habituation phase (2 days), mice received an intraperitoneal (i.p.) saline injection and placed into a black Plexiglass open field arena (25x25x40 cm) under low-light conditions and white noise for 60 minutes. 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 kg^{-1}), N-ethyl-pentedrone (1, 3 or 10 mg kg^{-1}), N,N-diethyl-pentedrone (3.5, 12.5 or 35 mg kg^{-1}), α-PVP (1, 3 or 10 mg kg^{-1}) or α-PpVP (7.5, 25 or 75 mg 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 am and 3:00 pm. On one hand, pentedrone and α-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 α-PpVP used in animal research. Therefore, these doses were chosen in pilot experiments and according to the psychostimulant effect induced by pentedrone or α-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 α-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 α-PVP (Hwang et al., 2017; Wojcieszak et al., 2018).

2.6. Conditioned place preference (CPP)

The potential of the five α-aminovalerophenone 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, α-PVP, or α-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-hours 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 preference.

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.

2.7. Δοςκινγ οφ α-αμινοαλεροπηενονε δεριατιες

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%), 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 GBVI/WSA ΔG score function was used for quantifying the free energy of binding of the 100 resulting conformations for each molecule. The drugs were built as (S)-enantiomers in protonated form and they were docked into the hDAT model using the previous protocol.

2.8. Data acquisition and statistical analysis

The data and statistical analysis in this study comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). Data from biochemical analyses were normalized with 100% defined as the mean of the technical replicates in the control group. During behavioural analysis, researchers were not aware of the drug that each animal had received. Competition curves were plotted and fitted by nonlinear regression. Data were best fitted to a sigmoidal dose-response curve and an IC_{50} or EC_{50}value was obtained. Transporter ratios were calculated as (1/DAT IC_{50} : 1/SERT IC_{50}), with higher values indicating greater selectivity for DAT. K_i(affinity) values were calculated using the Cheng-Prusoff equation:$K_i = IC_{50}/(1+[\text{radioligand concentration}/K_d])$. One-way ANOVA, and subsequent post hoc test (Tukey-Kramer) which was conducted only if F was significant, was used to determine overall α-aminovalerophenone derivatives effects on HLA, CPP as well as DA and 5-HT uptake in both rat synaptosomes and HEK cells. The α 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

[^{3}H]DA uptake inhibition by α-aminovalerophenone 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. [^{3}H]DA uptake inhibition in rat brain synaptosomes

Potencies of the tested compounds to inhibit DA uptake are depicted in Figure S1 and their respective IC_{50} values for [^{3}H]DA uptake inhibition at DAT are compiled in Table 1. The rank order of potency inhibiting [^{3}H]DA uptake was as follows: α-PVP>N-ethylpentedrone[?]α-PpVP>pentedrone>>N,N-diethyl-pentedrone ($F_{(4,14)}$=94.01).

3.1.2. Monoamine uptake inhibition in transfected HEK293 cells

The corresponding IC_{50} values and hDAT/hSERT inhibition ratios are presented in Table 1 and concentration-response curves are depicted in Figure S2. While assessing uptake-1 assays, all tested drugs displayed low IC_{50} values for hDAT and negligible activity at hSERT. Additionally, the order of potencies for hDAT was as follows: α-PVP[?]N-ethylpentedrone[?]α-PpVP>pentedrone>N,N-diethyl-pentedrone

;$F_{(4,10)}$=30.61). The order of potency for hSERT was as follows: pentedrone[?]N-ethyl-pentedrone>>α-PVP>N,N-diethyl-pentedrone[?]α-PpVP; $F_{(4,10)}$=179.1). It is important to point out the very low inhibitory potencies of N,N-diethyl-pentedrone, α-PVP and α-PpVP when studying [^{3}H]5-HT uptake inhibition at hSERT. However, an estimation of the IC_{50} 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 them with some QSAR parameters such as CLogP, total surface area, volume and CMR. As shown in Table 2, hSERT, but not hDAT IC_{50} 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; Figure S2), α-aminovalerophenone derivatives blocked hOCT-2 within the IC_{50}value range of 10–60 μM, with no considerable effect on hOCT-3.

3.2. Transporter binding affinities

The binding affinities of the α-aminovalerophenone 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 *(Ki)* are summarized in Table 1. All drugs exhibited higher binding affinity for hDAT in the medium-low nanomolar range (< 400 nM), when compared to hSERT (> 10000 nM). Therefore, all the compounds seem to be hDAT-selective. For instance, N-ethyl-pentedrone, α-PVP and α-PpVP were more potent than cocaine in binding to hDAT. Conversely, all drugs presented substantially lower affinity to hSERT, with pentedrone showing the highest affinity (*Ki*). 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, sterik bulk or total surface area of the amino-substituent.

3.3. Εφφεςτ οφ α-αμινοαλεροπηενονε δεριατιες ον ΗΛΑ

One-way ANOVA of HLA results revealed a significant effect of the variable Dose for all the drugs tested ([pentedrone], ($F_{(3,34)}$=22.7); [N-ethyl-pentedrone], ($F_{(3.36)}$=98.42); [N,N-diethyl-pentedrone], ($F_{(3,36)}$=52.89); [α-PVP], ($F_{(3,40)}$=72.09); [α-PpVP], ($F_{(3,36)}$=12.01). Overall, all compounds increased HLA in a dose-dependent manner in mice (Figure 2).

For pentedrone and α-PpVP, subsequent post-hoc Tukey-Kramer test demonstrated a significant increase in HLA after 10 and 30 mg kg^{-1} (pentedrone) and 25 and 75 mg kg^{-1} (α-PpVP) injections, respectively, compared to the saline group. As for N-ethyl-pentedrone (3 and 10 mg kg^{-1}), N,N-diethyl-pentedrone (12.5 and 35 mg kg^{-1}) and α-PVP (3 and 10 mg kg^{-1}), a similar increase in the hyperlocomotion 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.

When analysing the locomotor activity induced by the medium doses tested of each α-aminovalerophenone derivative, one-way ANOVA yielded no significant effect of the variable Compound ($F_{(4,46)}$=0.8724), which means that the medium doses used in this study were equally effective. Therefore, α-PVP and N-ethyl-pentedrone elicited the same locomotor response at a dose of 3 mg kg^{-1}, while pentedrone and N,N-diethyl-pentedrone induced the same effect at doses of 10 and 12.5 mg kg^{-1}, respectively and α-PpVP produced it at 25 mg kg^{-1}. Consequently, the rank order of potency according to the doses used in this is study for eliciting hyperlocomotion was α-PVP = N-ethyl-pentedrone > pentedrone > N,N-diethyl-pentedrone > α-PpVP.

3.4. Εφφεςτ οφ α-αμινοαλεροπηενονε δεριατιες ον ΤΠΠ

The CPP paradigm was used to study the rewarding effect of five α-aminovalerophenone derivatives. The percentages of time spent in 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 preference for one of the compartments (>70% of the total session time). Figure 3 shows the effects of α-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); [N-ethyl-pentedrone], ($F_{(3.49)}$=5.044); [N,N-diethyl-pentedrone], ($F_{(3.49)}$=2.802); [α-PVP], ($F_{(3.52)}$=4.555); [α-PpVP], ($F_{(3.52)}$=4.179)).

Particularly, animals conditioned with pentedrone and α-PVP at 3 and 10 mg kg^{-1} showed a significant increase in the preference score compared with the saline-treated animals (See Figure 3A and 3D). Moreover, a significant increase in the preference score after N-ethyl-pentedrone repeated administrations was observed for all doses tested (1, 3 and 10 mg kg^{-1}) compared to the control group (See Figure 3B). 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 mg kg^{-1} (See Figure 3C). Finally, after conditioning with α-PpVP, post hoc analysis only revealed an increase in the preference score at the lowest dose tested (7.5 mg kg^{-1}) (See Figure 3E).

3.5. Molecular docking with α-aminovalerophenone derivatives

The binding pocket can be divided into three subsites (A-C), (Figure 4A) (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 (Figure 4F), 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 (Figure 4B). This is in contrast to N-ethyl-pentedrone (Figure 4C), pentedrone (Figure 4D) and N,N-diethyl-pentedrone (Figure 4E), which showed similar binding mechanism establishing an hydrogen bond between the amino group and Asp79. The addition of an extra carbon atom in pentedrone seemed not to affect the binding mechanism (Figure 4C-D) but the high hydrophobicity of N,N-diethyl-pentedrone contrasted with the high polarity of subsite A and modified its interaction mechanism (Figure 4E). 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 (Figure 4F). Predicted binding affinities (approximated as docking score value) agreed with *Ki*experimental data ($R^2_{\mathrm{SCORE-(Ki)}} = 0.93$) and following the same order (Spearman correlation coefficient, *ρ=1).*

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 α-aminovalerophenone derivatives, which structurally differ only in their amino substituent (Figure 1).

A first evidence of the direct blockade of DA uptake by five different α-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 but with weak 5-HT uptake inhibition. However, the absolute 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; Saha et al., 2015; Mayer et al., 2016a; Sandtner et al., 2016). Moreover, drug selectivity for DAT vs SERT is a key determinant of the abuse potential of drugs targeting monoamine

transporters (Stevens Negus and Miller, 2014; Negus and Banks, 2017). High hDAT/hSERT ratios indicates 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 α-PVP pyrrolidine ring to a piperidine ring resulted in a progressive decrease in the DAT potency (Kolanos et al., 2015; Glennon and Young, 2016). 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 *Ki* 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 have to 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 (Koepsell et al., 1999; Jonker and Schinkel, 2004; 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. Particularly, a dose-response effect in locomotor activity was observed after synthetic cathinones injections and confirms previous reports about psychostimulant effects of α-PVP and pentedrone (Marusich et al., 2014, 2016; Gatch et al., 2015a, 2015b; Giannotti et al., 2017; Hwang et al., 2017; Javadi-Paydar et al., 2018; Wojcieszak et al., 2018). As expected, the substances that needed lower doses to produce the same behavioural effects (N-ethyl-pentedrone and α-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 α-PpVP was required to induce the same locomotor effect when compared to the other compounds. At this point, we can only hypothesize that some pharmacokinetic effect such as absorption and/or crossing blood-brain-barrier may be involved in such difference in *in vitro* vs *in vivo* effects.

Finally, our results also demonstrated that all the compounds tested induced rewarding effects and are consistent with previous studies (Gatch et al., 2015b; Hwang et al., 2017). Particularly, the lowest tested dose of α-PpVP, pentedrone and N-ethyl-pentedrone induced place-conditioning in spite of not producing hyperlocomotion, while the other two doses of N-ethyl-pentedrone induced both effects In contrast, the other two doses tested of α-PpVP 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.

In summary, all of the α-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_{50}, while changing

a pyrrolidine to a diethyl and piperidine ring increased the IC_{50} 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 substituent. Finally, our study also provides the first evidence that N-ethyl-pentedrone, N,N-diethyl-pentedrone and α-PpVP are able to induce psychostimulant and rewarding effects in mice, suggesting their abuse liability.

Author's Contribution

HHS, EE and RLA conceived and designed the research study. LDC, MM, BP, MN, SB and RLA performed *in vitro* and *in vivo* experiments. Synthesis and chemical characterization of compounds was carried out by NNG and XB. NNG and RET conducted computational docking analysis. LDC, EE, HHS and RLA contributed to analysis and interpretation of data. HHS, EE, DP, JC and RLA were involved in revising the manuscript critically for important intellectual content. LDC and RLA wrote the manuscript.

Conflict of 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.

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Table 1. Affinity and potency of substituted cathinones and standard compounds at monoamine transporters. Monoamine uptake-1 and uptake-2 inhibition: values are IC_{50} given as μM (mean and 95% confidence intervals (CI). Transporter binding affinities: values are *Ki* given as μM (mean ± SD).

	Monoamine uptake inhibition	**Monoamine uptake inhibition**	**Monoamine uptake inhibition**	**Monoamine uptake inhibition**	**Monoamine uptake inhibition**	**Monoamine uptake inhibition**	**Monoamine uptake inhibition**	**Monoamine uptake inhibition**	**Transporte binding affinities**
	Rat brain synaptosomes Uptake-1	Rat brain synaptosomes Uptake-1	Transfected HEK293 cells Uptake-1	Transfected HEK293 cells Uptake-1	Transfected HEK293 cells Uptake-1	Transfected HEK293 cells Uptake-1	Transfected HEK293 cells Uptake-2	Transfected HEK293 cells Uptake-2	Transfected HEK293 cells
Compound	[^{3}H]DA		[^{3}H]MPP$^+$ uptake at hDAT		[^{3}H]5-HT uptake at hSERT	hDAT/hSERT ratio	[^{3}H]MPP$^+$ uptake at hOCT-2	[^{3}H]MPP$^+$ uptake at hOCT-3	hDAT
Pentedrone	1.742 (1.371 – 2.112)		0.215 (0.163 – 0.267)		78.03 (69.270 – 86.790)	363	26.95 (23.996 – 29.90)	> 1000	0.146 ± 0.03

	Monoamine uptake inhibition	Monoamine uptake inhibition	Monoamine uptake inhibition	Monoamine uptake inhibition	Monoamine uptake inhibition	Monoamine uptake inhibition	Monoamine uptake inhibition	Monoamine uptake inhibition	Transporte binding affinities
N-ethyl-pentedrone	0.824 (0.718 – 0.930)		0.076 (0.041 – 0.111)		78.47 (77.997 – 78.94)	1029	57.58 (46.97 – 68.19)	> 1000	0.049 ± 0.02
N,N-diethyl-pentedrone	3.535 (3.175 – 3.895)		0.445 (0.325 – 0.565)		> 100	> 2000	47.81 (46.745 – 48.87)	> 1000	0.398 ± 0.05
α-ΠʺΠ	0.139 (0.104 – 0.174)		0.032 (0.027 – 0.037)		> 100	> 2000	17.49 (13.498 – 21.48)	> 1000	0.011 ± 0.004
α-Ππʺ Π	1.055 (0.917 – 1.193)		0.058 (0.046 – 0.070)		> 100	> 2000	12.88 (12.15 – 13.61)	> 1000	0.078 ± 0.03
Cocaine[a]	N.A.		1.189 (0.840 – 1.68)		7.94 (6.34 – 9.94)	6.68	N.A.	N.A.	0.217 ± 0.02
Imipramine[a]	N.A.		N.A.		N.A.	N.A.	N.A.	N.A.	N.A.

hDAT/hSERT ratio = 1/DAT IC_{50} : 1/SERT IC_{50}

N.A., not assessed; [a] Control compounds

Table 2. Correlation analysis between molecular or physicochemical descriptors and logIC_{50} or log hDAT/hSERT ratio values of monoamine uptake in transfected HEK293 cells.

	Log IC_{50} hDAT	Log IC_{50} hDAT	Log IC_{50} hSERT	Log IC_{50} hSERT	Log hDAT/hSERT ratio	Log hDAT/hSERT ratio
	r^2	p	r^2	p	r^2	p
CLogP	0.082	P > 0.05	**0.917**	**P < 0.05**	**0.806**	**P < 0.05**
Total Surface Area (Å^2)	0.013	P > 0.05	**0.922**	**P < 0.05**	0.639	P > 0.05
Volume (Å^3)	0.024	P > 0.05	**0.925**	**P < 0.05**	0.677	P > 0.05
CMR	0.045	P > 0.05	**0.926**	**P < 0.05**	0.734	P > 0.05

CLogP: partition coefficient

CMR: calculated molar refractivity

Legends of figure

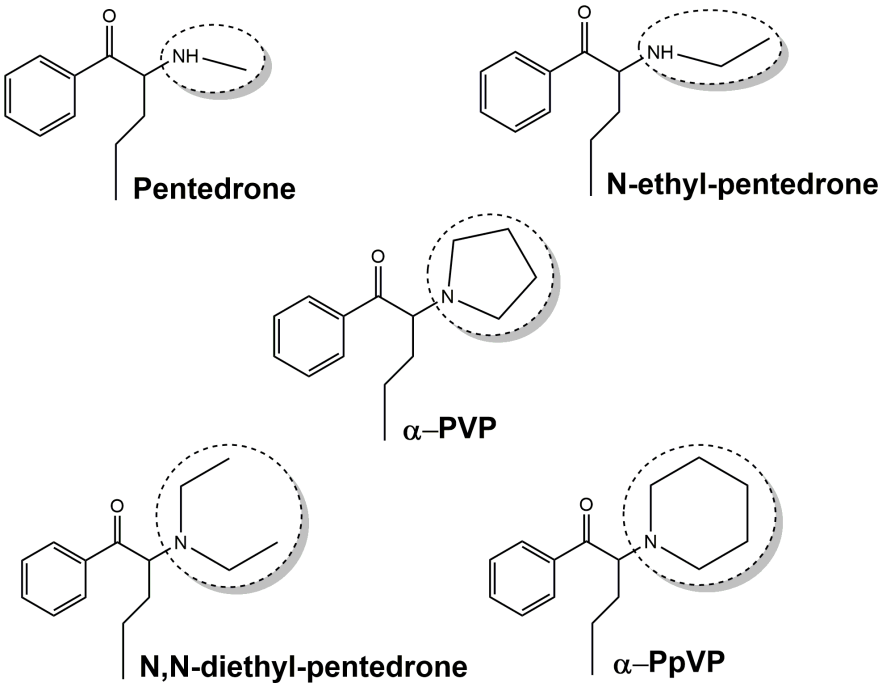
Figure 1. Chemical structure of the aminovalerophenone derivatives. Compounds share a similar chemical structure, only differing by their amino-substituent: methylamino- (pentedrone), ethylamino- (N-ethyl-pentedrone), diethylamino- (N,N-diethyl-pentedrone), pyrrolidine ring (α-PVP) or piperidine ring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

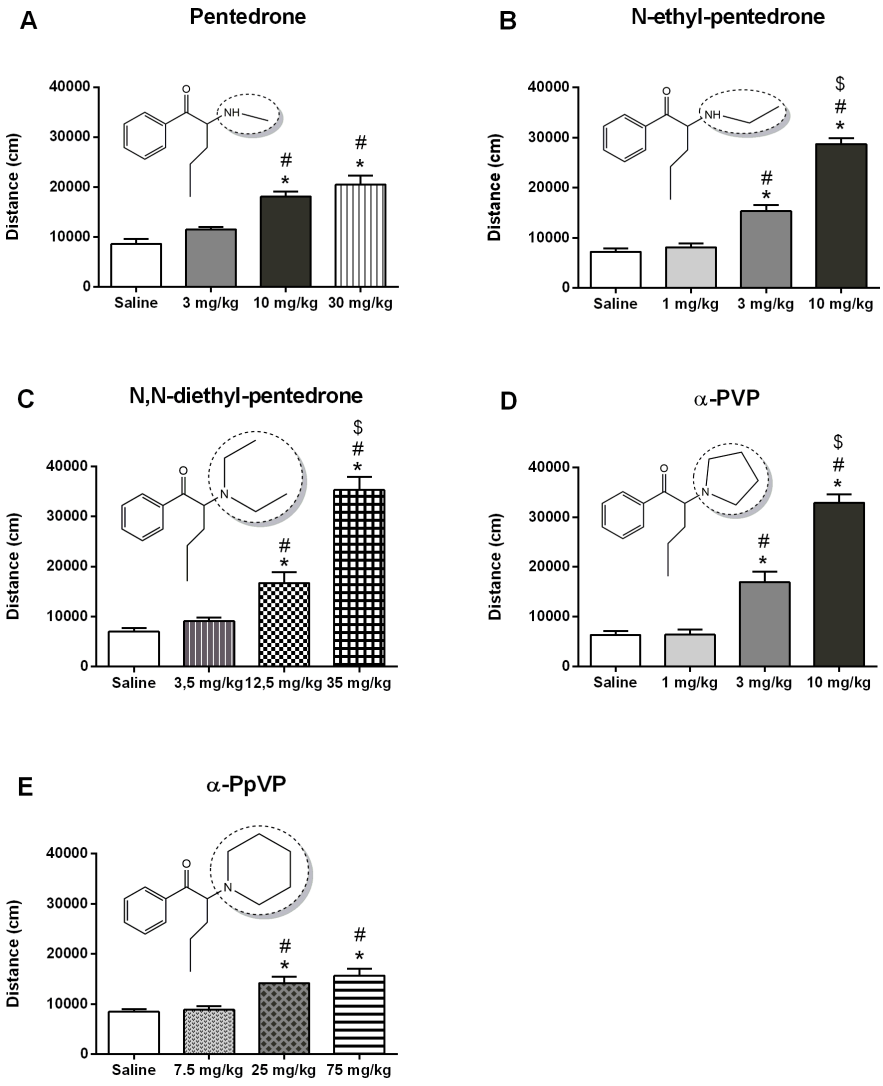
Figure 2. Effects of aminovalerophenone derivatives on cumulative locomotor activity in CD-1 mice. Bars

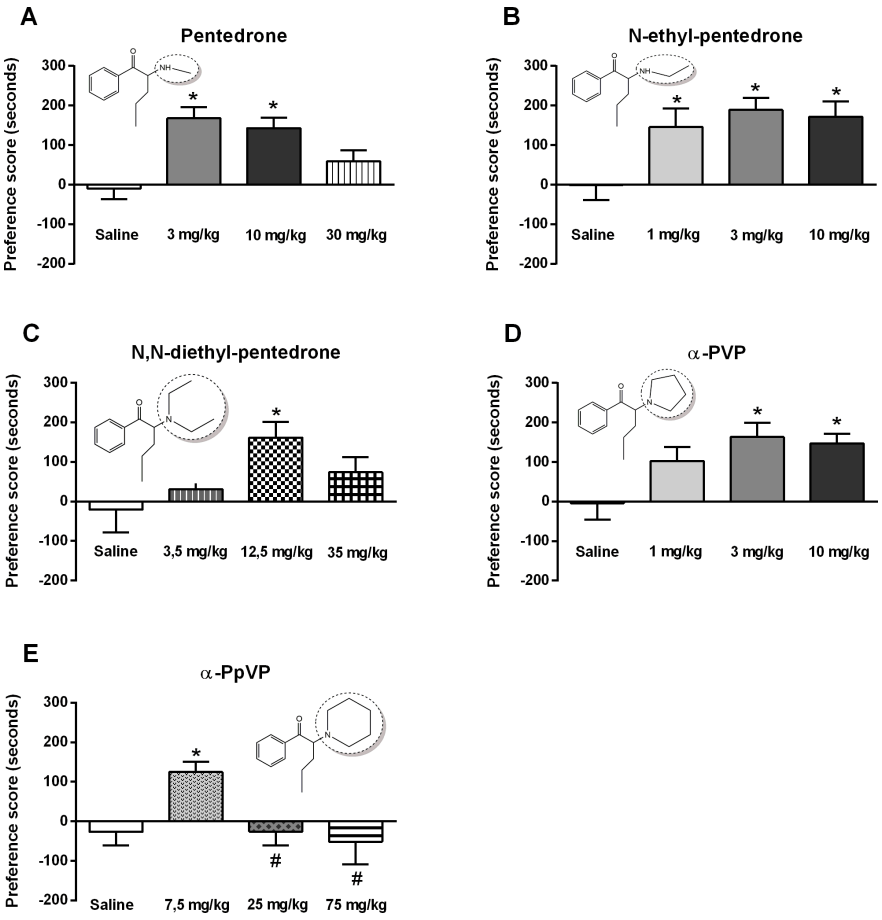
represent mean ± SEM of the distance travelled in 60 min. Panel A: saline and pentedrone 30 mg kg^{-1} groups, N=9/group; pentedrone 3 mg kg^{-1} and 10 mg kg^{-1} groups, N=10/group. Panel B: saline, N-ethyl-penedrone 1, 3, and 10 mg kg^{-1}, N=10/group. Panel C: saline, N,N-diethyl-pentedrone 3.5, 12.5, 35 mg kg^{-1}, N=10/group. Panel D: saline, α-PVP 1, 3, 10 mg kg^{-1}, N=11/group. Panel E: saline, α-PpVP 7.5, 25, 75 mg kg^{-1}, N=10/group. *P<0.05 vs saline; #P<0.05, vs the lower drug-dose; $P<0.05 vs the medium drug-dose.

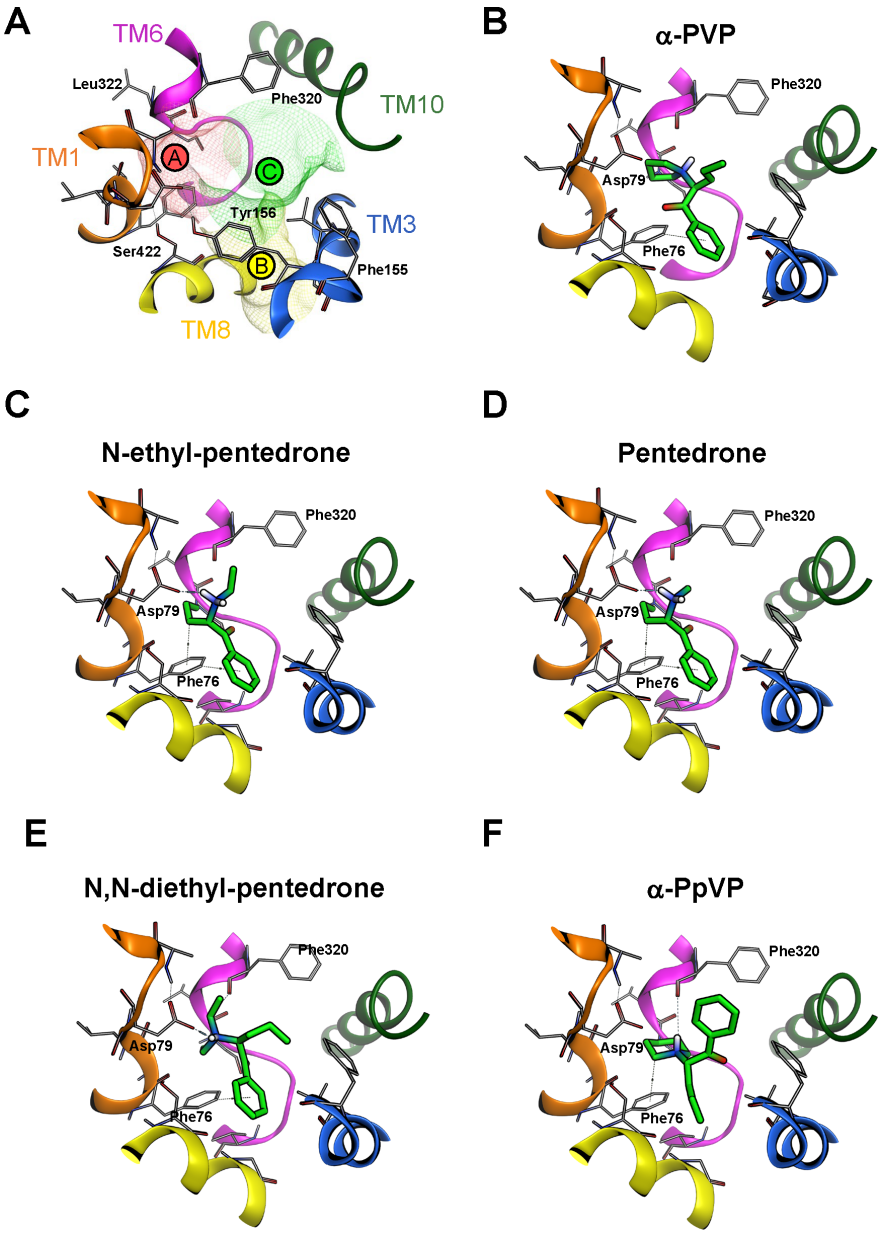
Figure 3. Effects of aminovalerophenone derivatives on conditioned place preference (CPP) test in CD-1 mice. Bars represent mean ± SEM of the preference score (difference between the time spent in the drug-paired compartment on the test day and the pre-conditioning day). Panel A: saline and pentedrone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

Figure 4. 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-pentedrone (C), pentedrone (D), N,N-diethyl-pentedrone (E) and α-PpVP (F).









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