The cathinones MDPV and α-PVP elicit different behavioral and molecular effects following acute exposure.

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1

Abstract

Since the mid-to-late 2000's, synthetic cathinones have gained popularity amongst drug users due to their psychostimulant effects greater than those produced by cocaine and amphetamine. Among them, MDPV and α-PVP are ones the most popular cathinones available in the clandestine market as "bath salts" or "fertilizers". Pre-clinical studies indicate that MDPV and α-PVP induced psychomotor stimulation, affected thermoregulation and promoted reinforcing properties in rodents. However, a direct comparative analysis on the effects caused by MDPV and α-PVP on the behavior and neuronal activation in rodents is still lacking. Behavioral analyses revealed that both MDPV and α-PVP affect spontaneous and stimulated motor responses. In particular, MDPV showed a greater psychomotor effect than α-PVP in line with its higher potency in blocking the dopamine transporter (DAT). Notably, MDPV was found to be more effective than α-PVP in facilitating spontaneous locomotion and it displayed a biphasic effect in contrast to the monophasically-stimulated locomotion induced by α-PVP. In addition to the behavioral results, we also found a different modulation of immediate early genes (IEGs) such as Arc/Arg3.1 and c-Fos in the frontal lobe, striatum and hippocampus, indicating that these drugs do impact brain homeostasis with changes in neuronal activity that depend on the drug, the brain area analyzed and the timing after the injection. These results provide the first discrimination between MDPV and α-PVP based on behavioral and molecular data that may contribute to explain, at least in part, their toxicity.

Introduction

Synthetic cathinones are an emerging class of designer drugs abused for their psychostimulant and empatogenic properties similar to cocaine, methylenedioxymethamphetamine or other amphetamines (German et al. 2014; Spiller et al. 2011) and commonly commercialized as "bath salts", "research chemicals" or "plant food" via the Internet market or smart shops (Meyers et al. 2015; Schneir et al. 2014). Among them, the methylenedioxy derivative of pyrovalerone MDPV (3,4-methylenedioxypyrovalerone) and its closely related derivative α-PVP ((1-Phenyl-2-(pyrrolidin-1-yl)pentan-1-one) also known as "flakka" or "gravel"), are in the top 5 among the most popular synthetic cathinones on the clandestine market (EMCDDA 2014; EMCDDA 2016; Glennon and Young 2016; WHO 2014; WHO 2015)

These two synthetic cathinones potently and selectively block dopamine (DA) and norepinephrine (NE) transporters with negligible affinity for the serotonin (5-HT) transporter (Baumann et al. 2013; Kolanos et al. 2015; Meltzer et al. 2006; Rickli et al. 2015; Simmler et al. 2013). Consistent with these pharmacodynamic features, acute MDPV and α-PVP treatment produces psychomotor effects in rodents and increased motor activity as well as the appearance of stereotypes (Aarde et al. 2015; Gatch et al. 2015; Gatch et al. 2013; Glennon and Young 2016; Marusich et al. 2012). Moreover, MDPV and/or α-PVP increased heart rate, blood pressure and differently affected thermoregulation (Glennon and Young 2016; Kiyatkin et al. 2015; Wakabayashi et al. 2015; WHO 2014; WHO 2015). In particular, MDPV's and α-PVP's effects on thermoregulation are variable, i.e., they sometimes have no effect, sometimes increase, and sometimes decrease temperature. Kiyatkin et al. (2015) and Miner et al. (2017) found MDPV had little effect on brain or body temperature. Aarde et al. (2015) found MDPV and α-PVP decreased temperature. These effects are different from temperature profiles of other methcathinone and psychostimulants, though they share the other properties mentioned of increased heart rate and blood pressure.

Furthermore, as typically reported for psychostimulants, they promoted reinforcing and rewarding properties in rodents (Aarde et al. 2015; Bonano et al. 2014; Fantegrossi et al. 2013; Gatch et al. 2015; Glennon and Young 2016; Naylor et al. 2015).

The major aim of our study was to map the neuronal activation brought about by MDPV and α-PVP in reward-related brain regions such as frontal lobe, striatum and hippocampus to provide novel information about brain region specificity and the timing of such activation. We focused our attention on two immediate early genes (IEGs), i.e. c-fos and Arc/Arg3.1. C-fos is the classic IEG, i.e. a transcription factor which indirectly alters the expression of a given target gene whereas Arc/Arg3.1 is an effector gene, which acts directly on cellular homeostasis and function. Thus, the analysis of these two IEGs could provide us with novel information about the acute profile of cathinone's actions. To complement the molecular analyses, we performed behavioral measures incorporating a wide range of doses (0.01-20 mg/kg i.p.) of these cathinones to evaluate their behavioral effects in CD-1 male mice on spontaneous and stimulated (drag test and accelerod) motor activity.

Materials and Methods

Animals

Male ICR (CD-1®) mice, 25-30 gr (ENVIGO Harlan Italy; S. Pietro al Natisone, Italy), were group-housed (8-10 mice per cage; floor area per animal was 80 cm^2; minimum enclosure height was 12 cm) on a 12:12-h light-dark cycle (light period from 6:30 AM to 6:30 PM), temperature of 20-22 °C, humidity of 45-55% and were provided ad libitum access to food (Diet 4RF25 GLP; Mucedola, Settimo Milanese, Milan, Italy) and water. Experimental protocols performed in the present study were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the European Communities Council Directive of September 2010 (2010/63/EU) and were approved by Italian Ministry of Health (license n. 335/2016-PR) and by the Ethics Committee of the University of Ferrara. Moreover, adequate measures were taken to minimize the number of animals used and their pain and discomfort.

Drug Preparation

MDPV and α-PVP were purchased from LGC Standards (LGC Standards S.r.l., Sesto San Giovanni, Milan, Italy). Drugs were initially dissolved in absolute ethanol (final concentration was 2%) and Tween 80 (2%) and brought to the final volume with saline (0.9% NaCl). The solution made of ethanol, Tween 80 and saline was also used as the vehicle. For the behavioral analysis, single intraperitoneal injections of five different doses (0.01, 0.1, 1, 10, 20 mg/kg) were tested at a volume of 4 µl/gr.

Behavioral motor studies and analysis of gene expression

Motor activity assessment.

Alterations of motor activity induced by MDPV and α-PVP were measured using the drag, accelerod tests and the analysis of spontaneous locomotor activity (Canazza et al. 2016; Ossato et al. 2016; Ossato et al. 2015; Vigolo et al. 2015).

Spontaneous locomotor activity was measured by using the ANY-maze video-tracking system (Ugo Basile, application version 4.99 g Beta). The mouse was placed in a square plastic

cage (60 X 60 cm) located in a sound- and light-attenuated room and its total distance travelled (m) was monitored for 240 min. Four mice were monitored at the same time in each experiment. The distance covered was analyzed every 15 min for a maximum of 240 min. To avoid mice olfactory cues, cages were carefully cleaned with ethanol solution (5%) and washed with water between animal trials. All experiments were performed between 9:00 AM to 1:00 PM.

In the *drag test,* the mouse was lifted by the tail, leaving the front paws on the table and dragged backward at a constant speed of about 20 cm/sec for a fixed distance (100 cm). The number of steps performed by each paw was recorded by two different observers. For each animal five to seven measurements were collected. The drag test was performed at 0, 40, 60, 95, 150, 210, 270, 330 min and 24 h post injection.

In the *accelerod test,* animals were placed on a rotating cylinder that automatically and constantly (0-60 rotations/min in 5 min) increased velocity. The time spent on the cylinder was measured. The accelerod test was performed at 0, 45, 70, 105, 160, 220, 280, 340 min and 24 h post injection.

To reduce the animal's stress induced by manipulation, and to confirm the stability and reproducibility over time of the responses of our tests, animals were trained 2 times per week for 2 weeks before the pharmacological treatment. All experiments were performed between 8:30 AM to 2:00 PM. Experiments were conducted in blind by trained observers working together in pairs (Redfern et al. 2005).

Analysis of gene expression

For the molecular analyses, the intermediate dose of 1 mg/kg was chosen based on the behavioral effects. At the scheduled times after the drug injection, mice were killed by cervical dislocation, their brains were quickly removed and the brain regions of interest were immediately dissected out, frozen on dry ice, and stored at -80°C until being processed. Dissections were performed according to the mice atlas of Paxinos and Franklin (2004); frontal lobe (approximately from Bregma +2.58 to +1.70) and striatum (approximately from Bregma +1.54 to 0.14) were

6

dissected from 2 mm coronal section while the hippocampi were dissected according to Spijker (2011).

Total RNA was isolated by single step guanidinium isothiocyanate/phenol extraction using PureZol RNA isolation reagent (Bio-Rad Laboratories, Segrate, Milan, Italy) according to the manufacturer's instructions and quantified by spectrophotometric analysis. Following total RNA extraction, the samples were processed for real-time reverse transcription polymerase chain reaction (real time RT-PCR) to assess mRNA levels. Briefly, an aliquot of each sample was treated with DNase to avoid DNA contamination. RNA was analyzed by TaqMan qRT-PCR instrument (CFX384 real time system, Bio-Rad Laboratories) using the iScriptTM one-step RT-PCR kit for probes (Bio-Rad Laboratories). Samples were run in 384 wells formats in triplicate as multiplexed reactions. Data were analyzed with the comparative threshold cycle ($\Delta\Delta$Ct) method using 36B4 as reference gene (Giannotti et al. 2014). The primer efficiencies were experimentally set up for each couple of primers. Thermal cycling was initiated with an incubation at 50°C for 10 min (RNA retrotranscription) and then at 95°C for 5 min (retrotranscriptase inactivation). After this initial step, 39 cycles of PCR were performed. Each PCR cycle consisted of heating the samples at 95°C for 10s to enable the melting process and then for 30s at 60°C for the annealing and extension reaction. Primers and probe for Arc/Arg3.1 were purchased from Life Technologies (Milan, Italy) (accession 240 number: EF125675, ASSAY ID: Rn02531967_s1). Primers and probes and for c-Fos and 36B4 were purchased from Eurofins MWG-Operon (Ebersberg, Germany) and their sequences are shown below:

c-Fos: forward primer 5′- TCCTTACGGACTCCCCAC -3′, reverse primer 5′-CTCCGTTTCTCTTCCTCTTCAG -3′, Probe 5′- TGCTCTACTTTGCCCCTTCTGCC -3′.

36B4: Forward primer 5'-AGATGCAGCAGATCCGCAT-3', Reverse Primer 5'-GTTCTTGCCCATCAGCACC-3', Probe 5'-CGCTCCGAGGGAAGGCCG-3'.

Data and statistical analysis

Data are expressed in absolute values (m, for distance traveled) in the analysis of spontaneous locomotion and as percentage of basal in drag and accelerod tests. Data represent the mean ± SEM of 8-10 animals for each treatment. All the numerical data are given as mean ± SEM of 4 independent experimental replications. Data were analyzed by utilizing repeated measures ANOVA. Results from treatments showing significant overall changes were subjected to *post hoc* Tukey tests with significance for $p<0.05$. The statistical analysis of the effects of the individual substances at different doses over time was performed by two-way ANOVA followed by Bonferroni's test for multiple comparisons. The analysis of the overall effect induced by treatments (expressed in histograms) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. Molecular data were collected in individual animals (independent determinations) and are presented as means ± standard errors. Changes produced by treatment and time after the injection alone as well as by their combination were analyzed using a two-way analysis of variance (ANOVA), with treatment and time after injection as independent variables. When appropriate, further differences between groups were analyzed by Fisher's PLSD test. Statistical significance was assumed at $p<0.05$. The statistical analysis was performed with the program Prism software (GraphPad Prism, USA).

Results

Spontaneous locomotor activity in mice.

Firstly, we investigated the effect of MDPV and α-PVP (0.01-20 mg/kg i.p.) on spontaneous locomotor activity in mice. Both psychostimulants at high doses (1-20 mg/kg) facilitate the spontaneous locomotor activity in mice within two hours after treatment in a dose dependent manner (MDPV, Fig 1 panel A: significant effect of treatment ($F_{5,672}$=151.2, p<0.0001), time ($F_{15,672}$=68.51, p<0.0001) and time x treatment interaction ($F_{75,672}$=4.066, p<0.0001) and α-PVP, Fig 1 panel B: significant effect of treatment ($F_{5,672}$=78.93, p<0.0001), time ($F_{15,672}$=44.70, p<0.0001) and time x treatment interaction ($F_{75,672}$=1.491, p=0.0064)). Moreover, MDPV was significantly greater than α-PVP at all 3 doses: 1, 10 and 20 mg/kg (Fig 1 panel C: significant effect of agonists ($F_{10,87}$=2316, p<0.0001)). From Figures 1 A and B it appears that locomotor activity of mice treated with MDPV and α-PVP was not significantly different from vehicle after 135 and 185 min, respectively.

Stimulated locomotor activity in mice.

Drag test

Differences were observed between MDPV and α-PVP in the number of steps performed by mice with the front legs: MDPV increased it only at 1 mg/kg and 10 mg/kg (Fig 1 panel D: significant effect of treatment ($F_{5,336}$=8.189, p<0.0001), time ($F_{7,336}$=1.062, p=0.3879) and time x treatment interaction ($F_{35,336}$=0.6315, p=0.9504)) while α-PVP only at the dose of 20 mg/kg (Fig 1 panel E: significant effect of treatment ($F_{5,336}$=2.786, p=0.0176), time ($F_{7,336}$=0.7956, p=0.5914) and time x treatment interaction ($F_{35,336}$=0.3993, p=0.9992)). Effects of both compounds vanished within 24 hours (data not shown).

Ultimately, the overall effect observed in 5 hours demonstrated that MDPV was more potent and effective than α-PVP in the range doses of 1-10 mg/kg (Fig 1 panel F: significant effect of MDPV and α-PVP ($F_{10,87}$=9.208, p<0.0001)).

Accelerod test

In the accelerod test, at the lowest doses (0.01 and 0.1 mg/kg i.p.), MDPV induced a prolonged increase in locomotion (Fig 1 panel G: significant effect of treatment ($F_{5,336}=9.038$, p<0.0001), time ($F_{7,336}=1.755$, p=0.0956) and time x treatment interaction ($F_{35,336}=0.3979$, p=0.9992)). A different situation was observed at the higher doses tested; in fact, MDPV at 1 and 20 mg/kg increased and reduced, respectively, locomotion, with non-significant effects. Conversely, injection of α-PVP, evoked a prolonged increase of locomotion only at the highest dose tested (10 and 20 mg/kg) (Fig 1 panel H: significant effect of treatment ($F_{5,336}=8.938$, p<0.0001), time ($F_{7,336}=0.7528$, p=0.6274) and time x treatment interaction ($F_{35,336}=0.3435$, p=0.9999)). The overall 5 hours effect demonstrated that MDPV was more potent than α-PVP in increasing stimulated locomotor activity (Fig 1 panel I: significant effect of MDPV and α-PVP ($F_{10,87}=36.18$, p<0.0001). Effects of both compounds vanished within 24 hours (data not shown).

Molecular analyses of the effects of MDPV and α-PVP on Arc/Arg3.1 and c-Fos *mRNA levels*

Frontal Lobe:

Panels A-B of Figure 2 show the effects of a single injection of MDPV and α-PVP (1 mg/kg) on Arc/Arg3.1 and c-Fos mRNA levels in the frontal lobe of mice at three different time points, i.e. 30 minutes, 2 and 24 hours after the drug exposure.

Arc/Arg3.1 mRNA levels:

Two-way ANOVA showed a main effect of treatment ($F_{2,48}=6.2985$, *p*=0.003724), time after injection ($F_{2,48}=4.5014$, *p*=0.016153) and a significant time x treatment interaction ($F_{4,48}=7.0529$, *p*=0.000131) on Arc/Arg3.1 mRNA levels (Fig 2 panel A). In detail, *post hoc* analysis revealed a temporal induction of the IEG, which depends on the time of sacrifice after injection. MDPV up-regulates Arc/Arg3.1 mRNA levels 30 min (+47%, *p*=0.006321 versus saline-treated mice), 2 hours (+53%, *p*=0.000479 versus saline-treated mice; +80%, p<0.000001 versus α-PVP treated mice) but not 24 hours after the injection.

A single injection of α-PVP, instead, up-regulates Arc/Arg3.1 mRNA levels 30 min after drug exposure (+34%, p=0.045181 versus saline-treated mice), an effect that disappears within 2 hours (-26%, p=0.068828 versus saline-treated) and is still at control level 24 hours later (+9%, p=0.573214 versus saline-treated mice).

c-Fos mRNA levels:

Two-way ANOVA revealed a main effect of treatment ($F_{2,49}$=4.9566; p=0.0110), time after injection ($F_{2,49}$=11.035; p=0.0001) and a significant time x treatment interaction ($F_{4,49}$=4.8629; p=0.0022) (Fig 2 panel B). Similarly, mice receiving a single injection of MDPV showed increased c-Fos mRNA levels 30 min later (+79%, p=0.0002 versus saline-treated mice), an effect that persisted up to 2 hours (+33%, p=0,0432 versus saline-treated mice; +54%, p=0.0013 versus α-PVP treated mice) but waned after 24 hours (-13%, p=0.4990 versus saline-treated mice).

Similarly to Arc/Arg3.1, a single injection of α-PVP up-regulates c-Fos mRNA levels 30 min later (+55%, p=0.0066 versus saline-treated mice), an effect that disappears within 2 hours (-21%, p=0.1906 versus saline-treated mice) and remains unaltered 24 hours later (+5%, p=0.7847 versus saline-treated mice).

Striatum:

Panels C-D of Figure 2 show the effects of a single injection of MDPV and α-PVP (1 mg/kg) on Arc/Arg3.1 and c-Fos mRNA levels in the striatum of mice at three different time points, i.e. 30 minutes, 2 and 24 hours after the drug exposure.

Arc/Arg3.1 mRNA levels:

Two-way ANOVA revealed a main effect of treatment ($F_{2,48}$=9.687; p=0.0003), time after injection ($F_{2,48}$=4.0982; p=0.0227) and a significant time x treatment interaction ($F_{4,48}$=2.9451; p=0.0295) on Arc/Arg3.1 mRNA levels (Fig 2 panel C). In detail, multiple comparisons revealed that a single injection of MDPV increases Arc/Arg3.1 mRNA levels after 30 min (+81%, p=0.0036 versus saline-treated mice), an effect that persists 2 hours later (+99%, p<0.0001 versus saline-

treated mice; +96%, $p<0.0001$ versus α-PVP treated mice) but subsides after 24 hours (+0%, $p=0.9850$ versus saline-treated mice). Conversely, Arc/Arg3.1 mRNA levels were not altered by α-PVP injection at the three time points considered.

c-Fos mRNA levels:

Two-way ANOVA revealed a main effect of the treatment ($F_{2,47}=7.4041$; $p=0.0016$), time after injection ($F_{2,47}=10.713$; $p=0.0001$) and a significant time x treatment interaction ($F_{4,47}=3.6034$; $p=0.0121$) for c-Fos mRNA levels (Fig 2 panel D). Multiple comparisons revealed a marked increase of c-Fos mRNA levels 30 min after a single injection of MDPV (+179%, $p=0.0002$ versus saline-treated mice), which persists up to 2 hours after the injection (+87%, $p=0.0172$ versus saline-treated mice; +91%, $p=0.0120$ versus α-PVP treated mice) but disappears 24 hours later (+3%, $p=0.9353$ versus saline-treated mice). Conversely, a single injection of α-PVP up-regulates c-Fos mRNA levels 30 min later (+161%, $p=0.0007$ versus saline-treated mice), an effect that fades away within 2 hours (-5%, $p=0.8876$ versus saline-treated mice) and remains unchanged 24 hours later (+14%, $p=0.7404$ versus saline-treated mice).

Hippocampus:

Panels E-F of Figure 2 show the effects of a single injection of MDPV and α-PVP (1 mg/kg) on Arc/Arg3.1 and c-Fos mRNA levels in the hippocampus of mice at three different time points, i.e. 30 minutes, 2 and 24 hours after the drug exposure.

Arc/Arg3.1 mRNA levels:

Two-way ANOVA revealed a main effect of treatment ($F_{2,49}=6.4063$; $p=0.0034$) and a significant time x treatment interaction ($F_{4,49}=4.8483$; $p=0.0023$) for Arc/Arg3.1 mRNA levels (Fig 2 panel E). Interestingly, at variance from the MDPV-induced expression profile observed in the frontal lobe and striatum, in the hippocampus the effect of the single injection of MDPV is short-lasting since it markedly increases Arc/Arg3.1 mRNA levels 2 hours later (+111%, $p<0.0001$ versus saline-treated mice; +118%, p<0.0001 versus α-PVP treated mice) but not 30 min after the drug

exposure (+32%, p=0.2451 versus saline-treated mice) and 24 hours later (+2%, p=0.9449 versus saline-treated mice). Of note, no significant changes were observed for α-PVP at the three time points analyzed.

c-Fos mRNA levels:

Two-way ANOVA revealed a main effect of treatment ($F_{2,49}$=4.5432; p=0.0155) and a significant time x treatment interaction ($F_{4,49}$=2.7941; p=0.0362) for c-Fos mRNA levels (Fig 2 panel F). Multiple comparisons revealed a selective increase of c-Fos mRNA levels 30 min after MDPV injection (+72%, p=0.0490 versus saline-treated mice) that further increases after 2 hours (+106%, p=0.0007 versus saline-treated mice; +107%, p=0.0006 versus α-PVP treated mice) while disappearing 24 hours later (-14%, p=0.7010 versus saline-treated mice). No significant changes were observed for α-PVP at the three time points analyzed.

Discussion

To the best of our knowledge, this is the first study comparing the effects of two cathinones, i.e. MDPV and α-PVP, in CD-1 male mice following a single exposure. Our data show that these cathinones rapidly change the expression of IEGs, with a specific temporal and regional profile, and alter the locomotor response as well differently.

We here demonstrate that MDPV not only was found to be more effective than α-PVP in facilitating both spontaneous and stimulated locomotion (primarily drag and accelerod tests), but also it displayed a biphasic effect as opposed to the monophasically-stimulated locomotion induced by α-PVP in both tests. Such difference might depend, at least in part, on the fact that MDPV is more potent than α-PVP in blocking the DAT (Marusich et al. 2014). Accordingly, it could be assumed that the inhibition of motor activity, caused by high doses of the cathinone, may rely on the synaptic overflow of DA in the dorsal striatum that causes the appearance of stereotypies and bizarre behaviors (Aliane et al. 2009). In fact, both cathinones, at the highest dose, induced stereotypies and bizarre behaviors, with MDPV being more potent than α-PVP (data not shown).

MDPV showed also a greater psychomotor effect than α-PVP, in line with its higher potency in blocking the dopamine transporter (DAT): a molecular correlate of such difference might be represented by the more persisting increase of Arc/Arg3.1 and c-Fos elicited by MDPV in striatum, i.e. the brain region with the highest density of DAT (Gainetdinov et al. 1998), while the up-regulation promoted by α-PVP's wanes within 2 hours. The psychomotor effects are consistent with the mechanism of action of cathinones that, as other psychostimulants, primarily inhibit monoaminergic transporters thereby increasing extracellular levels of monoamines (Baumann et al. 2013; Glennon and Young 2016). In particular, the increased synaptic levels of dopamine could activate D1 dopamine receptors, leading to increased Arc/Arg3.1 and c-Fos mRNA levels, as previously demonstrated (Fumagalli et al. 2006; Yoshida et al. 1995). However, we cannot rule out the possibility that increased levels of other neurotransmitters, such as glutamate and serotonin, may

come into play and drive IEG expression. It can be argued that the increased expression of IEGs may sustain, at least partially, the observed motor activity, given that motor activity over time reflects the time-dependent expression profile of the IEGs analyzed. However, we cannot rule out the possibility that such a difference may reflect the fact that α-PVP is more rapidly degraded to inactive metabolites compared to MDPV.

The picture in the frontal lobe is similar to that observed in the striatum, suggesting that these cathinones modulate dopamine levels also in cortical dopaminergic terminals. Since dopamine-induced activation of cortical regions drive and sustain nervousness, irritability, aggressiveness and paranoia that approximate schizophrenia (Morton 1999; Seo et al. 2008), it is reasonable to speculate that the increased neuronal activity in the frontal lobe may sustain some of the aggressive and bizarre behaviors herein observed. Of note, MDPV has been recently shown to alter functional connectivity between cortical and subcortical regions (Colon-Perez et al. 2016), further corroborating a drug-induced loss of control over the "*top-down*" neuronal connection, which is essential for the control of impulsive behaviors (Dalley et al. 2011) and may contribute to explain the high addictive properties of these drugs. Although the different pharmacokinetic profile does not result in different reinforcing properties of the drugs (Aarde et al. 2013; Watterson and Olive 2014), we cannot rule out the possibility that the more persistent activation of neuronal activity promoted by MDPV may initiate and sustain neuroadaptive changes caused by repeated exposure to the drug: this possibility will be investigated in the future by employing chronic treatment regimens with these two cathinones.

Cathinone-induced profile of IEGs expression is quite different in hippocampus. In fact, in this brain region, MDPV significantly up-regulates Arc/Arg3.1 and c-Fos mRNA levels, whereas α-PVP is ineffective. Moreover, it is important to note that c-Fos is immediately activated while Arc/Arg3.1 induction is delayed. This difference, which reflects the different nature of these IEGs, may rely on the fact that this brain region is not the primary target region of MDPV but, instead, it may be involved in secondary drug-related neuroadaptive mechanisms, perhaps involving the

15

glutamatergic neurotransmission, taking place over time. Moreover, since exposure to MDPV induces neuronal hippocampal loss (Adam et al. 2014), the possibility exists that a single dose of MDPV, but not α-PVP, is sufficient to drive a transient increase in neuronal activity as an attempt of the system to counterbalance the initial stage of cytotoxicity reported for these drugs (Wojcieszak et al. 2016).

Taken together, these data indicate that a single exposure to MDPV or α-PVP promotes changes in baseline neuronal activity, an effect that could be used as an early molecular signature of altered brain homeostasis following psychostimulant administration: this may also allow to discriminate between cathinones with different chemical structures as well as different pharmacodynamic properties.

In conclusion, we provide clear-cut evidence showing that the cathinones MDPV and α-PVP induce changes in locomotor and neuronal activity following a precise temporal and regional profile. Such rapid molecular effects may set the stage for the behavioral response observed and, perhaps, may account for their toxicity, at least partially. Although our analysis is limited to the acute effects of these cathinones, our data show that these drugs can modulate brain activity and functioning. Further studies employing chronic treatment regimens will be performed to determine their long-term effects as well as the effect of abstinence.

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

Ethical approval: All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All procedures performed in the studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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Figure Captions

Fig. 1 Effect of the systemic administration of MDPV (0.01-20 mg/kg i.p.) and α-PVP (0.01-20 mg/kg i.p.) on the distance travelled (panel A, B), the drag test (panel D, E) and accelerod test (panel G, H) in mice. The comparison of the total average effect observed was also reported (panel C, F, I). Data are expressed in absolute values (m, for distance travelled) in the analysis of spontaneous locomotion (panel A, B) and as percentage of basal in drag (panel D, E) and accelerod tests (panel G, H) (see material and methods) and represent the mean ± SEM of -10 determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for the dose response curve of each compounds at different times (panel A, B, D, E, G, H), while the statistical analysis of the comparison of the total average effect of the compounds (panel C, F, I) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons *p<0.05, **p<0.01, ***p<0.001 versus saline-treated mice and °°°p<0.001 versus α-PVP at same dosage.

Fig. 2 Time-dependent changes in Arc/Arg3.1 and c-Fos mRNA levels in the frontal lobe (Panel A and B), striatum (panel C and D) and hippocampus (panel E and F) of mice following a single IP injection of MDPV (1 mg/kg) or α-PVP (1 mg/kg). Mice were sacrificed at three different time points, i.e. 30 minutes, 2 and 24 hours after the drug administration. Results are expressed as percentages relative to saline-treated group and presented as mean ± standard error of the mean (SEM). *p<0.05, **p<0.01, ***p<0.001 versus saline-treated mice; [##]p<0.01, [###]p<0.001 versus α-PVP-treated mice (two-way ANOVA followed by Fisher LSD test).

Figures

Fig.1
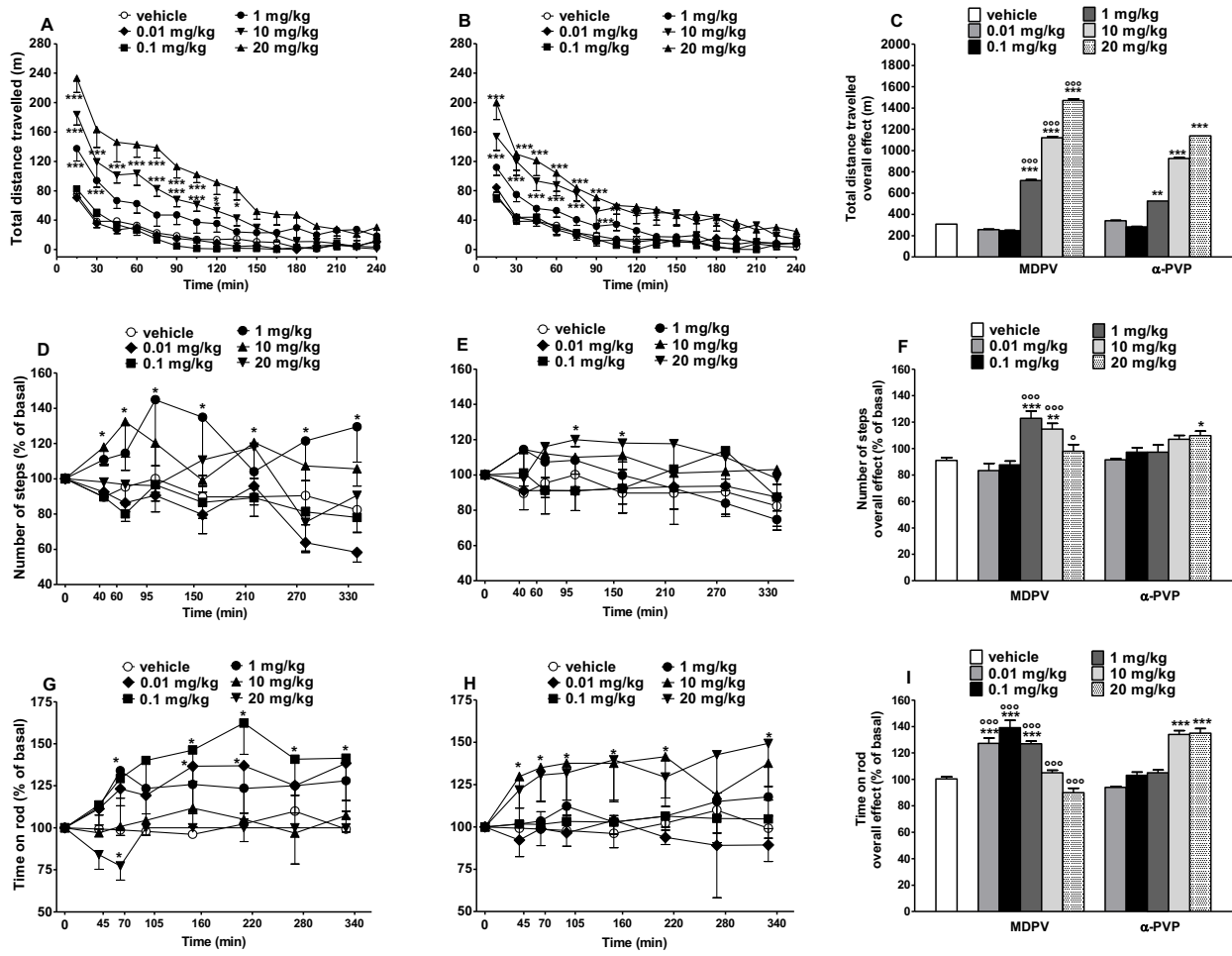
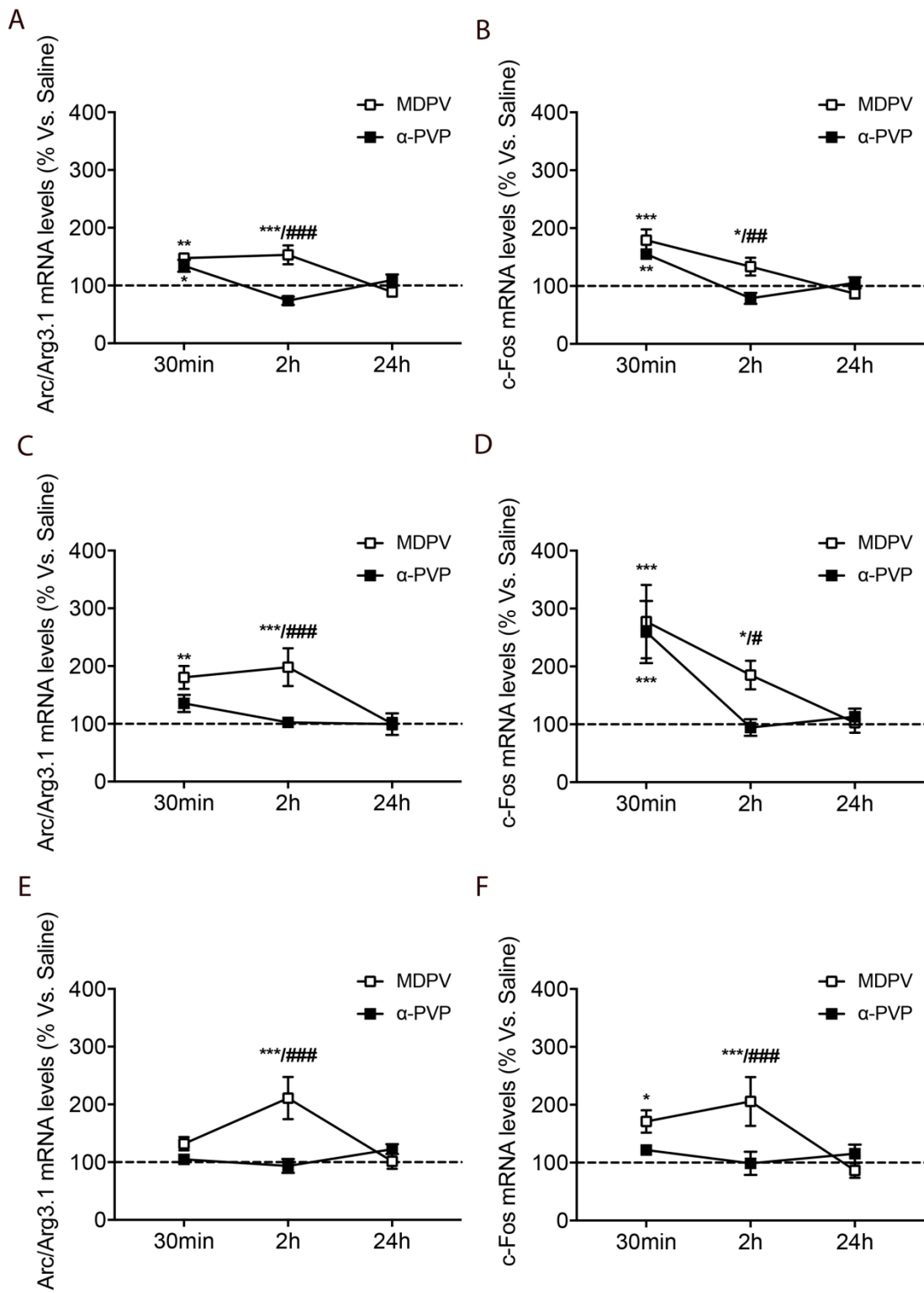**Fig. 2**