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Short communication

Induction of immediate early genes expression in the mouse striatum following acute administration of synthetic cathinones

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ABSTRACT

Background: Synthetic cathinones (SCs) form one of the most prominent group of the New Psychoactive Substances. SCs enhance central dopaminergic and noradrenergic neurotransmission, and are used as substitutes for illicit psychostimulants, namely cocaine, amphetamine, and methamphetamine. Changes in the expression of immediate early genes (IEGs) in the striatum underlie the addictive potential of drugs of abuse belonging to distinct pharmacologic groups. This work was aimed to assess the impact of acute administration of the prominent SCs on the mRNA levels of IEGs in the mouse striatum.
Methods: Effects of 3,4-MDPV, 2,3-MDPV, α-PVP, PV8, PV9, methcathinone (MC) and 3-fluoromethcathinone (3-FMC) on the mRNA levels of ten IEGs, one and two hours after exposure, were measured in the mouse striatum using the quantitative RT-PCR technique.
Results: All SCs used in the study produced increased mRNA levels of the following IEGs: *Areg, c-fos, Csrnp1, Dusp1, Dusp14, Egr2, Egr4* and *FosB*. Additionally, the majority of SCs increased the expression of *Homer1* and *c-jun*. The magnitude of observed changes varied by the drug, analyzed gene and, in many cases, by time after administration.
Conclusions: This study demonstrates that SCs increase the expression of IEGs in the mouse striatum, which may lead to a plethora of effects, as proteins encoded by the analyzed genes are involved in diverse actions, including an acute response to the drug and the neuroplasticity underlying the development of addiction.
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Introduction

The first decade of the 2000s witnessed an emergence and rapid development of the New Psychoactive Substances (NPS) market. In general, the term NPS refers to a wide group of compounds which mimic the effects of illegal drugs of abuse and are introduced into the market in order to circumvent legal regulations. Synthetic cathinones (SCs) are among the most prevalent groups of NPS [1]. They act mainly as dopaminergic and noradrenergic drugs *via* the inhibition of monoamine uptake or the induction of their release into the synaptic cleft. Some SCs are also able to stimulate serotonergic neurotransmission [2].

Owing to their mechanisms of action, SCs may produce differing effects. Cathinones selective for dopamine (DA) and noradrenaline (NA) neurotransmission exert psychostimulatory effects comparable to those of amphetamine, methamphetamine and cocaine, while more serotonergic drugs induce empathogenic effects, similar to those of MDMA [2]. By analogy to other psychostimulatory drugs, SCs are endowed with marked toxicity. Despite a relatively short presence on the recreational drug market, SCs have been associated with numerous acute intoxications, some of them fatal [3].

The social harm caused by drugs of abuse includes not only injuries and deaths resulting from acute intoxication, but also effects associated with the ability of drugs to induce long-term dependence. The addictive properties of old DA-ergic drugs of abuse, such as cocaine or methamphetamine, have been widely studied and well documented (e.g. [4,5]). Based on the similarity of pharmacological properties, it appears likely that SCs are endowed with abuse potential. The development of addiction is strongly related to the enhancement of DA-ergic transmission in the brain reward system, including the nucleus accumbens located within the ventral striatum [6]. It has been reported that various SCs evoke potent increases of extracellular DA levels in mouse and rat striata, and induce changes in behavior considered as addiction exponents: elevated spontaneous locomotor activity related to increased DA transmission in the striatum, drug self-administration

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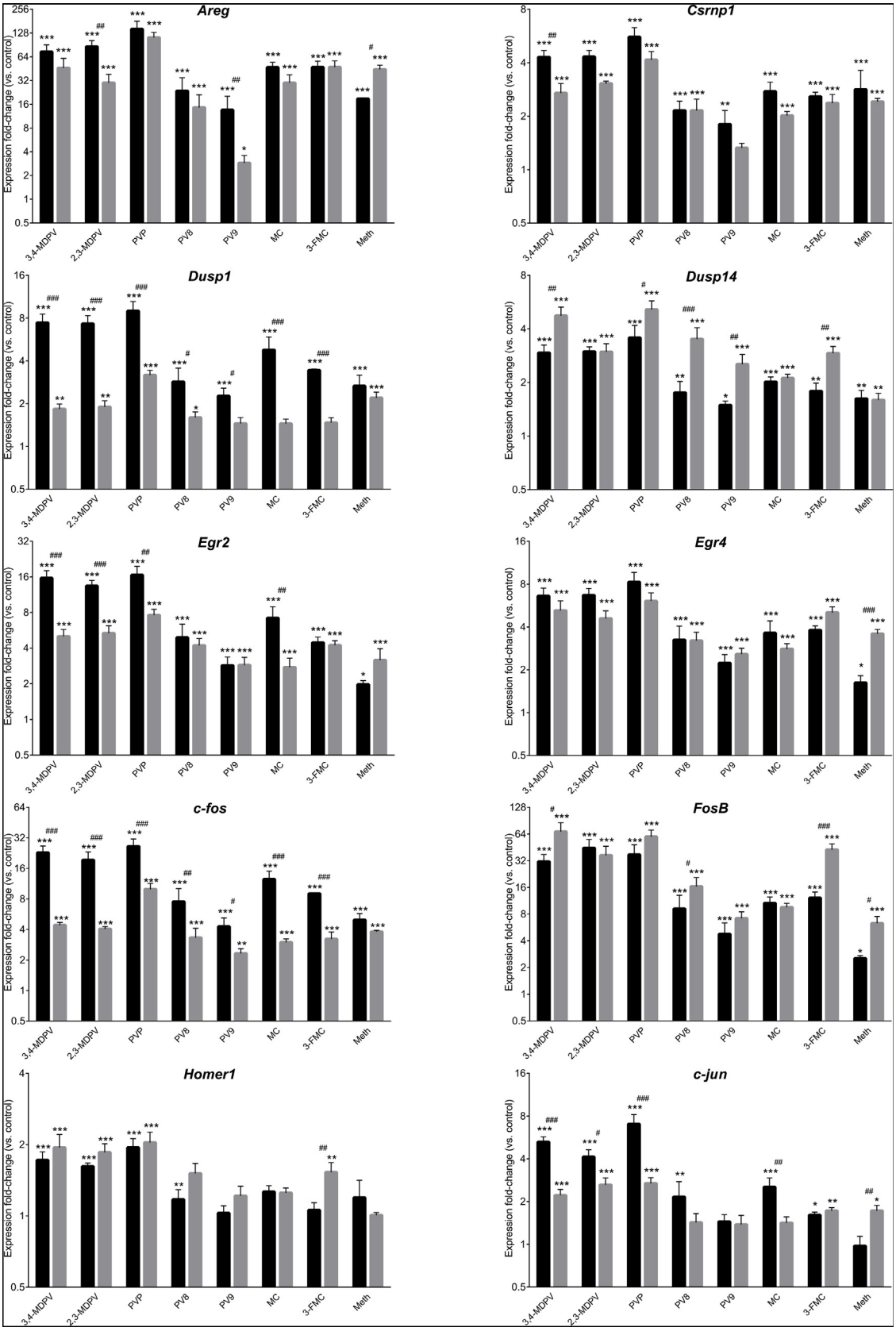

Fig. 1. Effects of SCs and methamphetamine on expression of IEGs, at the mRNA level, in the mouse striatum. Data represent fold changes in gene expression *vs*. the control group and are expressed as the mean ± SEM of four mice, each tested in duplicate. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ *vs.* control group. ### $p < 0.001$; ## $p < 0.01$; # $p < 0.05$ 1-hour exposure *vs.* 2-hour exposure.

Abbreviations used: 3,4-MDPV (3,4-methylenedioxypyrovalerone), 2,3-MDPV (2,3-methylenedioxypyrovalerone), α-PVP (α-pyrrolidinopentiophenone), PV8 (α-pyrrolidinoheptanophenone), PV9 (α-pyrrolidinooctanophenone), MC (methcathinone), 3-FMC (3-fluoromethcathinone), Meth (methamphetamine). Black bar – 1-h exposure; gray bar – 2-h exposure.

and discrimination from saline, conditioned place preference or lowered thresholds of intracranial self-stimulation that suggest the drug possesses rewarding/reinforcing properties [7–9].

Accumulating experimental evidence indicates that neuroadaptive processes in the brain circuitry, which are initiated by changes in the transcription of immediate early genes (IEGs), play an important role in the transition from the recreational to compulsive drug use. In general, IEGs code for transcription factors and effector proteins that are responsible for alterations in DA-ergic circuitry and signal transduction. The induction of IEGs expression has been demonstrated for several groups of drugs of abuse, e.g., DA-ergic drugs like cocaine, amphetamine and methamphetamine, opioids (morphine/heroin), alcohol, and nicotine [6,10–16].

Although several papers have analyzed the abuse potential of SCs using neurochemical and behavioral measures, little is known about the potential effects of these drugs on gene expression. Therefore, the aim of this work was to assess changes in the expression of IEGs associated with addiction as well as the acute effects experienced after a single dose of several prevalent SCs, with methamphetamine used as a reference compound. The present study focuses on IEGs whose expression was previously found to be elevated by an acute treatment with cocaine [14].

Materials and methods

Drugs and reagents

3,4-Methylenedioxypyrovalerone (3,4-MDPV), 2,3-methylenedioxypyrovalerone (2,3-MDPV), α-pyrrolidinopentiophenone (α-PVP), α-pyrrolidinoheptanophenone (PV8), α-pyrrolidinooctanophenone (PV9), methcathinone (MC), 3-fluoromethcathinone (3-FMC), and methamphetamine were purchased in the form of hydrochloride salts from Cayman Chemical (Ann Arbor, Michigan, USA). An isotonic solution of saline (0.9% NaCl) for injections was purchased from Polska Grupa Farmaceutyczna (Łódź, Poland). RNAlater® solution was purchased from Thermo Fisher Scientific (Warsaw, Poland).

Animals and treatment

All housing conditions and experimental procedures were performed in accordance with the European Union Directive 2010/63/UE and approved by the Local Ethical Commission for Experimentations on Animals (Łódź, Poland, permission number 76/ŁB/690/2013). Experiments were performed on male C57BL/6 J mice at approx. 9–12 weeks of age. The animals were housed in a sound-attenuated chamber, four per cage, with automatic 12 -h light/dark cycles (lights on at 06:00), free access to drinking water and standard food pellets. All procedures were performed during the light cycle (08:00 – 14:00).

All animals obtained one subcutaneous injection of an SC or methamphetamine solution in 0.9% NaCl, or a 0.9% NaCl injection for the control group (0.1 mL/10 g body mass) and were sacrificed one or two hours after injection. Immediately after sacrification, both striata were isolated from individual animals in anatomical borders, submerged in RNAlater® solution and frozen at −20 °C. A dose of each cathinone and methamphetamine was chosen based on its behavioral effects [7,8]: methamphetamine (3 mg/kg), 3,4-MDPV (3 mg/kg), 2,3-MDPV (10 mg/kg), α-PVP (10 mg/kg), MC (10 mg/kg), 3-FMC (10 mg/kg), PV8 (15 mg/kg), and PV9 (15 mg/kg).

Total RNA extraction and cDNA generation

Total RNA was extracted from tissue samples using RNeasy Mini Kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality of RNA samples, determined by measuring the absorption ratio at 260/280 nm, was 1.9 to 2.2. The purified total RNA was immediately used for cDNA synthesis or stored at −80 °C. For each sample, total RNA (1 μg) was subjected to reverse transcription (High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor; Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's specifications. The cDNA samples were kept frozen at −20 °C until used in the qPCR reaction.

Real-time PCR (qPCR)

Gene expression analysis was performed using standard TaqMan® Gene Expression Assays (Thermo Fisher Scientific Inc.): *Homer1* (Mm00516275_m1), *c-fos* (Mm00487425_m1), *FosB* (Mm00500401_m1), *Dusp1* (Mm00457274_g1), *Areg* (Mm00437583_m1), *Egr2* (Mm00456650_m1), *Egr4* (Mm00842279_g1), *c-jun* (Mm00495062_s1), *Csrnp1* (Mm00462723_m1). In addition, two assays were used as endogenous controls: *Actb* (Mm00607939_s1) and *Gapdh* (Mm99999915_g1). qPCR reactions were performed in 10 μL reaction volumes including 30 ng cDNA, 5 μL TaqMan Fast Advanced Master Mix and 0.5 μL TaqMan Gene Expression Assay (20x). Assays were performed on a 7900 H T Fast Real-Time PCR System (Thermo Fisher Scientific Inc.) in 96-well plates. Each sample was analyzed in duplicate (or in tetraplicate when the difference between Ct was greater than 0.5), together with standards and controls without templates. The following thermal cycling specifications were performed: 20s at 95°C and 40 cycles each for 3s at 95°C and 30s at 60°C. TaqMan PCR assays were performed on a 7900 H T Fast Real-Time PCR System (Thermo Fisher Scientific Inc.).

Data analysis

Statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad, San Diego, CA, USA). Gene expression was normalized using the mean of Ct values of both reference genes: *Actb* and *Gapdh*. For each gene, statistical significance was calculated using ΔΔCt input values by two-way ANOVA (treatment x time), followed by Fisher's LSD *post hoc* test. Each group consisted of four independent samples, each obtained from an individual animal. The results were considered statistically significant when $p < 0.05$. Fold change values were calculated for graphical presentation according to the $2^{-\Delta\Delta Ct}$ method [17].

Results

Fig. 1 presents the effects of the tested drugs on IEGs expression, at the mRNA level, in the mouse striatum.

Areg

Two-way ANOVA revealed there was a significant effect of treatment ($F_{8,54} = 51.57$; $p < 0.0001$), time ($F_{1,54} = 8.324$; $p = 0.0056$) and the treatment × time interaction ($F_{8,54} = 2.707$; $p = 0.0139$) on the expression of the *Areg* gene. All tested SCs and methamphetamine significantly increased *Areg* expression after one and two hours. Additionally, in the cases of 2,3-MDPV and PV9, the effect was stronger after one hour than after two hours, while methamphetamine produced a stronger effect after two hours. Of all the studied IEGs, *Areg* was associated with the highest fold changes, reaching more than a 143-fold increase one hour after treatment with α-PVP.

Csrnp1

Statistical analysis revealed a marked effect of treatment ($F_{8,54} = 27.61$; $p < 0.0001$) and time ($F_{1,54} = 11.66$; $p = 0.0012$) on the expression of the *Csrnp1* gene, while the treatment × time interaction ($F_{8,54} = 0.8928$; $p = 0.5290$) did not significantly affect its mRNA levels. All tested compounds significantly increased the expression of *Csrnp1* one hour after treatment. For all drugs, except for PV9, elevated expression of *Csrnp1* persisted for two hours. The expression of *Csrnp1* in animals treated with 3,4-MDPV was found to be significantly more pronounced after one hour than after two hours.

Dusp1

Expression of the *Dusp1* gene was found to be significantly affected by treatment ($F_{8,54} = 23.01$; $p < 0.0001$), time ($F_{1,54} = 128.5$; $p < 0.0001$) and the treatment × time interaction ($F_{8,54} = 6.294$; $p < 0.0001$). Significantly increased levels of mRNA were found for all tested substances after one hour and remained elevated after two hours in groups treated with 3,4-MDPV, 2,3-MDPV, α-PVP, PV8 and methamphetamine. Significant differences in *Dusp1* expression between the one-hour and two-hour time points were observed for all SCs, but not methamphetamine.

Dusp14

Two-way ANOVA revealed that *Dusp14* expression was significantly altered by treatment ($F_{8,54} = 26.18$; $p < 0.0001$), time ($F_{1,54} = 26.38$; $p = 0.0001$) and the treatment × time interaction ($F_{8,54} = 2.760$; $p = 0.0124$). All tested compounds caused a significant increase of *Dusp14* expression that persisted after one and two hours. A significant difference between the time points was observed in the case of 3,4-MDPV, α-PVP, PV8, PV9 and 3-FMC. In all cases, the induction of *Dusp14* expression was stronger after two hours.

Egr2

Expression of the *Egr2* gene was significantly affected by treatment ($F_{8,54} = 32.56$; $p < 0.0001$), time ($F_{1,54} = 18.37$; $p < 0.0001$) and the treatment × time interaction ($F_{8,54} = 4.315$; $p = 0.0005$). Elevated levels of *Egr2* mRNA after one and two hours were detected in animals treated with any of the tested substances, while differences between the two time points were found after treatment with 3,4-MDPV, 2,3-MDPV, α-PVP and MC, with significantly stronger effects observed after one hour.

Egr4

Statistical analysis revealed a significant effect of treatment ($F_{8,54} = 36.05$; $p < 0.0001$), and the treatment × time interaction ($F_{8,54} = 3.488$; $p = 0.0026$), but not time ($F_{1,54} = 0.07863$; $p < 0.7802$), on the expression of the *Egr4* gene. Similar to *Egr2*, the expression of *Egr4* was elevated at both time points in each group treated with SCs or methamphetamine. A significant difference between one and two hours was detected only in groups treated with methamphetamine.

c-fos

Expression of *c-fos* was significantly affected by treatment ($F_{8,54} = 38.03$; $p < 0.0001$), time ($F_{1,54} = 110.0$; $p < 0.0001$) and the treatment × time interaction ($F_{8,54} = 4.866$; $p = 0.0001$). All studied compounds caused elevation of *c-fos* mRNA levels, detectable after one and two hours. Expression of *c-fos* was stronger after one hour, compared to two hours post injection with SCs, but not methamphetamine.

FosB

Expression of *FosB* was significantly affected by treatment ($F_{8,54} = 51.12$; $p < 0.0001$) and time ($F_{1,54} = 16.36$; $p = 0.0002$), but not the treatment × time interaction ($F_{8,54} = 1.926$; $p = 0.0746$). All SCs and methamphetamine caused a significant elevation of *FosB* mRNA levels, detected one and two hours after treatment. Additionally, the induction of *FosB* expression evoked by 3,4-MDPV, PV8, 3-FMC and methamphetamine was found to be significantly higher after one hour than after two hours.

Homer1

Two-way ANOVA revealed that expression of *Homer1* was significantly affected by treatment ($F_{8,54} = 13.26$; $p < 0.0001$) and time ($F_{1,54} = 5.152$; $p = 0.0272$), but not the treatment × time interaction ($F_{8,54} = 1.214$; $p = 0.3085$). After treatment with either 3,4-MDPV, 2,3-MDPV or α-PVP, expression of *Homer1* was significantly elevated after one and two hours. Additionally, PV8 caused a significant increase in *Homer1* expression after one hour and 3-FMC after two hours. In the case of 3-FMC there was a significant difference in *Homer1* expression after one hour, compared to two hours.

c-jun

Statistical analysis revealed that expression of *c-jun* was significantly affected by treatment ($F_{8,54} = 24.62$; $p < 0.0001$), time ($F_{1,54} = 17.37$; $p < 0.0001$) and the treatment × time interaction ($F_{8,54} = 5.909$; $p < 0.0001$). 3,4-MDPV, 2,3-MDPV, α-PVP and 3-FMC caused a significant increase of *c-jun* expression after both one and two hours. In the case of 3,4-MDPV, 2,3-MDPV, α-PVP and MC expression was significantly more abundant after one hour than after two hours. Additionally, PV8 and MC caused an increase of *c-jun* expression only after one hour, while methamphetamine only resulted in an increased expression of *c-jun* after two hours. In the case of methamphetamine, the level of *c-jun* mRNA was significantly higher after two hours compared to one hour.

Discussion

Enhanced IEGs expression has been observed in response to various stimuli, including drugs of abuse from different pharmacologic groups, and is believed to be a substantial link between the external environment and long-term alterations in the cellular phenotype [6,10–16]. Among drugs of abuse, DA-ergic agents, such as methamphetamine and cocaine, have been demonstrated to evoke the highest induction of *c-fos* expression [14]. The drug-induced expression of IEGs is involved in a plethora of processes, including changes in cellular development and morphology, cellular growth and proliferation, cell-to-cell signaling, regulation of delayed expression of other genes that are responsible for acute behavioral changes and cell death, drug toxicity, homeostasis and reduction of the toxic effects associated with oxidative stress, as well as neuroplasticity and adaptive mechanisms that contribute to the development of tolerance, behavioral sensitization and addiction. It is believed that neuroadaptive changes in the brain circuitry related to the elevated expression of IEGs are crucial to the transition from casual to compulsive drug intake [6,10,13–15,18–20]. Therefore, it is important to examine the effects of drugs of abuse on the expression of IEGs in structures of the reward system as a part of the comprehensive assessment of addictive properties, complementary to behavioral and neurochemical studies.

IEGs, including genes involved in glutamatergic transmission and synaptic plasticity (*Homer1*) or genes encoding transcription factors that control the delayed expression of other genes, such as the *Fos*, *Jun* and *Egr* families, are used as markers of neuronal activity in the brain in response to various stimuli, including psychoactive substances [6,10,14,19,21].

The present study analyzed the effects of SCs on the expression of IEGs in the mouse striatum and found evidence for the up-regulation of genes known to be associated with an acute response to psychoactive drugs and development of a dependence on them. Genes belonging to the families *Fos*, *Egr* and *Jun* encode transcription factors that are responsible for the regulation of neurotransmission *via* genes coding for neurotransmitters (*Jun*) or promotion of memory formation and synaptic plasticity (*Egr*, *Fos*) [18]. *Dusp* genes are responsible for the synthesis of dual specificity protein phosphatases that act as major modulators of critical signaling pathways [14]. *Homer1* codes for a scaffold dendritic protein that regulates the function of cortico-striatal glutaminergic neurotransmission [19]. Up-regulation of the *Egr2* gene is believed to be associated with a loss of cognitive control over the use of the drug, resulting in its compulsive intake [19]. Altogether, the increased expression of these genes can be used as a marker of broad neuroplastic changes in the striatum, leading to altered neurotransmission and signal transduction and to the development of addiction. *Areg* is responsible for the synthesis of a protein that acts as an autocrine growth factor and mitogen for astrocytes. This gene also plays an important role in inflammation, immunity and tissue repair [22,23]. Thus, the up-regulation of *Areg* by SCs may indicate astroglial activation and ongoing neuroinflammation, or the homeostatic process directed at protecting brain neurons from drug-induced damage. *CSRNP1* codes for cysteine and serine rich nuclear protein 1, which is known to be a pro-apoptotic protein involved in Wnt signaling and is related to HIV-induced neurotoxicity; CSRNP1 acts as a tumor suppressing factor [24]. Thus, increased expression of *CSRNP1* may be considered a marker of SC-induced neurotoxicity.

It is hypothesized that increased expression of IEGs in the brain in response to drugs of abuse results from several processes occurring simultaneously. Among them, activation of the striatal D_1-DA receptors, leading to the elevation of neuronal cAMP levels, and the ongoing oxidative stress in this brain structure, are of crucial importance [11,13,15,16]. This theory is supported by numerous studies. For instance, a methamphetamine-dependent increase of *c-fos* expression in the rat striatum was completely blocked by pre-treatment with SCH23390, a D_1-DA receptor antagonist [6,18]. Furthermore, an increase of spontaneous locomotor activity in rodents in response to SCs was also blocked by SCH23390 [7–9]. It should be emphasized that of the three unsubstituted pyrovalerone SCs used in our study, namely α-PVP, PV8 and PV9, the first one produced the highest fold-increase in expression of the ten assessed IEGs, despite being used at a lower dose than the other two drugs. This observation is consistent with our previously published experimental data, where, compared to PV8 and PV9, α-PVP produced more pronounced stimulation of locomotor activity in mice and greater elevation of extracellular DA levels in the mouse striatum [7].

In conclusion, this study demonstrates that SCs increase expression of IEGs in the mouse striatum, the magnitude of which is dependent on the analyzed gene, drug tested and time after administration. Proteins encoded by the cluster of analyzed genes are involved in different processes, including neuroadaptation, development of addiction, neurotoxicity, and neuroprotection.

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