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RESEARCH ARTICLE

A New Look at an Old Drug: Cumulative Effects of Low Ribavirin Doses in Amphetamine-Sensitized Rats

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Abstract: ***Background*:** Psychotic states related to psychostimulant misuse in patients with hepatitis C virus infection may complicate acceptance and reaction to antiviral treatment. This observation equally applies to the widely used ribavirin therapy.

***Objective*:** We examined psychomotor and body weight gain responses to low ribavirin doses after cessation of intermittent amphetamine treatment in adult rats to assess its role in neurobehavioral outcome during psychostimulant withdrawal.

***Method*:** The model of amphetamine-induced (1.5 mg/kg/day, i.p., 7 consecutive days) motor sensitization and affected body weight gain was established in adult male Wistar rats. Then, additional cohort of amphetamine-sensitized rats was subjected to saline (0.9% NaCl; 1 mL/kg/day; i.p.) or ribavirin (10, 20 and 30 mg/kg/day, i.p.) treatment for 7 consecutive days. Animals' motor activity in a novel environment was monitored after the 1st and the 7th saline/ribavirin injection. Body weight gain was calculated as appropriate. Determination and quantification of ribavirin in the brain tissue were performed also.

***Results*:** The 1st application of ribavirin to amphetamine-sensitized rats affected/decreased their novelty-induced motor activity only at a dose of 30 mg/kg. After the 7th application, ribavirin 30 mg/kg/day still decreased, while 10 and 20 mg/kg/day increased novelty-induced motor activity. These behavioral effects coincided with the time required to reach maximum ribavirin concentration in the brain. Body weight gain during withdrawal was not influenced by any of the doses tested.

***Conclusion*:** Ribavirin displays central effects that in repeated treatment, depending on the applied dose, could significantly influence psychomotor response but not body weight gain during psychostimulant/amphetamine withdrawal.

Keywords: ribavirin, amphetamine, behavioral sensitization, motor activity, environmental novelty, body weight, rats.

1. INTRODUCTION

Drug addiction as a substance-use disorder is associated with severe health and social problems, and thus it deserves to be adequately treated. One of the leading health problems is a high rate of hepatitis C virus (HCV) infections in intravenous drug addicts [1]. Ribavirin is an integral component of HCV therapy [2], but its medication is quite often associated with pronounced psychiatric adverse effects and body weight loss [3-7].

It is well known that chronic amphetamine (AMPH) usage produces a long-lasting change in neural systems involved in psychomotor activity control. Specifically, the continuous administration of AMPH in high doses that produces and maintains its elevated brain concentrations for a few days produces a syndrome termed AMPH neurotoxicity, while the repeated discontinuous (intermittent) administration of AMPH by discrete daily injections of relatively low doses produces a phenomenon termed behavioral sensitization [8, 9]. Sensitization consists of the induction (or development) phase and the expression phase (behavioral responses performed after withdrawal following repeated treatment with the drug) [8, 9]. The induction phase of AMPH-induced psychomotor sensitization is associated with activation of the ventral tegmental area (VTA), and the expression phase is linked to activation of the nucleus accumbens [9]. Along with behavioral sensitization, AMPH and related drugs might inhibit feeding by reducing the motivation for food (anorexia) or by inducing responses that compete with the ability to locate, approach, and make contact with food [10]. AMPH treatment influences cortico-basal ganglia circuits that control both movement and appetitive motivation, making them sensitized to further drug action and less responsive to biologically meaningful stimuli [11, 12]. The fact that repeated exposure to psychostimulants induces alterations that progress from ventral to dorsal areas of the striatum (which likely mediates augmented stereotypy) and that capable of activating mesoaccumbens dopamine should also be able to promote stereotypy, are well-known for some time now [13, 14].

Ribavirin at low doses has already been proven to be effective in some animal models [15-20]. It seems that in conditions of disturbed central neurotransmission, the primary role of ribavirin could be to maintain a steady state and prevent functional and metabolic activation. Most previous studies have found that A1 agonists, including ribavirin [21], do not significantly affect the release of neurotransmitters or motor activity in basal conditions, but at the same time, they show an evident attenuating effect when these processes are stimulated [15, 16, 20, 22-24]. Also, the microinjection of A1

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agonist into the nucleus accumbens was sufficient to reverse the expression of cocaine sensitization in rats [25]. These effects could be realized through the activation of pre- and/or postsynaptic A1 receptors. Stimulation of presynaptic A1 receptors leads to a reduction of Ca^{2+} influx [26] and inhibition of presynaptic release of neurotransmitters, predominantly excitatory ones [27, 28], while stimulation of postsynaptic A1 receptors reduces the affinity of dopamine for D1 receptors [29-32].

Peculiarities in dose-related cumulative effects of ribavirin related to acute AMPH exposure have been accentuated in a previous study of our group [20]. However, those effects have never been deliberately examined in subjects/animals that passed repeated intermittent administration of psychostimulant/AMPH, as they already have an affected brain neurochemistry and behavior. This topic seems to be exciting as ribavirin has an affinity for adenosine A1 receptors [21] that antagonistically modulate the activity of the dopamine D1 receptors [29, 33, 34], which play a critical role in the development of behavioral sensitization to AMPH [35]. Thus, the current study addresses psychomotor and body weight gain responses to low ribavirin doses (10, 20 and 30 mg/kg/day, i.p.) on the 1st and 7th day of withdrawal from intermittent exposure to moderately low AMPH doses (1.5 mg/kg/day, i.p., 7 consecutive days) in adult rats. It is important to note that the difference in withdrawal times is an important point to consider since it is related to a time-dependent cascade of different neurochemical/molecular changes [36]. In our study, ribavirin treatment was present during the entire withdrawal period, permanently influencing neurochemical and molecular signaling related to it, so the obtained data should be viewed as highly specific for the model.

2. MATERIALS AND METHOD

2.1. Animals and Drugs

All experiments were performed on adult 2.5- to 3.5-month-old male Wistar rats. The animals were maintained in groups of 4-5 rats per cage under standard conditions (23±2°C, 60-70% relative humidity, 12 h light/dark intervals, food, and water provided *ad libitum*). All animal procedures were in compliance with Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade, Serbia.

D-amphetamine sulfate (AMPH) and ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide; Virazole) were acquired from ICN Pharmaceuticals (Costa Mesa, USA). The appropriate dose of each substance was dissolved in 1.0 mL of saline solution (0.9% NaCl) and injected once a day in a volume of 1 mL per kg of body weight intraperitoneally (i.p.). The daily doses of ribavirin in adult rats were 10, 20 and 30 mg/kg/day, and they corresponded to human daily doses of 1.6, 3.2, and 4.8 mg/kg/day, or 100, 200 and 300 mg/day, respectively (assumed for a 60 kg human). The daily dose of AMPH in adult rats was 1.5 mg/kg/day, and it corresponded to a human daily dose of 0.25 mg/kg/day, or 15 mg/day (assumed for a 60 kg human). Dose translation, which provides conversion of the animal dose to a human dose and *vice versa*, is based on body surface area [37].

2.2. Experimental Procedure

Two series of experiments were performed to establish a model of behavioral sensitization (Experiment 1) and to evaluate the effects of ribavirin on motor behavior in the novel environment and body weight gain of AMPH-sensitized rats (Experiment 2).

In Experiment 1 (schematic presentation is given in Fig. **1A**), a cohort of 12 adult male rats was used. It was divided into two groups (n = 6 per group) and i.p. injected once daily with saline (0.9% NaCl; 1 mL/kg/day; control group) or AMPH (1.5 mg/kg/day) for 7 consecutive days in home cages between 09.00 and 10.00 h (the induction or development phase of behavioral sensitization). After a 1-day withdrawal period (*i.e.*, on the 8th day of the experiment), both groups received a challenge injection of AMPH (1.5 mg/kg, i.p.) (the expression phase of behavioral sensitization). Immediately after that, the animals' behavior was monitored in the open field arena for 120 min. Such an experimental procedure enabled us to establish and validate a model of behavioral sensitization in rats.

In Experiment 2 (schematic presentation is given in Fig. **1B**), a cohort of 24 adult male rats was used. Animals were i.p. injected once daily with AMPH (1.5 mg/kg/day) for 7 consecutive days in the home cages between 09.00 and 10.00 h. After a 1-day withdrawal period (*i.e.*, on the 8th day of the experiment), the animals were divided into four groups and i.p. injected once daily with saline (0.9% NaCl; 1 mL/kg/day; control group, n = 6) or ribavirin at doses of 10, 20 and 30 mg/kg/day (n = 6 per dose) for 7 consecutive days. Animals' behavior was monitored immediately after saline/ribavirin injection on day 1 and day 7 of withdrawal (*i.e.*, on the 8th and 14th day of the experiment) in the open field arena for 120 min. Such an experimental procedure enabled us to evaluate the impact of single or repeated treatment with low doses of ribavirin in AMPH-sensitized rats.

In both experiments, behavioral testing was performed between 09.00 and 15.00 h in an isolated room under controlled conditions, and the body weight of animals was measured every day before the treatment. In Experiment 1, body weight gain was calculated by subtracting the weight of the rat measured at the beginning of the experiment from that measured on the day of behavioral testing (*i.e.*, on the 8th day of the experiment). In Experiment 2, body weight gain was calculated by subtracting the weight of the rat measured at the beginning of the experiment from that measured on day 1 of withdrawal (*i.e.*, on the 8th day of the experiment, to assess changes in body weight gain related to the sensitization period). Moreover, body weight gain was calculated by subtracting the weight of the rat measured on day 1 (*i.e.*, on the 8th day of the experiment) from that measured on day 7 of withdrawal (*i.e.*, on the 14th day of the experiment, to assess changes in body weight gain related to the withdrawal period).

2.3. Behavioral Monitoring

Animals' behavior was monitored in the open field by an automatic device Columbus Auto-Track System (Version 3.0 A, Columbus Institute, OH, USA) (described in detail by Janać *et al.* [16]). Each monitoring instrument (Opto-Varimex), consisted of a plexiglass cage (44.2 x 43.2 x 20 cm) intersected by horizontal and vertical infrared beams, was placed into the light- and sound-attenuated chamber with artificially regulated ventilation and illumination (100 lx), and connected to the Auto-Track interface. The type of activity was determined by a user-defined box size (set to 5 beams). The following parameters were considered: locomotor activity or locomotion (distance traveled in cm), stereotypy-like movements (such as sniffing, self-grooming, licking, and head waving), and vertical activity or rearing (lifting both forepaws off the floor). Locomotion was defined as a trespass of 5 consecutive infrared beams, stereotypy-like movements as the number of repeated breaks of the same beam, and vertical activity as the number of infrared beams that were broken by the rearing of the animal. The described parameters were defined by Auto-track system for IBM-PC/XT/AT version 3.0A (Instruction Manual 0113-005L, 1990). Each plexiglass cage was washed with fresh water and dried with disposable paper towels between consecutive recordings to eliminate any scent traces from previously used animals.

2.4. High-performance Liquid Chromatography Analysis

Determination and quantification of ribavirin in the brain tissue were performed in animals after i.p. injection of 125 mg/kg. Considering our previous work with tiazofurin [38], this dose was used

to achieve a detectable concentration of ribavirin in the brain. Animals were sacrificed 20, 40 and 60 min, as well as 24 h after ribavirin injection (n = 5 per time point), and their brains were dissected out and prepared for high-performance liquid chromatography (HPLC) analysis. Samples were homogenized in 0.15 M KCl (0.1 mL KCl per 1 g of tissue), then centrifuged at 40000 rpm for 25 min at 4°C. The supernatant was separated, and the proteins were denatured by boiling for 3 min. The supernatant was again centrifuged at 40000 rpm and filtered through a Sartorius filter (0.2 μm). Clear supernatant was used for HPLC analysis.

The analyses were performed on HPLC system Hewlett-Packard 1100 with the binary pump and diode-array detector (chromatographic conditions: column – Zorbax SB-C18, 4.6 x 250 mm, 5 μm; mobile phase – 0.5 mM KH_2PO_4, pH 6.5; temperature – 25°C; flow – 1 mL/min; injection volume – 100 μL; UV detector – 215 nm).

2.5. Statistical Analysis

The normality of data sets was estimated by the Kolmogorov-Smirnov test. The Kruskal-Wallis ANOVA followed by the Mann-Whitney U test, and the Friedman ANOVA followed by the Wilcoxon matched-pairs test were used to analyze between-group and within-group differences in behavior and body weight, respectively. When the two sets of data were compared, analyses were performed by the Mann-Whitney U, and Wilcoxon matched-pairs tests. The one-way ANOVA followed by the Fisher LSD test was used to analyze ribavirin concentration in the brain tissue measured at different time points after its i.p. administration. The results were considered significant when $p < 0.05$.

3. RESULTS

3.1. Behavioral Sensitization and Body Weight Gain due to Intermittent Exposure to Moderately Low Amphetamine Dose

The expression of behavioral sensitization to intermittent AMPH treatment (1.5 mg/kg, i.p., one injection per day for 7 consecutive days) was confirmed using a challenge injection of AMPH (AMPH/AMPH) after the 1-day withdrawal period (Figure 2). Compared to the control group, which was intermittently injected with saline before AMPH challenge (saline/AMPH), the AMPH/AMPH group showed a significant increase in the locomotor activity in the first 60 min of testing (Fig. **2A**; the first 30 min: $U = 2.0$, $p < 0.05$; the second 30 min: $U = 4.0$, $p < 0.05$), but not in stereotypy-like (Fig. **2B**) and vertical (Fig. **2C**) activity. Considering motor activity across time, an expected decrease was observed in both groups for all three parameters (results of the Friedman ANOVA and Wilcoxon test are given in Tables **1** and **2**, respectively).

Importantly, although the Friedman ANOVA revealed increase in body weight across time in both groups (Fig. **3A**; saline: $\chi^2_{(6,7)} = 24.70$, $p < 0.001$; AMPH: $\chi^2_{(6,7)} = 16.29$, $p < 0.05$), AMPH-sensitized group had significantly lower body weight gain compared to saline-injected group (Fig. **3B**; $U = 1.5$, $p < 0.01$), as assessed after the 1-day withdrawal period.

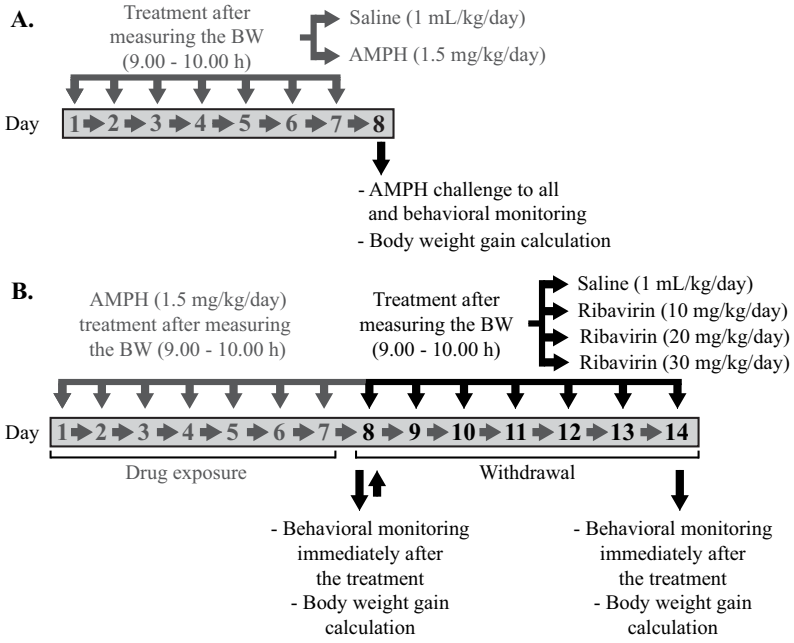

Fig. (1). Schematic presentation of the experimental procedure. **A** – Two groups of adult male Wistar rats (n = 6 per group) were injected with saline (0.9% NaCl; 1 mL/kg/day, i.p.; control group) or amphetamine (AMPH; 1.5 mg/kg/day, i.p.) for 7 consecutive days in the home cages. After a 1-day withdrawal period (*i.e.*, on the 8th day of the experiment), both groups received a challenge injection of AMPH (1.5 mg/kg, i.p.) and animals' behavior was monitored in the open field arena for 120 min. Body weight of the animals was measured every day before the treatment and body weight gain was calculated by subtracting the weight of the rat measured at the beginning of the experiment from that measured at the day of behavioral testing. Such an experimental procedure enabled to confirm the expression of behavioral sensitization and affected body weight gain in rats due to the previous drug experience.
B – A cohort of 24 adult male Wistar rats was injected once daily with AMPH (1.5 mg/kg/day, i.p.) for 7 consecutive days in the home cages. After a 1-day withdrawal period (*i.e.*, on the 8th day of the experiment), the animals were divided into four groups and i.p. injected once daily with saline (0.9% NaCl; 1 mL/kg/day; control group, n = 6) or ribavirin at doses of 10, 20 and 30 mg/kg/day (n = 6 per dose) for 7 consecutive days. Animals' behavior was monitored immediately after saline/ribavirin injection on day 1 and day 7 of withdrawal (*i.e.*, on the 8th and 14th day of the experiment) in the open field arena for 120 min. Body weight of the animals was measured every day before the treatment. Body weight gain was calculated by subtracting the weight of the rat measured at the beginning of the experiment from that measured on day 1 of withdrawal (*i.e.*, on the 8th day of the experiment). Also body weight gain was calculated by subtracting the weight of the rat measured on day 1 (*i.e.*, on the 8th day of the experiment) from that measured on day 7 of withdrawal (*i.e.*, on the 14th day of the experiment). Such an experimental procedure enabled to evaluate psychomotor and body weight gain response to ribavirin during withdrawal from intermittent AMPH treatment. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

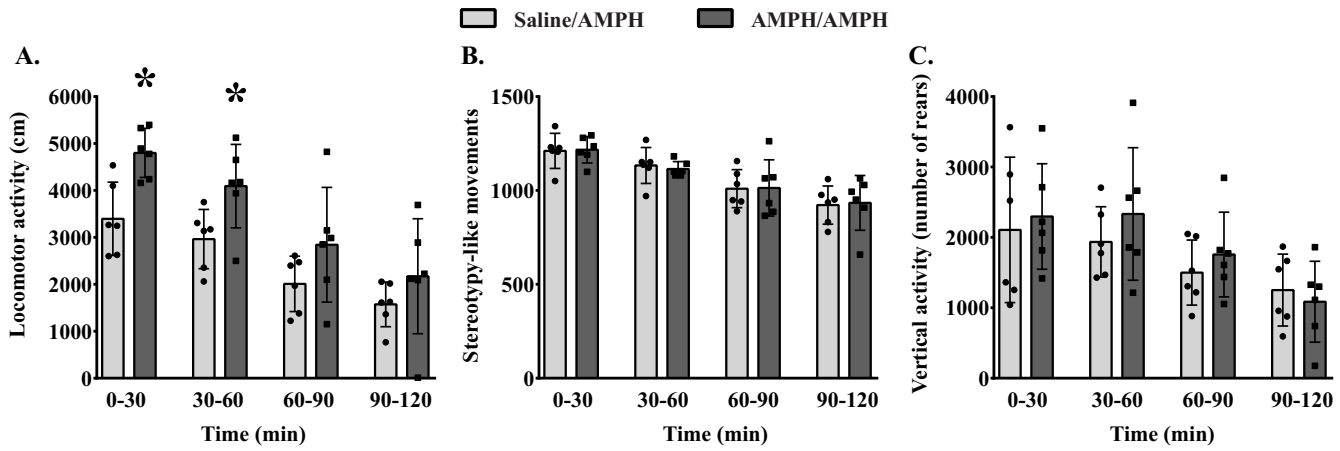

Fig. (2). The expression of behavioral sensitization to intermittent exposure to moderately low AMPH dose in adult male Wistar rats. Challenge injection of amphetamine (AMPH; 1.5 mg/kg, i.p.) was given to rats after the 1-day withdrawal from intermittent (7 consecutive days) treatment with saline (0.9% NaCl; 1 mL/kg/day, i.p.) or AMPH (1.5 mg/kg/day, i.p.). The animals' locomotor (**A**), stereotypy-like (**B**), and vertical (**C**) activity was monitored for 120 min and the data were further expressed as the total for four 30-min consecutive periods, which allowed the detection of fundamental behavioral differences during particular intervals, as well as an appropriate comparison between groups. *p< 0.05 *vs.* saline/AMPH group (Mann-Whitney U test). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Table 1. Results of Friedman ANOVA for given behavioral parameters of specified experimental groups across time (four consecutive periods, 30 min each).

-	LOCOMOTION		STEREOTYPY-LIKE MOVEMENTS		VERTICAL ACTIVITY	
-	$\chi^2_{(6,3)}$	p	$\chi^2_{(6,3)}$	p	$\chi^2_{(6,3)}$	p
Saline/AMPH	16.40	***	17.00	***	13.40	**
AMPH/AMPH	12.60	**	15.00	**	13.40	**
AMPH/Saline	-	-	-	-	-	-
1st day of withdrawal	13.20	**	13.20	**	13.20	**
7th day of withdrawal	11.95	**	12.97	**	12.20	**
AMPH/RBV 10	-	-	-	-	-	-
1st day of withdrawal	13.40	**	14.60	**	12.60	**
7th day of withdrawal	12.20	**	13.40	**	11.60	**
AMPH/RBV 20	-	-	-	-	-	-
1st day of withdrawal	12.60	**	13.20	**	12.20	**
7th day of withdrawal	14.80	**	14.60	**	14.80	**
AMPH/RBV 30	-	-	-	-	-	-
1st day of withdrawal	14.39	**	12.20	**	12.20	**
7th day of withdrawal	11.40	**	12.60	**	12.20	**

$^{**}p < 0.01$, $^{***}p < 0.001$; χ^2 (number of animals, degrees of freedom)

Abbreviations: AMPH - amphetamine; RBV 10 - ribavirin 10 mg/kg/day; RBV 20 - ribavirin 20 mg/kg/day; RBV 30 - ribavirin 30 mg/kg/day.

Table 2. Results of Wilcoxon test for given behavioral parameters of specified experimental groups across time.

-	LOCOMOTION		STEREOTYPY-LIKE MOVEMENTS				VERTICAL ACTIVITY	
-	1st day of with-drawal	7th day of with-drawal	1st day of with-drawal		7th day of with-drawal		1st day of with-drawal	7th day of with-drawal
Saline/AMPH								
30 *vs.* 60 min	*			*				
30 *vs.* 90 min	*			*				
30 *vs.* 120 min	*			*			*	
60 *vs.* 90 min	*			*			*	
60 *vs.* 120 min	*			*			*	
AMPH/AMPH								
30 *vs.* 60 min				*				
30 *vs.* 90 min	*			*			*	
30 *vs.* 120 min	*			*			*	
60 *vs.* 90 min								
60 *vs.* 120 min	*			*			*	
90 *vs.* 120 min							*	
AMPH/Saline								
30 *vs.* 60 min	*	*		*	*		*	*
30 *vs.* 90 min	*	*		*	*		*	*
30 *vs.* 120 min	*	*		*	*		*	*
60 *vs.* 90 min								
60 *vs.* 120 min	*							
AMPH/RBV 10								
30 *vs.* 60 min	*	*		*	*		*	*
30 *vs.* 90 min	*	*		*	*		*	*
30 *vs.* 120 min	*	*		*	*		*	*
60 *vs.* 90 min	*			*	*			
60 *vs.* 120 min				*				
AMPH/RBV 20								
30 *vs.* 60 min	*	*		*	*		*	*
30 *vs.* 90 min	*	*		*	*		*	*
30 *vs.* 120 min	*	*		*	*		*	*
60 *vs.* 90 min		*		*	*			*
AMPH/RBV 30								
30 *vs.* 60 min	*	*		*	*		*	*
30 *vs.* 90 min	*	*		*	*		*	*
30 *vs.* 120 min	*	*		*	*		*	*

*$p < 0.05$

Abbreviations: Abbreviations: AMPH - amphetamine; RBV 10 - ribavirin 10 mg/kg/day; RBV 20 - ribavirin 20 mg/kg/day; RBV 30 - ribavirin 30 mg/kg/day.

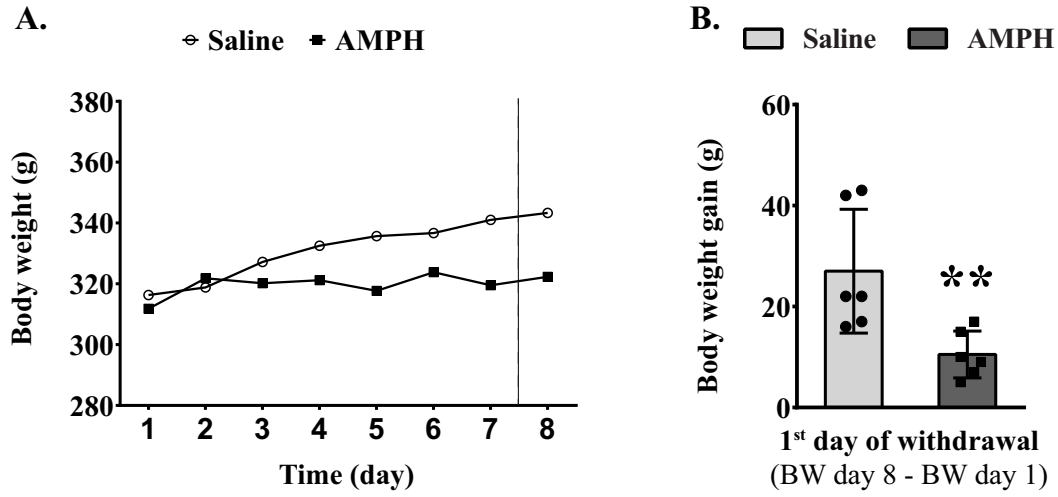

Fig. (3). Body weight gain due to intermittent exposure to moderately low AMPH dose in adult male Wistar rats. The animals were injected with saline (0.9% NaCl; 1 mL/kg/day, i.p.; control group) or amphetamine (AMPH; 1.5 mg/kg/day, i.p.) for 7 consecutive days in the home cages. Body weight (**A**) of the animals was measured every day before the treatment and after a 1-day withdrawal period (*i.e.*, on the 8th day of the experiment). (**B**) Body weight gain was calculated by subtracting the weight of the rat measured at the beginning of the experiment from that measured on the 8th day (the day of behavioral testing, *i.e.*, confirmation of behavioral sensitization). **$p< 0.01$ *vs.* saline-injected group (Mann-Whitney U test). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.2. The Effects of Ribavirin on Motor Behavior of Amphetamine-sensitized Rats - The Role of Dose and Treatment Duration

The influence of ribavirin injection after the 1-day withdrawal period to AMPH-sensitized rats is presented in Fig. **4**, left panel. The Kruskal-Wallis ANOVA revealed significant effects of ribavirin on motor activity of AMPH-sensitized rats during the second 30 min of testing (locomotion: $H_{(3,24)} = 12.13$, $p < 0.01$; stereotypy-like movements: $H_{(3,24)} = 14.57$, $p < 0.01$; vertical activity: $H_{(3,24)} = 14.24$, $p < 0.01$). For this period, the dose of 30 mg/kg/day decreased all three parameters of motor activity (Figs. **4A-C**; locomotion: $U = 2.0$, $p < 0.05$; stereotypy-like movements: $U = 5.0$, $p < 0.05$; vertical activity: $U = 0.5$, $p < 0.01$), while the other two lower tested doses (10 and 20 mg/kg, i.p.) were without effects.

The influence of repeated application of ribavirin to AMPH-sensitized rats during the 7-day withdrawal period is presented in Fig. **4**, right panel. The Kruskal-Wallis ANOVA revealed significant effects of ribavirin on motor activity of AMPH-sensitized rats during the first 30 min (locomotion: $H_{(3,24)} = 7.85$, $p < 0.05$) and second 30 min of testing (locomotion: $H_{(3,24)} = 10.64$, $p < 0.05$; stereotypy-like movements: $H_{(3,24)} = 11.89$, $p < 0.01$; vertical activity: $H_{(3,24)} = 12.91$, $p < 0.01$). While the dose of 30 mg/kg/day decreased locomotor activity during the first 30 min of testing ($U = 3.0$, $p < 0.05$) without influencing stereotypy-like and vertical activity, the other two lower tested doses (10 and 20 mg/kg/day) significantly increased all three parameters of motor activity specifically during the second 30 min of testing (Figs. **4D-F**; AMPH/RBV 10 – locomotion: $U = 2.0$, $p < 0.05$; stereotypy-like movements: $U = 2.0$, $p < 0.05$; vertical activity: $U = 0.0$, $p< 0.01$; AMPH/RBV 20 – locomotion: $U = 4.0$, $p < 0.05$; stereotypy-like movements:$U = 4.5$, $p < 0.05$; vertical activity: $U = 5.0$, $p < 0.01$).

As for motor activity across time, an expected decrease was observed in all groups for all three parameters (results of the Friedman ANOVA and Wilcoxon test are given in Tables **1** and **2**, respectively).

Comparison of motor activity on days 1 and 7 of withdrawal revealed a marked decrease of locomotion and stereotypy-like movements in AMPH/saline group on the 7th day during the second 30 min of testing ($p< 0.05$, Wilcoxon test). Importantly, no changes in AMPH/RBV groups were observed between motor activity parameters detected on day 1 and day 7 of withdrawal.

3.3. The Effects of Ribavirin on Body Weight of Amphetamine-sensitized Rats - The Role of Dose and Treatment Duration

In AMPH-sensitized rats, the Friedman ANOVA did not reveal relevant changes in body weight across time in all examined groups during the 7-day withdrawal period accompanied by ribavirin treatment (Fig. **5A**; experimental days 8 – 14; AMPH/Saline: $\chi^2_{(6,6)} = 9.24$, $p = 0.16$; AMPH/RBV 10: $\chi^2_{(6,6)} = 9.27$, $p = 0.16$; AMPH/RBV 20: $\chi^2_{(6,6)} = 9.05$, $p = 0.17$; AMPH/RBV 30: $\chi^2_{(6,6)} = 13.13$, $p = 0.04$). There were no significant changes in body weight gain of animals injected with ribavirin (AMPH/RBV groups) compared to saline-treated (AMPH/saline) rats during this period (Fig. **5B**; $H_{(3,24)} = 1.31$, $p = 0.73$), as assessed on day 7 of withdrawal.

3.4. The Concentration of Ribavirin in the Brain After i.p. Injection

Ribavirin concentration in the brain tissue after i.p. administration of 125 mg/kg changed across time ($F = 218.4$, $df = 3$, $p < 0.001$). As can be seen in Fig. (**6**), ribavirin was detected 20 min after administration, reached a peak after 60 min, and was still present in the brain tissue after 24 h. Significant differences were revealed by comparing any two ribavirin concentrations measured at different time points after its i.p. administration.

4. DISCUSSION

This study examined for the first time peculiarities in dose-related cumulative effects of ribavirin in the condition of already developed behavioral sensitization due to intermittent usage of psychostimulant AMPH. Obtained findings strongly suggest that in adult rats sensitized to AMPH, ribavirin displays central effects that depending on the applied dose, could result in either suppression or potentiation of motor/exploratory response to novelty. Importantly, body weight gain during the withdrawal period was not influenced by ribavirin treatment. In line with our previous findings [20], these data point to the critical role of the previous drug experience for the behavioral outcome during low-dose ribavirin therapy after cessation of intermittent AMPH usage.

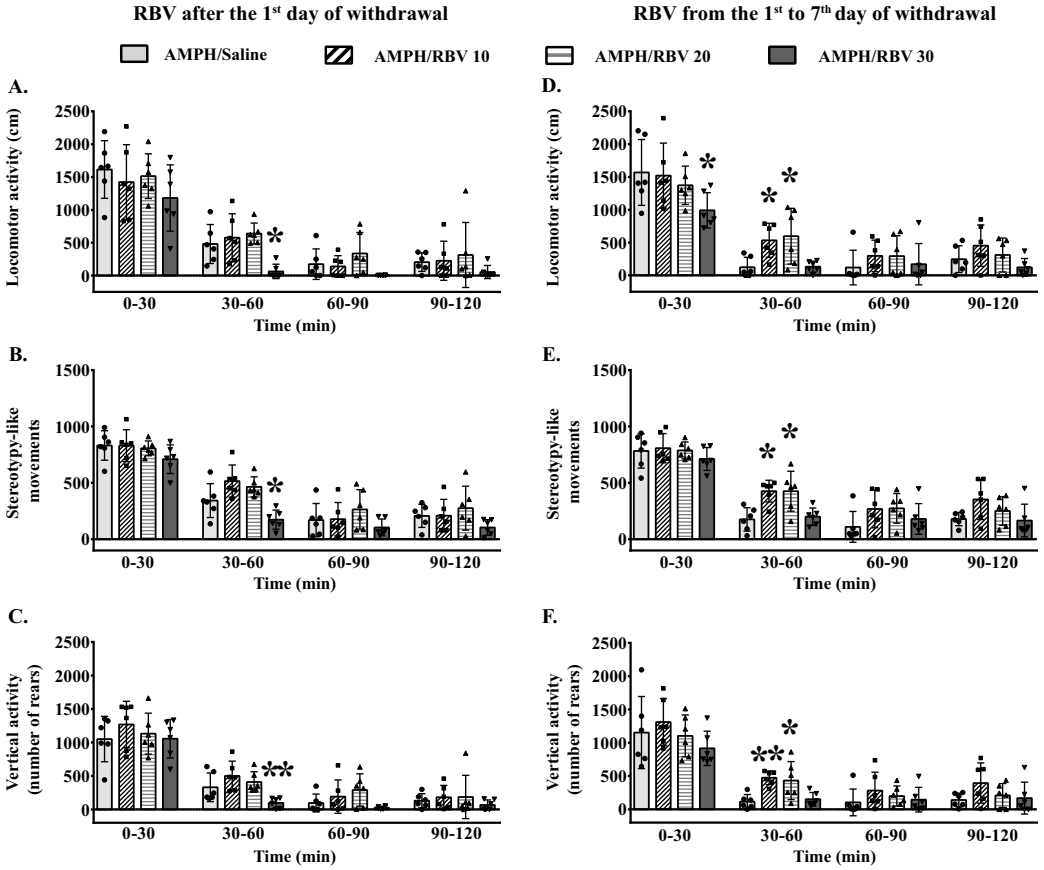

Fig. (4). The influence of ribavirin injection during withdrawal period on novelty-induced motor activity in AMPH-sensitized rats. Compared to the control, saline-injected group (AMPH/Saline), ribavirin (RBV) application to amphetamine (AMPH) sensitized rats on the 1st day of withdrawal (left panel) significantly decreased locomotor (**A**), stereotypy-like (**B**) and vertical (**C**) activity during the second 30 min of testing at a dose of 30 mg/kg/day (AMPH/RBV 30), while the other two tested doses of ribavirin (10 mg/kg/day, AMPH/RBV 10 and 20 mg/kg/day, AMPH/RBV 20) were without significant influence. After a seven-day application (from the 1st to 7th day of withdrawal; right panel) the dose of 30 mg/kg/day decreased locomotor activity (**D**) during the first 30 min of testing without influencing stereotypy-like (**E**) and vertical (**F**) activity. In contrast, the other two lower tested doses (10 and 20 mg/kg/day) significantly increased all three parameters of motor activity specifically during the second 30 min of testing. **p*< 0.05, ***p*< 0.01 *vs.* AMPH/Saline group (Mann-Whitney U test). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

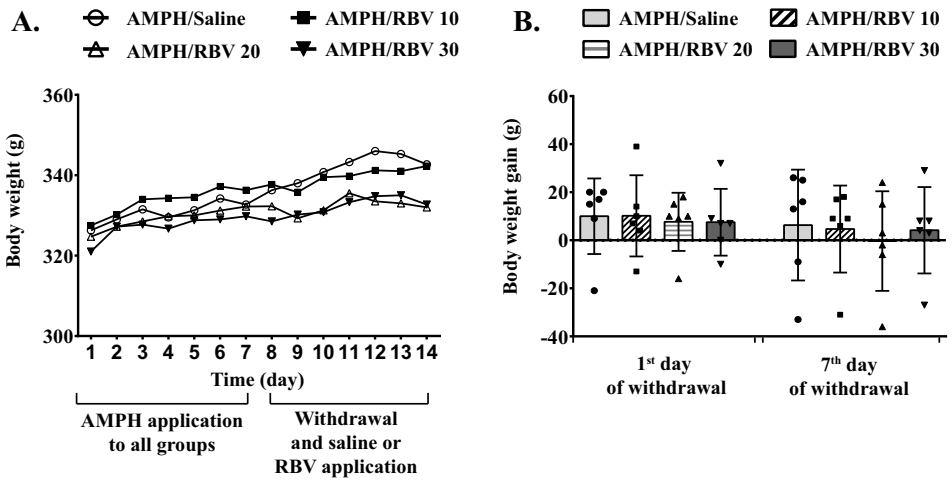

Fig. (5). The influence of ribavirin injection during withdrawal period on body weight in AMPH-sensitized rats. Panel **A** gives mean values of body weights of all experimental groups during induction of sensitization (AMPH application once daily to all animals), and withdrawal period that was accompanied by saline (AMPH/Saline, control) or ribavirin (RBV) i.p. application at a dose of 10 mg/kg/day (AMPH/RBV 10), 20 mg/kg/day (AMPH/RBV 20) or 30 mg/kg/day (AMPH/RBV 30). Panel **B** gives body weight gain calculated for the 1st and the 7th day of withdrawal. Please note no difference in body weight gain between four groups of AMPH-sensitized rats on the 1st day of withdrawal, prior to injections of saline or RBV. No significant changes in body weight gain between AMPH/Saline group and AMPH/RBV groups were detected on the 7th day of withdrawal. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

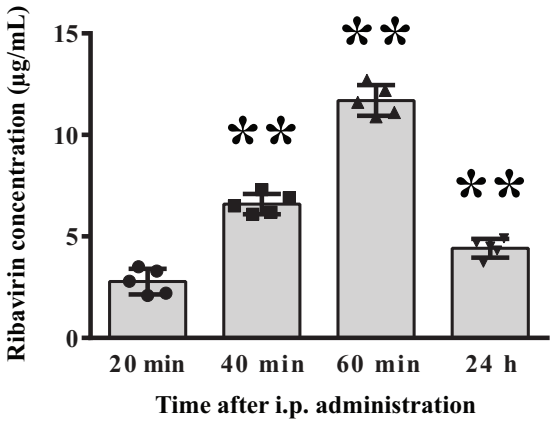

Fig. (6). Concentration of ribavirin in the brain after intraperitoneal injection. Ribavirin was i.p. applied in the dose of 125 mg/kg. $^{**}p < 0.01$ *vs.* concentration detected 20 min after i.p. administration (Fisher LSD test). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

It is known that the acute administration of AMPH produces a wide range of dose-dependent behavioral changes, including increased arousal or wakefulness, anorexia, hyperactivity, perseverative movements and, in particular, a state of pleasurable effect, elation and euphoria, which can lead to the abuse of the drug [39]. Prolonged use of AMPH can culminate in addiction (the loss of control over drug-taking) and psychosis with tolerance and sensitization, depending on the dose and the interval between the drug treatments (for details see [40]). In the current study, AMPH injection at a dose of 1.5 mg/kg/day administered for 7 consecutive days to adult rats led to the induction/development of behavioral/locomotor sensitization (expression tested after the 1-day withdrawal period to challenge injection of AMPH, 1.5 mg/kg). The same dose of the drug was used as a challenge considering evidence that sensitization should be more robustly expressed when the challenge dose is the same or lower than the inductive dose [8]. We did not detect large variations in response to AMPH challenge dose within the group that was repeatedly exposed to AMPH. This observation is in agreement with previous findings that regardless of the initial level of sensitivity to AMPH (which predicts high and low responders, especially in response to doses that are equal or lower than 1 mg/kg), the same levels of sensitization would develop at AMPH dose of 1.5 mg/kg i.p. [41]. Overall, this information could be of clinical importance as it helps to understand variations in both behavioral responses to low doses of AMPH ($\leq$ 0.25 mg/kg/day) and the incidence of behavioral sensitization after intermittent usage of low doses in humans [39, 42, 43].

Observed selective sensitization of locomotor, but not stereotypy-like and vertical activity suggests that in adult rats, the mesolimbic dopaminergic system is more responsive to repeated intermittent exposure to moderately low doses of AMPH (0.5 - 2 mg/kg; [44]) than the extrapyramidal dopaminergic system, which contributes to the altered behavioral profile seen after the challenge dose. Finally, our findings indicate that the potency of intermittent exposure to AMPH at a dose of 1.5 mg/kg/day to induce psychomotor sensitization is not dependent on the environmental novelty, as during the treatment, animals were in their home cages the whole time (it has previously been shown that environmental novelty facilitates robust sensitization to AMPH and that this phenomenon is independent of the effects of the novel environment on the acute response to these drugs, [45]).

In addition to locomotor sensitization, intermittent AMPH treatment decreased body weight gain in the drug-exposed compared to the control (saline-injected) group. These findings for the first time accentuate that AMPH interferes with weight gain in rats even when given about 10 h before the dark phase when most of the feeding behavior takes place. It is essential to underline this fact because earlier studies showed that AMPH-induced anorectic response appeared to be prominent at 2 - 5 h after drug injection [46], which is, considering the time of AMPH treatment in our model, still far from the active phase. Findings of our research also indicate that the prevention of weight gain in animals exposed to intermittent AMPH treatment is a phenomenon associated with diminished motivation for food and not with sensitized stereotypy-like activity, which as such could affect the feeding process [47]. We have to accentuate the fact that single housing of animals and direct measurement of food consumption per animal were not performed in our experiment, in order to avoid stress reaction and behavioral consequences of social isolation [48], as they could interfere with the parameters of interest. Water intake was not monitored for the same reason, although it has been reported that in rats, chronic exposure to AMPH produces a positive hydric balance by increasing water intake (patients suffering from AMPH-induced psychosis show a positive hydric balance as well) [49, 50]. Therefore, the findings of our study highlight body weight gain (and behavioral/locomotor sensitization) related to intermittent AMPH treatment in socially undisturbed, group-housed adult male rats.

The weight changes after the usage of AMPH depend on the dose used, as indicated in rodent studies [40, 51]. The appetite reduction and weight loss in response to continuous AMPH use present a health problem as well, which is observed even in clinically approved psychostimulant medication [52]. There is a question about whether the decreased food intake after AMPH represents a real loss of appetite or whether it is a consequence of facilitation of behaviors that are physically incompatible with feeding, but this debate could rather be related to the moderate and high doses (that produce psychomotor excitement; for review see [40]) than to low doses for which variations in palatability and nutritive content of offered food should be taken into account [51]. The facilitatory effects of AMPH on feeding without evidence of an increase in general activity in rats have been observed with doses less than 0.5 mg/kg. Using the dose of 0.25 mg/kg for the treatment of experimental rats in paradigms where animals have a choice of several foods, Evans and Vaccarino [51] showed that AMPH was most effective at increasing the intake of foods which contained carbohydrates, which implicated reward mechanisms in the expression of AMPH-induced feeding.

In AMPH-sensitized rats, the initial i.p. injection of ribavirin after the 1-day withdrawal period influenced the motor activity of the animals in a novel environment in a dose-dependent manner: the dose of 30 mg/kg decreased both exploratory (locomotion, vertical activity) and stereotypy-like activity specifically during the second 30 min of testing, while the other two lower doses tested (10 and 20 mg/kg) were without effects. As ribavirin has a moderate affinity for A1 receptors [21], the lower level of motor activity in rats injected with ribavirin before the placement into the novel environment could be due to a decrease in the VTA stimulation by novelty. Considering antagonistic interactions between adenosine A1 and dopamine D1 receptors and the abundant presence of A1 receptors in the basal ganglia [53], the potential influence of systemically administered ribavirin on accumbal/striatal tissue should not be excluded either. Importantly, observed time-related effect of ribavirin on novelty-provoked motor activity highly correlated with the time-dependent changes in its concentration in the brain after i.p. administration (the peak during the second 30 min after the treatment). Overall, these findings strongly suggest that, if existent, the action of ribavirin on novelty-provoked motor activity in AMPH-sensitized rats after the 1-day withdrawal period is largely related to the time course of ribavirin concentrations in the brain. Obtained results are in accordance with findings indicating the ability of A1 agonist injected into the nucleus accumbens to reverse the

expression of cocaine sensitization in rats [25]. Unlike Hobson *et al.* observation [25], the attenuating effect of ribavirin on the behavior of AMPH-sensitized rats in our experiment was achieved after its i.p. administration, which could be of even greater significance considering the practical aspects of pharmacotherapy.

One more interesting observation is the significant decrease in the intensity of locomotor and stereotypy-like responses to the novel environment in AMPH-sensitized group between days 1 and 7 of withdrawal (vertical activity was not significantly affected, indicating that this parameter of exploratory activity recovers differently from locomotor activity that is a representative of horizontal exploration). Importantly, repeated application of two lower doses of ribavirin (10 and 20 mg/kg/day, i.p) to AMPH-sensitized rats from days 1 to 7 of withdrawal prevented changes in novelty-induced motor activity (there was no significant difference between motor activity observed on days 1 and 7 of withdrawal), thus resulting in a significant increase of all three parameters of motor activity in the ribavirin-exposed compared to the control group on day 7 of withdrawal. Seven-day administration of the highest tested ribavirin dose (30 mg/kg/day, i.p.) facilitated earlier appearance (during the first 30 min) of the diminution of locomotor activity in the novel environment than that seen after the 1-day withdrawal period (differences were observed during the second 30 min of testing). To the best of our knowledge, these data for the first time accentuate cumulative and dose-related effects of low ribavirin doses on novelty-provoked behavior in AMPH-sensitized rats during withdrawal.

Intermittent AMPH injection that affected body weight gain in adult rats during the drug exposure period was not related to significant weight gain during the 7-day withdrawal period (actually, the extent of weight gain was highly similar to that registered for the drug exposure period). These findings indicate that used model/schedule of intermittent exposure to moderately low AMPH doses is not related to an increase in the rewarding effect of chow and consequent increase in food consumption, as in the used experimental conditions (unforced energetic utilization, socially undisturbed every-day living environment), body weight gain primarily reflected energetic intake. Results of this study contribute to the view of psychostimulant withdrawal-induced reductions in motivation for a natural reward, such as food (reviewed in [40]). By comparing the influence of 7-day exposure to ribavirin (10, 20 and 30 mg/kg/day; [20]) and AMPH (1.5 mg/kg/day, current study) on body weight gain in drug-naive animals, we obtained a surprising finding regarding highly similar potency of these two drugs to slow down/diminish body weight gain during the injection period, with the important notion of U-shaped dose-response relationship in ribavirin treatment [20]. Such experimental findings have not been addressed previously, though clinical findings accentuate the relationship between combined interferon (IFN)-ribavirin therapy and decreased appetite/weight loss in patients (*e.g.*, [5, 54]), viewing the observed phenomenon as IFN-related without questioning the contribution of ribavirin by itself. Underlying mechanisms of ribavirin-related changes in body weight gain remain to be elucidated, as contrary to AMPH whose anorexigenic effects are well known (discussed above), the influence of pure ribavirin on body weight changes is poorly understood and rarely reported. Our current findings accentuate the complexity of this issue, as in rats sensitized to AMPH, there were no additional changes in weight gain due to the administration of low ribavirin doses during the early withdrawal period, contrary to what was seen in drug-naive animals [20]. Moreover, novel findings indicate that ribavirin carried by selenium nanoparticles has greater therapeutic efficacy and fewer side effects in terms of reduced body weight and appetite [55].

Certain limitations of the study should be mentioned. It is well known that experimental research should be performed using an optimal number of animals, *i.e.*, avoiding both too small sample sizes (as it can miss the real effect) and too large sample sizes (questioning ethical principles of reduction of animal use). In the designs where no previous findings are available, multiple endpoints are measured, and testing hypotheses is not the main objective, the usage of "resource equation" method is suggested [56]. Furthermore, in biomedical research practice, scientists quite often reach for non-parametric tests mainly if there exist departures from normality, and if the data being examined is based on a small population sample or does not have a clear Gaussian function [57, 58]. Being aware of the fact that an increase in statistical power is generally achieved by larger sample sizes and considering suggestions that "researchers should describe not only the data analyses that produced statistically significant results but all statistical tests because this way of statistical analysis presentation lends weight and visibility to longstanding concerns over undue reliance on the p-value" [59], we performed an extensive statistical analysis and reported it in detail. Thus, the observed effects can be used to design a larger study with greater power.

CONCLUSION

Prolonged usage of moderately low AMPH doses and the incidence of behavioral sensitization after intermittent usage in humans have been recognized as a current research priority, along with intravenous drug practice that brings the risk for viral infections led by HCV. Already present neurochemical disturbances induced by prolonged psychostimulant usage may further complicate reaction to standard antiviral therapy (an integral component of which is ribavirin) that by itself has side effects including depression, anxiety, and body weight loss. Using a rodent model, our study showed that intermittent exposure to a moderately low AMPH dose (1.5 mg/kg/day; corresponds to the dose of 0.25 mg/kg/day in the adult human) subtly influences neural circuits that control appetitive motivation making them sensitized to further drug action and less responsive to biologically meaningful stimuli such as chow. Application of ribavirin during withdrawal from intermittent AMPH usage significantly influences novelty-provoked behavior, in relation to the dose and duration of ribavirin treatment, with the outcome ranging from suppression to potentiation, but without affecting expected body weight gain. Current findings accentuate that the use of ribavirin/antiviral therapy during withdrawal from intermittent AMPH/psychostimulant drug exposure could influence physiological and affective withdrawal responses, additionally complicating everyday life of medicated subjects.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All animal procedures were in compliance with Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade, Serbia.

HUMAN AND ANIMAL RIGHTS

No human subjects were used in the study. The reported experiments on animals were in accordance with the Standards set forth in the 8th Edition of Guide for the Care and Use of Laboratory Animals (http://grants.nih.gov/grants/olaw/Guide-for-thecare-and-use-of-laboratory-animals.pdf) published by the National Academy of Sciences, The National Academies Press, Washington DC, United States of America.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data that supports the findings of this study is available within the article.

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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