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Influence of calcium channel antagonists on nonsomatic signs of nicotine and D-amphetamine withdrawal in mice

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ABSTRACT

Background: Nonsomatic signs of psychostimulant withdrawal, difficult to demonstrate in animal paradigms, may appear to promote drug seeking and drug relapse in humans; thus, it is important to understand the mechanisms that mediate this kind of behaviors. The present study was undertaken to examine the calcium-dependent mechanism of negative nonsomatic and anhedonia-related symptoms of acute and protracted withdrawal of nicotine and D-amphetamine.

Methods: Mice were chronically treated with nicotine (seven days, three times daily, 3.35 mg/kg, *sc*) or D-amphetamine (14 days, once daily, 2.5 mg/kg, *ip*). Then, at the first, seventh or 14th day of withdrawal, anxiety- or depression-related effects, as well as cognition or nociception were studied.

Results: Our results demonstrated that, at the seventh or 14th day of D-amphetamine or nicotine withdrawal, respectively, mice exhibited increased anxiety and depression-like effects, memory impairment and hyperalgesia. Further, major findings showed that calcium channel antagonists, i.e., nimodipine, verapamil and flunarizine (10 and 20 mg/kg, *ip*), injected before the test, attenuated above-mentioned signs of drug withdrawal.

Conclusions: As an outcome, these findings support the hypothesis that similar calcium-dependent mechanisms are involved in an aversive nonsomatic component, associated with nicotine or D-amphetamine withdrawal. We can suggest that calcium channel blockers have potential to alleviate drug withdrawal and may thus be beneficial as pharmacotherapy of drug cessation and relapse.

Introduction

Nicotine, a natural alkaloid present in tobacco, is largely responsible for the acquisition and maintenance of tobacco addiction which prompts continued tobacco use despite the addicted subject's awareness about harmful consequences for his/her health. Nicotine dependence affects millions of individuals all over the world. The main obstacle in the treatment of nicotine addiction is the strong withdrawal syndrome, experienced after cessation of repeated exposure to nicotine [14].

In general, nicotine withdrawal syndrome is a sum of somatic and affective (motivational) symptoms, observed within a few hours after discontinuation of nicotine intake [26,58,66]. These symptoms reflect an imbalanced brain functionality, resulting from nicotine absence. The most common withdrawal symptoms in humans include nicotine craving, irritability, sleep disturbance, anxiety, problems with concentration, depressed mood and increased appetite [34,58,63]. Although many cigarette smokers report an intention to quit smoking, few are successful. Therefore, there is a great need of effective pharmacotherapy to aid smokers who really desire to quit their smoking habit. Similarly as humans, rodents manifest both somatic and affective signs of nicotine withdrawal [8,27,34,38,62]. In rodents, after a sudden discontinuation of nicotine treatment or administration of nicotinic cholinergic receptor (nAChR) antagonists (i.e., mecamylamine), somatic signs of physical dependence can be observed, including teeth chattering, chewing, gasps, ptosis, tremors, shaking, grooming and yawns [8,39,42] which become repetitive and long-lasting.

Basically, drug withdrawal syndrome is a common problem among all psychostimulant addicts. For example, approximately 87% of amphetamine users report withdrawal signs. For this reason, understanding of the mechanisms that underlie psychostimulant drug withdrawal, as well as the behavioral alterations

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that occur during this state, is relevant, particularly because the intense craving, related to this process, may be a critical factor leading to drug relapse.

Thus, amphetamine withdrawal has been much less studied than nicotine, ethanol and morphine withdrawal. In humans, amphetamine can increase arousal, reduce fatigue and appetite and induce hyperactivity, mood elevation as well as euphoria after an acute administration [18,21]. Amphetamine withdrawal effects, however, have been reported to peak within a few days, with the most characteristic signs, including anhedonia with depressive state [18]. The rather small number of studies on psychostimulant withdrawal, along with the absence of clear-cut physical withdrawal symptoms, makes it difficult to demonstrate, as in case of other drugs of abuse, especially in animal models. Therefore, there has been growing concern over the relevance of valid and reliable animal paradigms that express the behavioral consequences of this kind of withdrawal.

Concerning the principal mechanisms of action of both mentioned drugs, it is widely accepted that systemic nicotine administration stimulates nAChRs in the ventral tegmental area (VTA), consequently enhancing the firing of VTA dopamine neurons and the release of dopamine to targets, including the nucleus accumbens (NAC). When neuronal nAChRs are activated by nicotine, several other neurotransmitters are released, i.e., dopamine, noradrenaline, serotonin (5-HT), and γ-amino butyric acid (GABA), activating multiple neuronal systems which may control nicotine addiction [15,63,64]. In turn, the acute pharmacological effects of amphetamine are to increase central monoamine neurotransmission by influencing the processes of release, re-uptake and metabolism, leading to increased extracellular levels of dopamine, 5-HT and noradrenaline [60,61].

Negative affect and increased sensitivity to stress during psychostimulant abstinence appear to promote drug seeking and drug relapse [2,35,36]. This study was aimed to evaluate the effects of withdrawal from both nicotine and D-amphetamine on four different tasks in mice in order to identify animal models of nonsomatic manifestation of acute or protracted withdrawal, like heightened anxiety and depression-like states, as well as memory impairment and hyperalgesia. This kind of comparative study has not yet been fully described but it seems to be necessary to examine the nicotine and D-amphetamine withdrawal underlying neurobiology. Then, as changes in calcium channel function are considered to play an essential role in drug tolerance and dependence development, we investigated the effect of pretreatment with nimodipine, verapamil and flunarizine, three representative L-type voltage-dependent calcium channel (VDCC) antagonists (CCAs) with high lipophility, on the expression of above-mentioned affective withdrawal syndromes in mice, chronically pretreated with nicotine or D-amphetamine. The range of doses and timing was selected, taking into account our previous studies of behavioral effects of nicotine, D-amphetamine and CCAs on rodents [3–8,11]. Accordingly, when administered at the specified doses the drugs did not affect locomotor activity measured either in the elevated plus maze (EPM) test or in the actimeter cages and open field test. Moreover, hypotension and a resultant decrease in cerebral blood flow could be implicated in the behaviors of rodents, however, CCAs, e.g., nimodipine have cerebrovasodilatory and neuronal effects at doses that have little effect on systemic circulation [24,33].

Uncovering the calcium-dependent molecular and receptor-mediated mechanisms, which underly the complex withdrawal-related states, would support the search for an effective, drug addiction eliminating medication, as well as to better understand the issues of long-lasting drug seeking behavior and relapse.

Materials and methods

Animals

The experiments were conducted using adult naive Swiss mice (Farm of Laboratory Animals, Warszawa, Poland) weighing 20–25 g at the beginning of the experiments. The animals were group-housed and maintained under standard laboratory conditions (12-h light/dark cycle, room temperature 21 ± 1 °C, humidity 55 ± 5%) with free access to tap water and laboratory chow (Bacutil, Motycz, Poland) in their home cages, and were adapted to the laboratory conditions for at least one week. Each experimental group consisted of 8–12 animals. Additionally, all efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data. All behavioral experiments were conducted according to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and to the European Community Council Directive for the Care and Use of Laboratory Animals of 24 November 1986 (86/609/EEC), and approved by the local ethics committee (license Nº 47/2010).

Drugs

The compounds tested were: (−)nicotine hydrogen tartrate (3.35 mg/kg; Sigma–Aldrich, St. Louis, MO, USA); nimodipine (10 and 20 mg/kg; RBI, Natick, MA, USA), flunarizine dihydrochloride (10 and 20 mg/kg; Sigma–Aldrich, St. Louis, MO, USA), verapamil (10 and 20 mg/kg; Knoll, Germany), and D-amphetamine sulphate (2.5 mg/kg; Sigma–Aldrich, St. Louis, MO, USA). The drugs were dissolved in saline (0.9% NaCl). Nimodipine was dissolved in one drop of Tween 80 and diluted in 0.9% NaCl. Other drugs were dissolved in saline solution and refer to the salt forms. The pH of the nicotine solution was adjusted to 7.0. Fresh drug solutions were prepared on each day of experimentation. All agents were administered intraperitoneally (*ip*) or subcutaneously (*sc*) at a volume of 1 ml/100 g. Control groups received vehicle injections at the same volume and by the same route.

Apparatus

Anxiety-related behavior

Anxiety responses were measured in the EPM test. The procedure was chosen based on our recently published data [5,7] and it was similar to the method of Lister [40]. The experimental apparatus is shaped like a "plus" sign and consists of a central platform (5 cm × 5 cm), two open arms (30 cm × 5 cm) and two equal-sized closed (30 cm × 5 cm × 15 cm) arms opposite to each other. The maze is made of dark Plexiglas, elevated to a height of 50 cm above the floor and illuminated by a dim light. The test consisted of placing a mouse in the central platform facing an enclosed arm and allowing it to freely explore the maze for 5 min. The entry into one arm was defined as the stage when the animal placed all its four paws past the line dividing the central square from the open arms. The test arena was wiped with a damp cloth after each trial. The number of entries into the open and enclosed arms and also the time spent in open arms were measured by an observer blind to drug treatment. Anxiolytic activity was indicated by an increase in time spent in the open arms or in the number of open arms entries; anxiogenic effects were characterized by a decrease in those measures. The percentage of time spent on the open arms was calculated, just as was the percentage number of open arm entries. Additionally, the number of entries into the enclosed arms was recorded as the indicator of motor activity of tested animals.

Memory-related behavior

Memory and learning responses were measured using the modified elevated plus maze (mEPM) test. The commonly used

procedure to measure cognitive effects, was selected following our recently published data [6,7]. The experimental apparatus was similar to that described above. Recently, this test, originally developed to estimate anxiety in rodents [40], was modified to evaluate spatial learning and memory. In both cases the parameters measured are different, i.e., the number of entries into the open and closed arms and the time spent in open arms for anxiety, or the transfer latency (TL) on the second trial, i.e., the time which the mice took to move from the open arm to either of the enclosed arms for the memory processes.

In the mEPM test, the mice were placed individually at the end of one open arm, facing away from the central platform. Each group was submitted to the same procedure twice (interval between trials of 24 h). In the first (acquisition) trial, the time during which each mouse moved from the open arm to either of the enclosed arms was recorded (TL1). If the mice failed to enter the enclosed arm within 90 s, they were placed at an enclosed arm and permitted to explore the plus maze for additional 60 s. In such a case, TL1 was recorded as 90 s. In the subsequent (retention) trial, 24 h later, the test was performed in the same manner as the first one, and the TL was recorded (TL2). If the mouse did not enter the enclosed arm within 90 s, the test was stopped and TL2 was recorded as 90 s. Any animal that fell off the maze was excluded from the experiment.

In the mEPM test, we used the TL2 values on the retention trial as the index of memory and learning effects. An improvement in memory was characterized by reduced time which a mouse needed to move from the open arm to either of the enclosed arms on the second day (TL2), *vs.* the control group. Impairments in memory and learning were characterized by increases values of above-mentioned time.

Nociceptive responses

The hot plate test was performed as described previously [19]. The heated surface of the plate was kept at a constant temperature of 52 °C and the latency of pain response (paw licking, jump) was measured by a blind observer. The cut off time of 30 s was used to prevent tissue damage.

Depression-like behavior

Immobility was evaluated in the forced swimming (FST) paradigm (Porsolt test) [53]. In this test, the mice were placed individually in a glass beaker (height 25 cm, diameter 10 cm) filled with 25 ± 1 °C water to a depth of 12 cm. The total duration of immobility (that is, the time during which the mice make only minimal movements to stay afloat without any attempts to escape and show only slow movements to keep its head above the water) was recorded during the last 4 min of the 6 min testing period, after 2 min of habituation.

Experimental procedure

Nicotine was injected *sc* for seven days, three times daily (8.00, 14.00, 20.00) at the dose of 3.35 mg/kg, accordingly to Suzuki et al. [59] and Biala and Weglinska [8]. Amphetamine was injected *ip* for 14 days, once daily at the dose of 2.5 mg/kg (according to our pilot study). To determine whether withdrawal-related behavior measured using the paradigms described above emerges immediately upon nicotine or D-amphetamine abstinence or during protracted cessation, mice were allocated to one, seven or 14 days withdrawal groups. The 24 h period represents the shortest withdrawal period that can be easily distinguished. An appropriate, control group was injected with vehicle by the same route. To determine whether antagonism of L-type VDCCs could reverse increased anxiety- or depression-like behavior as well as memory disturbance and increased nociception exhibited by nicotine or D-amphetamine-withdrawal, mice were injected with CCAs, i.e., nimodipine, flunarizine and verapamil (10 and 20 mg/kg, *ip*) or saline 15 min before behavioral testing at 14 days or seven days following the last nicotine or D-amphetamine injection, respectively.

Statistical analysis

The data are expressed as the means ± standard error of the mean (SEM). The statistical analysis was performed using two-way or one-way analysis of variance (ANOVA). Post hoc comparison of means was carried out with the Tukey's test for multiple comparisons, when appropriate. The confidence limit of $p < 0.05$ was considered statistically significant. All statistical tests were performed using GraphPad Prism version 5.01 for Windows (GraphPad Software, USA).

Results

Anxiety-related behavior after D-amphetamine withdrawal in mice

D-Amphetamine was administered for 14 days, once daily at the dose of 2.5 mg/kg. Anxiety-related behavior was measured one, seven and 14 days after its withdrawal. Two-way ANOVA revealed that there was significant effect of the percentage of time spent on the open arms [day effect: $F(2,52) = 7.372$, $p = 0.0015$; treatment effect: $F(1,52) = 7.372$, $p < 0.0001$; interactions: $F(2,52) = 6.746$, $p = 0.0025$] (Fig. 1A) and the percentage of open arm entries [treatment effect: $F(1,52) = 14.81$, $p = 0.0003$; without day effect: $F(2,52) = 0.9145$, $p = 0,4071$ nor interactions: $F(2,52) = 0.1593$, $p = 0.8532$] (Fig. 1B). The post hoc analysis showed that after the first and the seventh day of withdrawal, significant decrease in the percentage of time spent on the open arms was observed ($p < 0.01$ and $p < 0.001$) (Fig. 1A) indicating an anxiogenic effect as

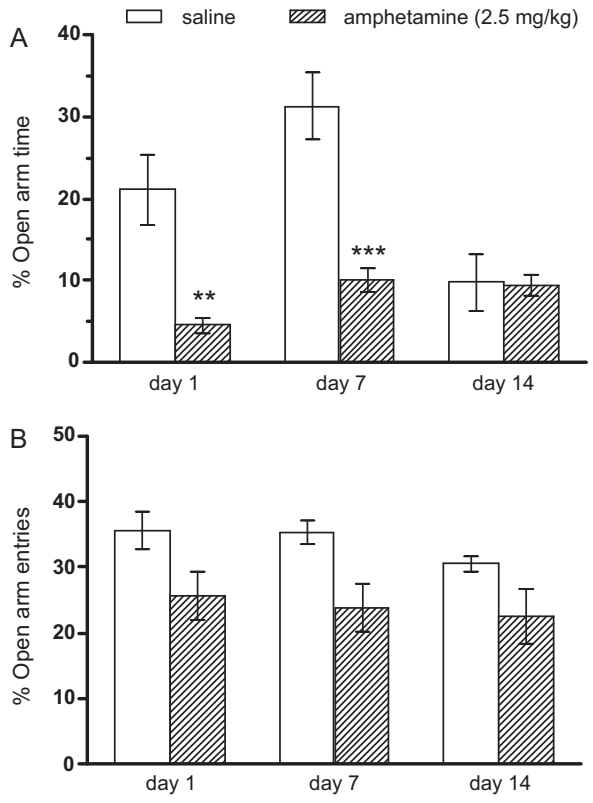

Fig. 1. Mean (±SEM) percentage of time spent on the open arms (A) and percentage of open arm entries (B) in the EPM, on the first, seventh and 14th day of D-amphetamine withdrawal in mice; $n = 8$–12. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. saline control group, Tukey's test.

Table 1
Mean (±SEM) number of enclosed arms entries in the EPM test in mice on the seventh day of D-amphetamine (or saline) withdrawal; $n = 8–10$.

Chronic administration (days 1–14)	Acute administration (7th day of withdrawal – the test day)	The number of enclosed arm entries
Saline	Saline	9.90 ± 1.29
Amphetamine (2.5 mg/kg)	Saline	9.62 ± 0.40
	Verapamil (10 mg/kg)	9.67 ± 0.79
	Verapamil (20 mg/kg)	7.15 ± 0.73
	Nimodipine (10 mg/kg)	9.47 ± 1.03
	Nimodipine (20 mg/kg)	7.62 ± 0.51
	Flunarizine (10 mg/kg)	11.46 ± 0.66
	Flunarizine (20 mg/kg)	9.71 ± 0.73

compared with saline-treated mice. Moreover, D-amphetamine withdrawal did not provoke any changes in number of enclosed arm entries in the EPM test (Table 1) causing no changing in locomotor activity of animals.

Influence of CCAs on anxiety-related behavior during D-amphetamine withdrawal in the EPM in mice

As shown in Fig. 2, after seven days of D-amphetamine withdrawal, when anxiogenic effect was the most significant, administration of verapamil, nimodipine or flunarizine (10 and 20 mg/kg), 15 min before the test, diminished these anxiogenic reactions. In D-amphetamine-pretreated mice, one-way ANOVA revealed that there was significant treatment effect of the percentage of time spent on the open arms [$F(6,94) = 27.45$, $p < 0.0001$] (Fig. 2A) and the percentage of open arm entries [$F(6,94) = 16.04$, $p < 0.0001$] (Fig. 2B). The post hoc analysis showed that all CCAs used significantly increased the percentage of time spent on the open arms ($p < 0.001$) (Fig. 2A) as well as the percentage of open arm entries at the doses of 10 mg/kg ($p < 0.001$) and 20 mg/kg ($p < 0.05$ for flunarizine and nimodipine; $p < 0.001$ for verapamil) as compared with D-amphetamine-pretreated and saline-tested mice (Fig. 2B).

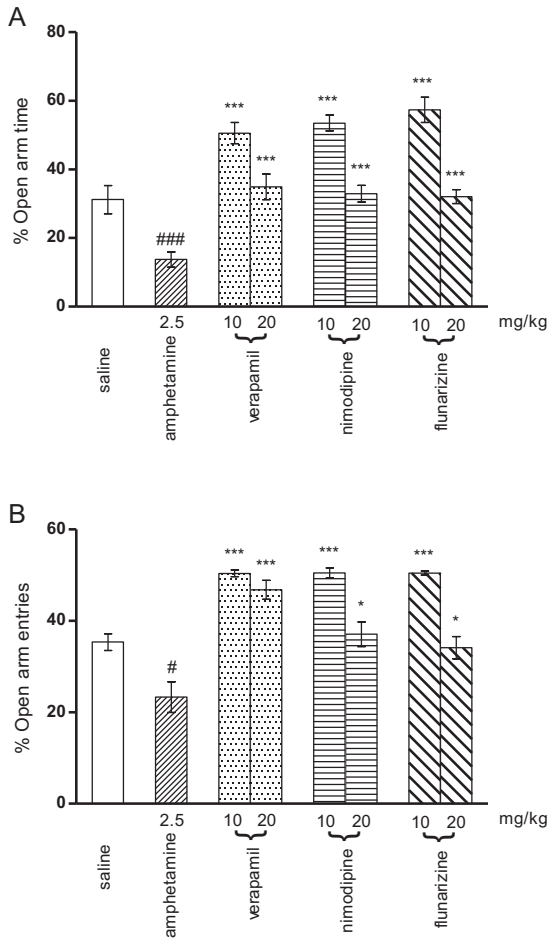

Fig. 2. Influence of calcium channel antagonists on percentage of time spent on the open arms (A) and percentage of open arm entries (B) after seven days of D-amphetamine withdrawal in the EPM in mice. Verapamil (10 and 20 mg/kg, *ip*), nimodipine (10 and 20 mg/kg, *ip*), flunarizine (10 and 20 mg/kg, *ip*), or saline were administered 15 min prior to test; $n = 8–12$. $^{*}p < 0.05$; $^{***}p < 0.001$ vs. amphetamine-pretreated and saline-tested group; $^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$ vs. saline-pretreated and saline-tested control group, Tukey's test.

Anxiety-related behavior after nicotine withdrawal in mice

Nicotine was administered for seven days, three times daily at the dose of 3.35 mg/kg. Anxiety-related behavior was measured one, seven and 14 days after its withdrawal. Two-way ANOVA analysis revealed that there was significant effect of the percentage of time spent on the open arms [treatment effect: $F(1,47) = 7.373$, $p = 0.0092$; without day effect: $F(2,47) = 1.94$, $p = 0.1550$ nor interactions: $F(2,47) = 2.118$, $p = 0.1316$] (Fig. 3A) and the percentage of open arm entries [treatment effect: $F(1,64) = 12.85$, $p = 0.0007$; day effect: $F(2,64) = 8.715$, $p = 0.0004$; interactions: $F(2,64) = 19.63$, $p < 0.0001$] (Fig. 3B). The *post hoc* analysis showed that after the 14th day of withdrawal, significant decrease in time spent on the open arms ($p < 0.05$) as well as in percentage of open arm entries was observed ($p < 0.001$) indicating an anxiogenic effect as compared with saline-pretreated mice. Moreover, nicotine withdrawal did not provoke any changes in number of enclosed

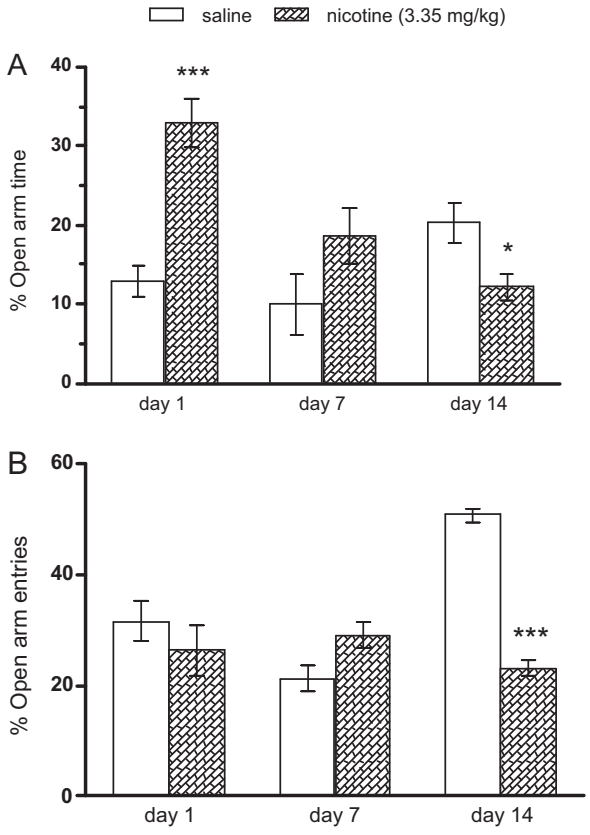

Fig. 3. Mean (±SEM) percentage of time spent on the open arms (A) and percentage of open arm entries (B) in the EPM, on the first, seventh and 14th day of nicotine withdrawal in mice; $n = 8–12$. $^{*}p < 0.05$ and $^{***}p < 0.001$ vs. saline control group, Tukey's test.

Table 2
Mean (±SEM) number of enclosed arms entries in the EPM test in mice on the 14th day of nicotine (or saline) withdrawal; $n = 8–10$.

Chronic administration (days 1–7)	Acute administration (14th day of withdrawal – the test day)	The number of enclosed arm entries
Saline	Saline	8.42 ± 0.65
Nicotine (3.35 mg/kg)	Saline	8.67 ± 0.53
	Verapamil (10 mg/kg)	7.00 ± 0.71
	Verapamil (20 mg/kg)	8.58 ± 0.77
	Nimodipine (10 mg/kg)	6.15 ± 0.46
	Nimodipine (20 mg/kg)	8.07 ± 0.46
	Flunarizine (10 mg/kg)	6.62 ± 0.55
	Flunarizine (20 mg/kg)	8.57 ± 1.03

arm entries in the EPM test (Table 2) causing no changing in locomotor activity of animals.

Influence of CCAs on anxiety-related behavior during nicotine withdrawal in the EPM in mice

As shown in Fig. 4, after 14 days of nicotine withdrawal, when anxiogenic effect was the most significant, administration of verapamil, nimodipine or flunarizine (10 and 20 mg/kg), 15 min before the test, diminished these anxiogenic reactions. In nicotine-pretreated mice, one-way ANOVA analysis revealed that there was significant treatment effect of the percentage of time spent on the open arms [F (6,91) = 7.419, $p < 0.0001$] (Fig. 4A) and the percentage of open arm entries [F (6,91) = 14.66, $p < 0.0001$] (Fig. 4B). The *post hoc* analysis showed that all CCAs used significantly increased the percentage of time spent on the open arms ($p < 0.05$ for verapamil 20 mg/kg; $p < 0.01$ for nimodipine and flunarizine 20 mg/kg; $p < 0.001$ for flunarizine 10 mg/kg) (Fig. 4A) as well as the percentage of open arm entries at the doses of 20 mg/kg ($p < 0.001$) and 10 mg/kg ($p < 0.05$ for nimodipine and $p < 0.01$ for flunarizine) as compared with nicotine-pretreated and saline-tested mice (Fig. 4B).

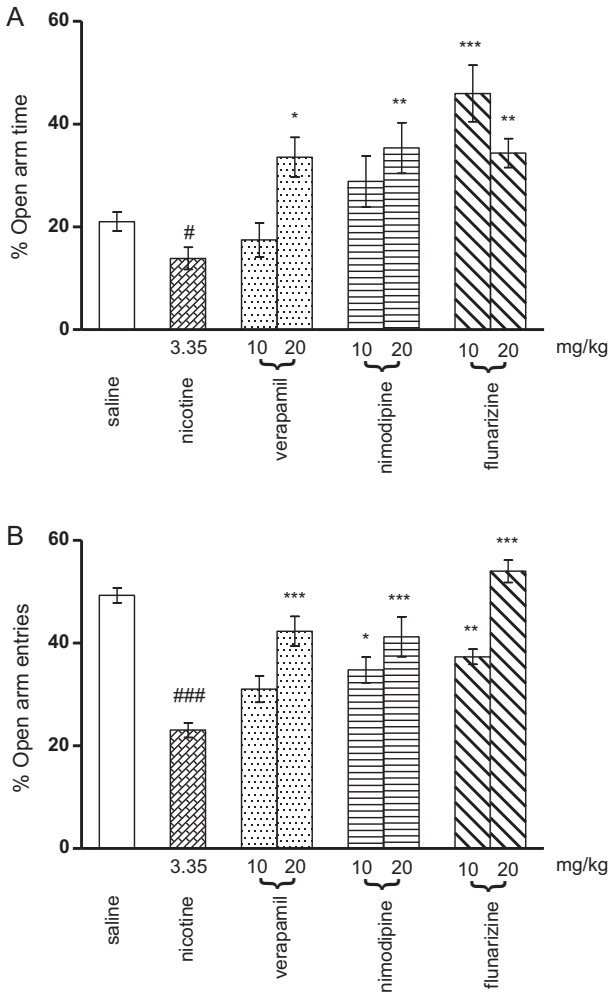

Fig. 4. Influence of calcium channel antagonists on percentage of time spent on the open arms (A) and percentage of open arm entries (B) after 14 days of nicotine withdrawal in the EPM in mice. Verapamil (10 and 20 mg/kg, *ip*), nimodipine (10 and 20 mg/kg, *ip*), flunarizine (10 and 20 mg/kg, *ip*), or saline were administered 15 min prior to test; $n = 8–12$. $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$ vs. nicotine-pretreated and saline-tested group; $^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$ vs. saline-pretreated and saline-tested control group, Tukey's test.

Memory-related behavior

Across all experiments, the time (in s) that each mouse took to move from the open arm to either of the enclosed arms on the first trial (pre-test), i.e., TL1, did not significantly differ among groups (mean TL1 = 43.25 ± 3.45).

Memory-related behavior after D-amphetamine withdrawal in the mEPM test in mice

D-Amphetamine was administered for 14 days, once daily at the dose of 2.5 mg/kg. Memory-related behavior was measured one, seven and 14 days after its withdrawal. Two-way ANOVA analysis revealed that there was significant effect of the TL2 values [treatment effect: F (1,45) = 27.46, $p < 0.0001$; without day effect: F (2,45) = 0.4403, $p = 0.6466$ nor interactions: F (2,45) = 2.033, $p = 0.1428$]. Indeed, the *post hoc* analysis showed that after the seventh and the 14th day of withdrawal, significant increase in the TL2 values was observed ($p < 0.01$) (Fig. 5A) indicating a disturbance in memory processes as compared with saline-pretreated mice.

Influence of CCAs on memory-related behavior during D-amphetamine withdrawal in the mEPM in mice

As shown in Fig. 5B, after seven days of D-amphetamine withdrawal, when amnestic effect was the most significant, administration of verapamil, nimodipine or flunarizine (10 and 20 mg/kg), 15 min before the test, diminished these mnemonic disturbances. In D-amphetamine-pretreated mice, one-way ANOVA analysis revealed that there was significant treatment effect [F (6,61) = 7.112, $p < 0.0001$] (Fig. 5B). The post hoc analysis showed that all CCAs used significantly decreased the TL2 values ($p < 0.05$ for both doses of verapamil; $p < 0.001$ for other CCAs) as compared with D-amphetamine-pretreated and saline-tested mice (Fig. 5B).

Memory-related behavior after nicotine withdrawal in the mEPM test in mice

Nicotine was administered for seven days, three times daily at the dose of 3.35 mg/kg. Memory-related behavior was measured one, seven and 14 days after its withdrawal. Two-way ANOVA analysis revealed that there was significant effect of the TL2 values [treatment effect: F (1,61) = 21.73, $p < 0.0001$; day effect: F (2,61) = 4.485, $p = 0.0152$ without interactions: F (2,61) = 1.122, $p = 0.3323$]. Indeed, the post hoc analysis showed that after the seventh and the 14th day of withdrawal, significant increase in the TL2 values was observed ($p < 0.01$) (Fig. 6A) indicating a disturbance in memory processes as compared with saline-pretreated mice.

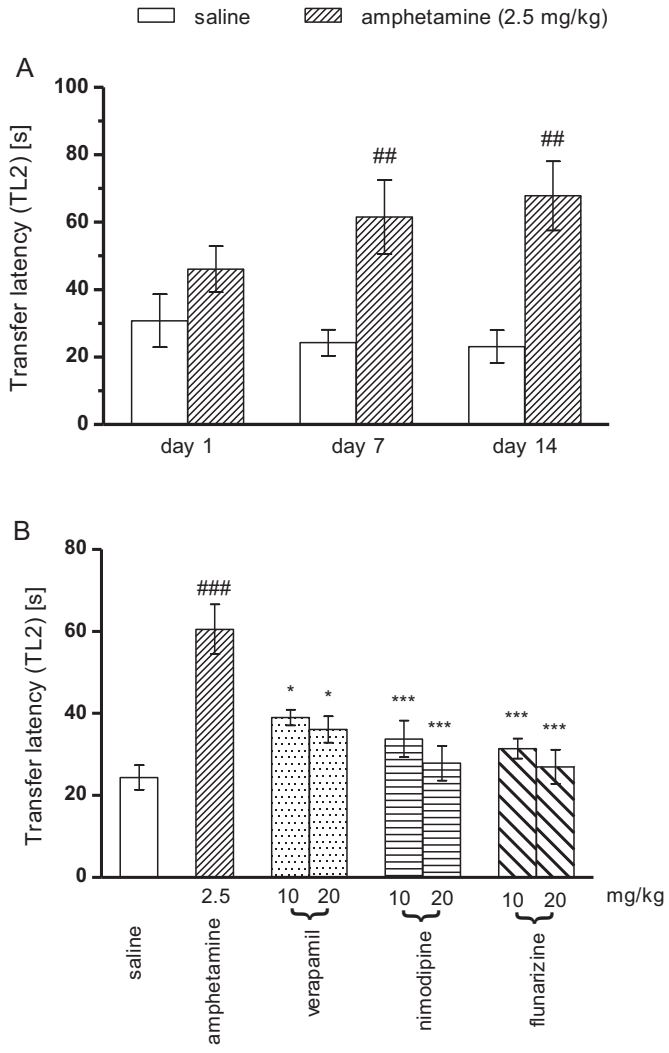

Fig. 5. Mean (±SEM) transfer latency to the enclosed arm on the first, seventh and 14th day of D-amphetamine withdrawal (A) and influence of calcium channel antagonists on TL2 value after seven days of D-amphetamine withdrawal (B) in the mEPM in mice. Verapamil (10 and 20 mg/kg, *ip*), nimodipine (10 and 20 mg/kg, *ip*), flunarizine (10 and 20 mg/kg, *ip*), or saline were administered 15 min prior to test; $n = 8–12$. $^{*}p < 0.05$; $^{***}p < 0.001$ vs. D-amphetamine-pretreated and saline-tested mice; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. saline-pretreated and saline-tested control group, Tukey's test.

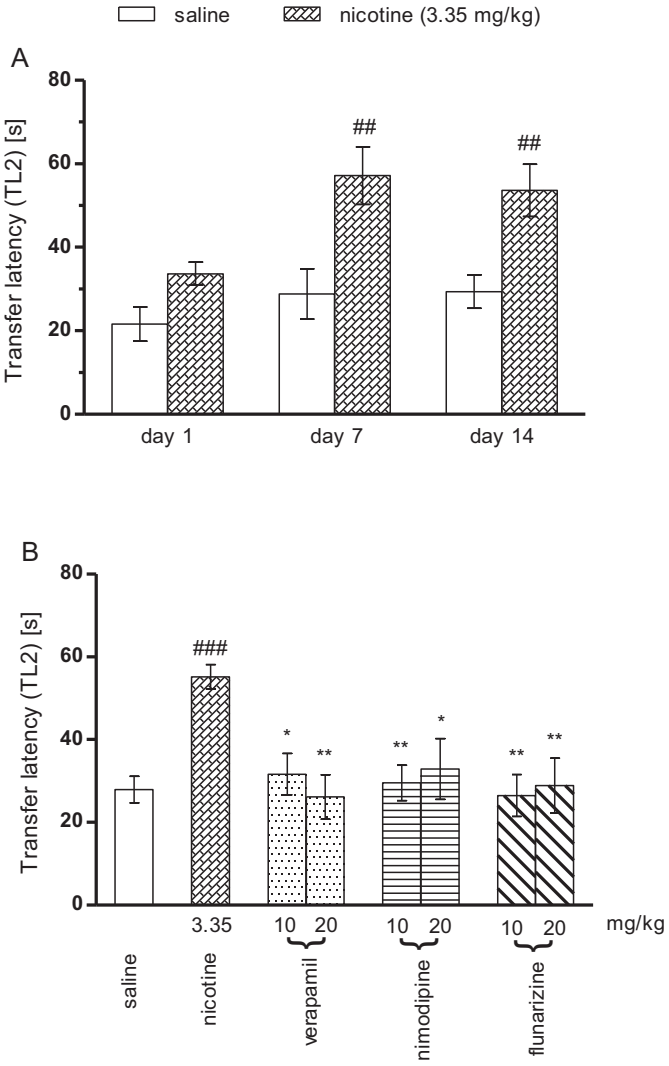

Fig. 6. Mean (±SEM) transfer latency to the enclosed arm on the first, seventh and 14th day of nicotine withdrawal (A) and influence of calcium channel antagonists on TL2 value after 14 days of nicotine withdrawal (B) in the mEPM in mice. Verapamil (10 and 20 mg/kg, *ip*), nimodipine (10 and 20 mg/kg, *ip*), flunarizine (10 and 20 mg/kg, *ip*), or saline were administered 15 min prior to test; $n = 8–12$. $^{*}p < 0.05$; $^{**}p < 0.01$ vs. nicotine-pretreated and saline-tested mice; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. saline-pretreated and saline-tested control group, Tukey's test.

Influence of CCAs on memory-related behavior during nicotine withdrawal in the mEPM in mice

As shown in Fig. 6B, after 14 days of nicotine withdrawal, when amnestic effect was the most significant, administration of verapamil, nimodipine or flunarizine (10 and 20 mg/kg), 15 min before the test, diminished these mnemonic disturbances. In nicotine-pretreated mice, one-way ANOVA analysis revealed that there was significant treatment effect [F (6,55) = 5.855; $p < 0.0001$] (Fig. 6B). The *post hoc* analysis showed that all CCAs used significantly decreased the TL2 values ($p < 0.05$ for nimodipine 20 mg/kg and verapamil 10 mg/kg; $p < 0.01$ for other CCAs) as compared with nicotine-pretreated and saline-tested mice (Fig. 6B).

Nociceptive responses after D-amphetamine withdrawal in the hot plate test in mice

D-Amphetamine was administered for 14 days, once daily at the dose of 2.5 mg/kg. Nociception was measured one, seven and 14 days after its withdrawal. Two-way ANOVA analysis revealed that there was significant effect of the time of latency [treatment effect: $F(1,77) = 14.60$, $p < 0.0003$; day effect: $F(2,77) = 3.365$, $p = 0.0397$; interactions: F (2,77) = 3.139, $p = 0.0489$]. Indeed, the *post hoc* analysis showed that after the seventh and the 14th day of withdrawal, significant decrease in the time of latency was observed ($p < 0.05$) (Fig. 7A) indicating an hyperalgesia as compared with saline-pretreated mice.

Influence of CCAs on nociceptive responses during D-amphetamine withdrawal in the hot plate test in mice

As shown in Fig. 7B, after seven days of D-amphetamine withdrawal, when hyperalgesia was the most significant, administration of verapamil, nimodipine or flunarizine (10 and 20 mg/kg), 15 min before the test, diminished these reactions. In D-amphetamine-pretreated mice, one-way ANOVA analysis revealed that there was significant treatment effect [F (7,104) = 6.047, $p < 0.0001$] (Fig. 7B). The *post hoc* analysis showed that all CCAs used, at the higher dose, significantly increased the time of latency ($p < 0.05$ for flunarizine, $p < 0.01$ for verapamil and $p < 0.001$ for

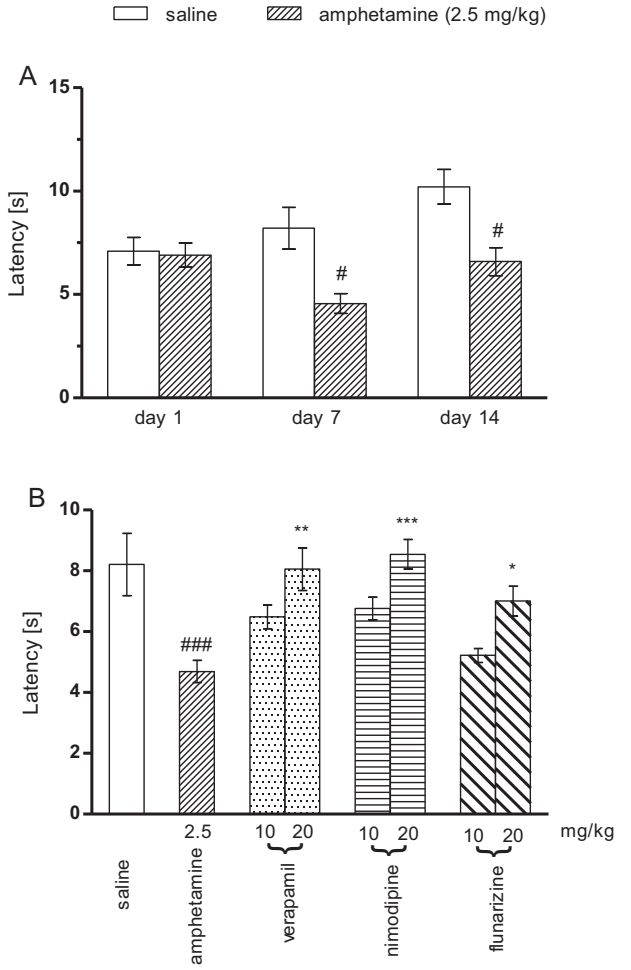

Fig. 7. Mean (±SEM) latency time on the first, seventh and 14th day of D-amphetamine withdrawal (A) and influence of calcium channel antagonists on latency time after seven days of D-amphetamine withdrawal (B) in the hot plate test in mice. Verapamil (10 and 20 mg/kg, *ip*), nimodipine (10 and 20 mg/kg, *ip*), flunarizine (10 and 20 mg/kg, *ip*), or saline were administered 15 min prior to test; $n = 8–12$. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ vs. D-amphetamine-pretreated and saline-tested mice; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. saline-pretreated and saline-tested control group, Tukey's test.

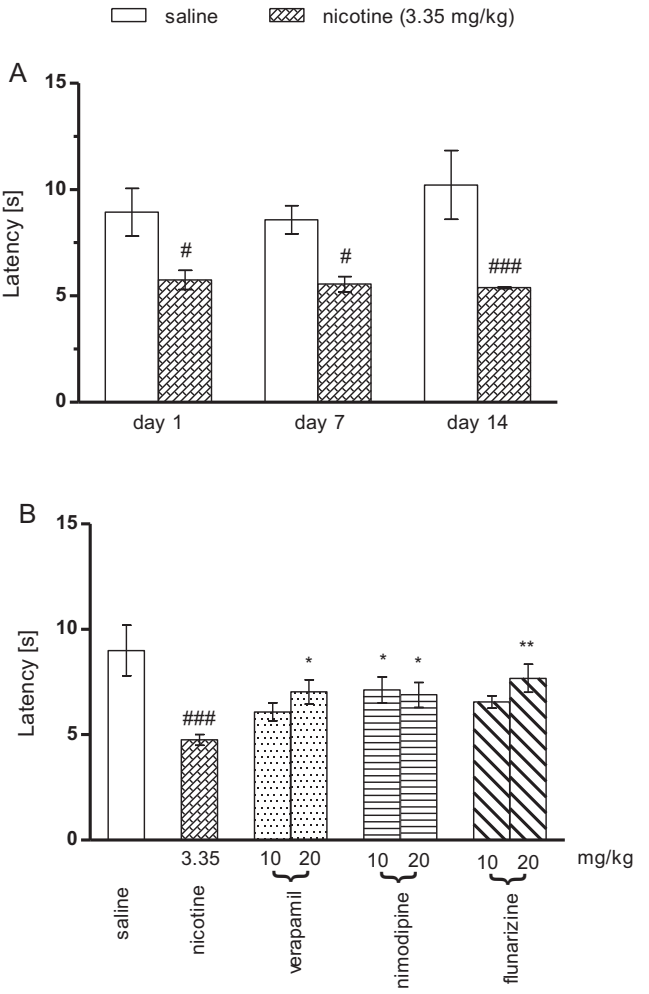

Fig. 8. Mean (±SEM) latency time on the first, seventh and 14th day of nicotine withdrawal (A) and influence of calcium channel antagonists on latency time after 14 days of nicotine withdrawal (B) in the hot plate test in mice. Verapamil (10 and 20 mg/kg, *ip*), nimodipine (10 and 20 mg/kg, *ip*), flunarizine (10 and 20 mg/kg, *ip*), or saline were administered 15 min prior to test; $n = 8–12$. $^{*}p < 0.05$; $^{**}p < 0.01$ vs. nicotine-pretreated and saline-tested mice; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. saline-pretreated and saline-tested control group, Tukey's test.

nimodipine) as compared with D-amphetamine-pretreated and saline-tested mice (Fig. 7B).

Nociceptive responses after nicotine withdrawal in the hot plate test in mice

Nicotine was administered for seven days, three times daily, at the dose of 3.35 mg/kg. Nociception was measured one, seven and 14 days after its withdrawal. Two-way ANOVA analysis revealed that there was significant effect of the time of latency [treatment effect: $F(1,68) = 32.29$, $p < 0.0001$; without day effect: $F(2,68) = 0.4665$, $p = 0.6292$ nor interactions: $F(2,68) = 0.7895$, $p = 0.4582$]. Indeed, the *post hoc* analysis showed that after the first, the seventh and the 14th day of withdrawal, significant decrease in the time of latency was observed ($p < 0.05$ and $p < 0.001$, respectively) (Fig. 8A) indicating an hyperalgesia as compared with saline-pretreated mice.

Influence of CCAs on nociceptive responses during nicotine withdrawal in the hot plate test in mice

As shown in Fig. 8B, after 14 days of nicotine withdrawal, when hyperalgesia was the most significant, administration of verapamil, nimodipine or flunarizine (10 and 20 mg/kg), 15 min before the test, diminished these reactions. In nicotine-pretreated mice, one-way ANOVA analysis revealed that there was significant treatment effect [$F(6,85) = 3.812$; $p = 0.0021$] (Fig. 8B). The *post hoc* analysis showed that all CCAs used significantly increased the time of latency ($p < 0.05$ for verapamil 20 mg/kg, nimodipine 10 and 20 mg/kg; $p < 0.01$ for flunarizine 20 mg/kg) as compared with nicotine-pretreated and saline-tested mice (Fig. 8B).

Depression-like behavior after D-amphetamine withdrawal in the FST in mice; influence of CCAs

D-Amphetamine was administered for 14 days, once daily, at the dose of 2.5 mg/kg. As above-mentioned experiments have shown that after seven days of D-amphetamine withdrawal, the withdrawal-related effects in mice were the most significant, including the depression-like effect ($p < 0.05$, Fig. 9), we measured the depression-like effects of D-amphetamine withdrawal when verapamil, nimodipine, flunarizine (10 and 20 mg/kg) or saline were administered 15 min before the test. In D-amphetamine-pretreated mice, one-way ANOVA analysis revealed that there was significant treatment effect [$F(6,76) = 4.394$, $p = 0.0007$]. The *post hoc* analysis showed that nimodipine and flunarizine significantly

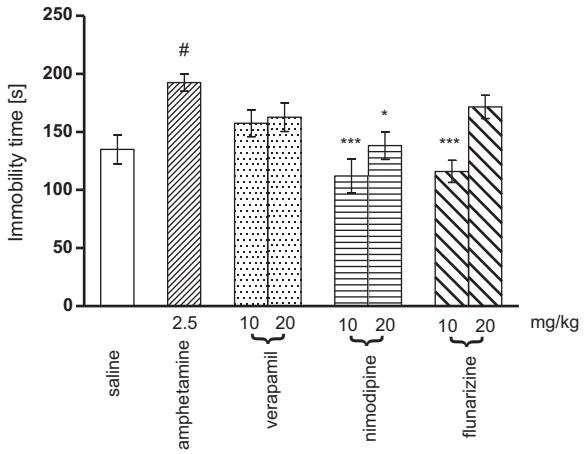

Fig. 9. Influence of calcium channel antagonists on immobility time after seven days of D-amphetamine withdrawal in the FST in mice. Verapamil (10 and 20 mg/kg, *ip*), nimodipine (10 and 20 mg/kg, *ip*), flunarizine (10 and 20 mg/kg, *ip*), or saline were administered 15 min prior to test; $n = 8–12$. $^{*}p < 0.05$; $^{***}p < 0.001$ vs. D-amphetamine-pretreated and saline-tested group; $^{\#}p < 0.05$ vs. saline-pretreated and saline-tested control group, Tukey's test.

decreased the total immobility duration ($p < 0.05$ for nimodipine 20 mg/kg, $p < 0.001$ for flunarizine and nimodipine 10 mg/kg) as compared with D-amphetamine-pretreated and saline-tested mice (Fig. 9).

Depression-like behavior after nicotine withdrawal in the FST in mice; influence of CCAs

Nicotine was administered for seven days, three times daily, at the dose of 3.35 mg/kg. As above-mentioned experiments have shown that after 14 days of nicotine withdrawal, the withdrawal-related effects in mice were the most significant, including the depression-like effect ($p < 0.001$, Fig. 10), we measured the depression-like effects of nicotine withdrawal when verapamil, nimodipine, flunarizine (10 and 20 mg/kg) or saline were administered 15 min before the test. In nicotine-pretreated mice, one-way ANOVA analysis revealed that there was significant treatment effect [$F(6,76) = 4.394$, $p = 0.0007$]. The *post hoc* analysis showed that nimodipine and flunarizine significantly decreased the total immobility duration ($p < 0.05$ for flunarizine 20 mg/kg, $p < 0.01$ for flunarizine and nimodipine 10 mg/kg) as compared with nicotine-pretreated and saline-tested mice (Fig. 10).

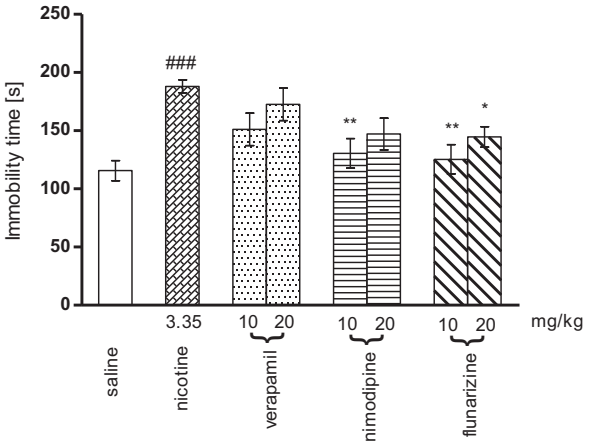

Fig. 10. Influence of calcium channel antagonists on immobility time after 14 days of nicotine withdrawal in the FST in mice. Verapamil (10 and 20 mg/kg, *ip*), nimodipine (10 and 20 mg/kg, *ip*), flunarizine (10 and 20 mg/kg, *ip*), or saline were administered 15 min prior to test; $n = 8–12$. $^{*}p < 0.05$; $^{**}p < 0.01$ vs. nicotine-pretreated and saline-tested group; $^{\#\#\#}p < 0.001$ vs. saline-pretreated and saline-tested control group, Tukey's test.

Any of the calcium channel antagonists, at the doses tested caused no significant changes in the behavioral tests performed by themselves (not shown), as it was also revealed in our previous experiments already cited.

Discussion

It is commonly accepted that motivational nonsomatic drug withdrawal symptoms play a pivotal role in relapse and continued drug use; thus, it is important to understand the molecular and neurophysiological mechanisms that mediate these kinds of withdrawal behaviors. Our previous work has suggested that nicotine withdrawal is associated with a negative affective state, and place aversion to previously neutral environmental stimuli could represent a motivational component in the maintenance of drug use [11]. In the present study, we further investigated animal model in mice repeatedly treated with two drugs, nicotine or D-amphetamine in which different nonsomatic withdrawal symptoms could be revealed. Moreover, in this study we intended to describe the role of calcium homeostasis and calcium channels in the above-mentioned aversive effects of nicotine and D-amphetamine withdrawal. Our data showed that nimodipine, flunarizine and verapamil, L-type VDCC antagonists, blocked nicotine- and amphetamine-induced withdrawal signs after seven or 14 days of spontaneous cessation of drug administration, *i.e.*, heightened anxiety and depression-like state, memory disturbance and hyperalgesia in mice at the doses that did not have any effects in naive mice in those behavioral paradigms by themselves.

Unlike other drugs of abuse, such as alcohol or opioids, where withdrawal symptoms have a significant physical component, psychostimulant withdrawal demonstrates predominantly psychological and affective elements. Withdrawal from cocaine, amphetamine, methamphetamine and nicotine results in anhedonia-like symptoms, such as increased anxiety, dysphoric mood, depressive state, hypersomnolence, fatigue, sadness, and suicidal attempts in humans [13,18,25,26,34,43]. In animal tests, in addition to anhedonia, other depression-like symptoms during psychostimulant withdrawal can also be observed, such as decreased locomotor activity, decreased appetite, decreased grooming behavior and mild somatic symptoms [2]. Concerning nicotine, withdrawal effects can be induced either by discontinuing of its administration (i.e., spontaneous withdrawal) or by administering a nAChR antagonist (i.e., precipitated withdrawal after mecamylamine injection).

In humans, there is a high comorbidity of psychostimulant addiction with psychiatric disorders, such as anxiety and depression, which have also been shown to increase the risk for relapse. In our behavioral study, we demonstrated that both nicotine and D-amphetamine withdrawal, following repeated drug exposure, increased immobility time in the FST on day seven and 14, in comparison to vehicle-treated mice. This observation is in line with previous studies that suggest that nicotine withdrawal can lead to a negative mood state [10,18]. The enhanced time of immobility following psychostimulant withdrawal, as previously suggested, may be attributed to depression-like behavior in rodents [2], accordingly to human withdrawal from chronic nicotine, amphetamine or cocaine [13,34,36] that can contribute to poor outcomes of drug cessation. Regarding possible mechanisms, which may underly these effects, increased plasma corticosterone levels have been reported in rats, as well as increased cortisol levels in patients, who suffer from endogenous depression [35,36]. Serotoninergic pathways could also play some role, as the 5-HT_{2A} receptor antagonists, as well as 5-HT_{2C} receptor agonists, have been shown to facilitate tobacco cessation by relieving nicotine

withdrawal symptoms [65]. Accordingly, the corticotropin-releasing factor increases 5-HT release in the central nucleus of the amygdala and this neurochemical circuitry may support mediating fear and anxiety states [48,56]. Moreover, in the context of anhedonia, enhanced anxiety linked to nicotine and D-amphetamine withdrawal can interfere with the behavioral results revealed using other tasks [34,37]. Data showed increased anxiety-related behaviors after both nicotine and amphetamine withdrawal as the EPM paradigm was also performed in the present study. Importantly, heightened state of anxiety during methamphetamine or cocaine abstinence is often a provoking factor, leading to relapse to drug use in human addicts. The acquired evidence suggests that dysregulation of the mesocorticolimbic brain reward pathway, which plays a critical role in mediating the rewarding effects of all drugs of abuse, may have an important role in anhedonia and its constituent symptoms, such as anxiety and depression [9,18,34,50], observed during withdrawal from psychostimulant drug. As the serotoninergic and adrenergic systems may also be engaged in mediating anhedonia during withdrawal, deficits in serotoninergic transmission, i.e., decreased 5-HT levels in the NAC and other brain regions, such as the hippocampus, striatum, and prefrontal cortex, as well as decreased noradrenaline levels in the prefrontal cortex, hypothalamus, and caudate accompanied with alteration in 5-HT and α receptors has been revealed [52,60,61]. The latter effect is important as the biochemical effects of psychostimulants include blockade of noradrenaline reuptake and enhancement of noradrenaline release, or both as already stated [25].

An increasing evidence also suggests that glutamatergic substrates with changes in glutamatergic receptors and neuroadaptation in the GABAergic system, following a chronic administration of nicotine and amphetamine, may mediate depression/anhedonia/anxiety symptoms, observed during drug withdrawal [38,43]. Concerning nicotine, it should be pointed out that acetylcholine is another neurotransmitter that plays an important role in regulating the mesocorticolimbic reward circuit [15,17]. Altogether, the anticholinergic effect of currently available antidepressant drugs and the regulation of the mescorticolimbic reward pathway by cholinergic neurons suggest that acetylcholine may be an important substrate that may need further evaluation with respect to psychostimulant withdrawal-induced anhedonia.

Other nonsomatic symptoms of drug withdrawal can be related to cognitive impairment also confirmed in our study by using the mEPM paradigm in mice. Accordingly, methamphetamine addiction often includes a number of neurocognitive impairments in the domains of episodic memory, executive functions and information processing [55,57]. While some of these deficits may subside with time, memory impairments are among the most pronounced and persistent problems in methamphetamine dependence in both humans and animals [45,46,57]. Methamphetamine abusers show dysfunction in the cortex, related to cognition and attention [51] and dopamine signaling is reduced in the prefrontal cortex (i.e., downregulation) of mice, treated with methamphetamine, one of animal models of psychosis and schizophrenia. Also in case of nicotine addiction, cognitive changes in abstinent smokers such as deficits in working memory, verbal memory, digit recall, and associative learning are observed as a serious withdrawal syndrome [30,44]. More specifically, these withdrawal deficits in learning can be mediated by upregulation of β2 subunits-containing nAChR, especially in the dentate gyrus of the hippocampus also implicated in the pathophysiology of affective disorders, fear and anxiety behavior and in the relapse stage of addiction [16,22,54].

In the context of our study, it has been established that chronic drug administration provokes subsequent molecular and cellular effects that lead to long-lasting neuroadaptations [47,49]. On this basis, VDCCs are known to contribute to synaptic plasticity, including long-term potentiation (LTP) and long-term depression [31,41]. Accordingly, Ca^{2+} influx plays some role in drug-induced behavioral and neurochemical changes [3–6,31,32]. In particular, Ca^{2+}-dependent signaling mechanisms such as Ca^{2+}/calmodulin-dependent kinase II (CaMKII), the most abundant calcium-dependent kinase in the central nervous system, and protein kinase C (PKC), are stimulated by increased intracellular Ca^{2+} concentration by VDCCs and participate in drug addiction [47]. Accordingly, nicotine, by activation of nAChRs, opens calcium channel and directs Ca^{+2} influx through these receptors, leading to an indirect influx of Ca^{2+} ions through calcium channels. Such an effect is sufficient to activate CaMKII, leading to the induction of LTP. Interestingly, VDCC antagonists have been shown to diminish nicotine-induced LTP in the hippocampus [23]. Moreover, in the context of hyperalgesia observed in our study after nicotine and D-amphetamine withdrawal, it is important to note that enhanced activation of both CaMKII and PKC, mediating Ca^{2+} signaling, has been reported in nociceptive enhancement called opioid-induced hyperalgesia after repeated administration of opioid receptor agonists [12]. In case of nicotine, however, the α6 nAChR subtype also seems to be involved in nicotine-stimulated dopamine release in the striatum in affective but not physical nicotine withdrawal behaviors, including response to a nociceptive stimulus [29].

According to our previous and present data we can state that these calcium-dependent processes might represent a general mechanism for developing drug-induced positive and negative conditioned responses. Interestingly, in humans, in both normotensive and hypertensive smokers, CCAs (i.e., isradipine and nicardipine), particularly after long-term treatment, reduced blood pressure variability which might be useful in the treatment of hypertensive smokers unable to stop smoking [20]. Currently, the role of calcium-dependent mechanisms in physical and affective withdrawal remain less clear and more complex as the results suggest L-type VDCCs and the indirect Ca^{+2} influx to be involved only in somatic signs of physical nicotine withdrawal without any influence on affective withdrawal behavior [28], contrary to our research. We can speculate that after chronic exposure to nicotine or amphetamine, which alters intracellular calcium level, greater Ca^{+2} influx, through both NMDA receptors and VDCCs, is needed to trigger LTP in dependence state. Also, assuming that chronic drug exposure causes alterations in NMDA receptor activity as well as an increase in the number of L-type Ca^{+2} channels in different brain regions, both NMDA and VDCC antagonists could prevent the induction of synaptic neuroadaptation processes and withdrawal syndrome expression of in mice, as demonstrated in the present study.

In conclusion, our results show that withdrawal from nicotine and D-amphetamine in mice resulted in an anhedonic state, i.e., heightened state of anxiety and depression, but also in memory disturbance and hyperalgesia, both seven and 14 days from the last repeated injection of drugs. It should be acknowledged, however, that although acute drug withdrawal can be better understood and treated correctly, treatment of the protracted abstinence syndrome should also be further studied because it may help prevent drug relapse. Simple and practical animal tests, such as those, proposed in the present study, may represent important tools to obtain the goal. Moreover, our data showed that all the three used L-type VDCC antagonists, i.e., nimodipine, verapamil and flunarizine, blocked the expression of the above-mentioned nicotine- and D-amphetamine nonsomatic withdrawal signs. Thus, our results have provided a new insight into the role for the calcium-dependent mechanisms, not only in the expression of somatic sings of nicotine or morphine withdrawal, as previously described [1,8], but also in affective or nonsomatic symptoms of drug dependence, what contributes to relapse and continued drug use.

Such studies may further suggest VDCC antagonism to be a possible trend for therapeutic treatment to reduce different drug withdrawal syndromes.

Conflict of interest

All authors declare that they have no conflict of interest to disclose including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

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