Neuroscience Letters 541 (2013) 144–149

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Neurosteroid allopregnanolone attenuates development of nicotine withdrawal behavior in mice

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HIGHLIGHTS

- Allopregnanolone and progesterone prevents development of nicotine withdrawal behavior.
- Neurosteroid biosynthesis inhibitors attenuate this effect of progesterone.
- Allopregnanolone does not affect expression of nicotine withdrawal behavior.

ARTICLE INFO

Article history:
Received 22 November 2012
Received in revised form 29 January 2013
Accepted 13 February 2013

Keywords:
Nicotine withdrawal behavior
Progesterone
Allopregnanolone

ABSTRACT

Avoidance of the nicotine withdrawal syndrome as well as the positive subjective effects of nicotine is the major predisposing factor to motivate nicotine abuse. However, its underlying neurobehavioral mechanisms remain perplexing. In the present study, we investigated the influence of the neurosteroid allopregnanolone (ALLO; 0.5–2 mg/kg) on the development of nicotine withdrawal in mice. Chronic nicotine injections (2 mg/kg, four times daily, 10 days) followed by its withdrawal, elicited severe somatic signs, anxiety and marked reduction in locomotion. However, these withdrawal signs were not evident in animals pretreated with ALLO or progesterone (Day 8–10) daily before 1st injection of nicotine. This effect of neurosteroid on the nicotine withdrawal signs was reversed by indomethacin and finasteride the inhibitors of neurosteroid biosynthesis. On the contrary, single or repeated dose administration of ALLO or progesterone during nicotine withdrawal (Day 11) did not affect the expression of nicotine withdrawal signs. Thus, compounds that modulate endogenous neurosteroid ALLO are likely to have therapeutic potential for treating various aspects of nicotine dependence and withdrawal.

1. Introduction

Nicotine, the primary component of tobacco produces powerful addiction in humans. Tobacco users or smokers experience considerable withdrawal symptoms, which make them highly susceptible to relapse during cessation attempts [8]. Withdrawal symptoms include irritability, anxiety, and difficulty in concentration, insomnia, fatigue, depressed mood, restlessness, impatience, hostility, weight gain and craving [11]. In animals after prolonged administration, cessation of nicotine, exhibits most prominently somatic withdrawal signs [12] as well as anxiety like symptoms [14]. Although significant progress has been achieved in the recent years, existing medications are only partially effective in treating tobacco abuse. It has been proposed that neurosteroid ALLO represent a new therapeutic target for the drug dependence [13,20] and notably, nicotine dependence [19].

Previous evidences suggest that nicotine after acute, chronic administration or during withdrawal alters brain and plasma concentrations of neurosteroids including ALLO [5,22]. Moreover, serum ALLO levels were positively correlated with salivary nicotine levels in male smokers [19]. It was also suggested that increase in the brain ALLO concentration may be relevant to the modulation of reinforcing effect during acquisition phase of nicotine addiction [5]. Thus these reports further support that changes in the synthesis of neurosteroid ALLO in the brain might play a role in the development of drug addiction.

ALLO (3α, 5α-tetrahydroprogesterone) is synthesized in the brain from progesterone by the sequential action of two enzymes 5α-reductase and 3α-hydroxysteroidoxidoreductase [4,7]. Similarly, clinical and preclinical studies have demonstrated

Abbreviations: ALLO, allopregnanolone; 5α-DHP, 5α-dihydroprogesterone; 3α-HSOR, 3α-hydroxysteroid oxidoreductase; ANOVA, analysis of variance.

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http://dx.doi.org/10.1016/j.neulet.2013.02.023

modulatory effects of progesterone on the subjective effects of nicotine as well as urges to smoke cigarettes [17,26]. Women who quit smoking during the follicular (high estrogen) phase of the menstrual cycle had shorter times to relapse than women who quit during the luteal (high progesterone) phase [1]. Interestingly, the progestins including ALLO and pregnanolone remain increased during the luteal phase [3]. Moreover therapeutic use of PROG has been proposed as a potential relapse prevention treatment in nicotine addiction, especially in female smokers [25]. However, whether the beneficial effect is due to the progesterone or its metabolite ALLO is still intriguing.

ALLO rapidly alters the neuronal excitability by bi-directional allosteric modulation of the $GABA_A$ receptor Cl^- ionophore complex (GRC). Administration of neurosteroid ALLO in rodents evokes broad range of behavioral effects including anxiolysis, sedation, analgesia, antiseizure and antidepressant activity [9,10,15].

In present study, we have described the effects of ALLO, its precursor progesterone and selective neurosteroid biosynthesis inhibitors (indomethacin and finasteride) on the development of nicotine withdrawal somatic signs, anxiety and locomotion in mice.

2. Materials and methods

2.1. Animals

Young healthy (8–10 weeks) male Swiss albino mice (20–25 g) were housed (six per cage) under controlled temperature ($24 \pm 2\,^{\circ}C$) and light conditions (lights on 0700–1900 h) and were allowed free access to rodent chow and water. All treatments protocols and procedures were approved by Institutional Animal Ethical Committee of S. K. B. College of Pharmacy, Kamptee (M.S.) India and complied with the guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals, Government of India. All the behavioral observations were recorded from 9.00 am until the end of experiment.

2.2. Drugs and administration

(−)Nicotine hydrogen tartarate, ALLO, and progesterone were purchased from Sigma (St. Louis, MO, USA). Indomethacin (Micro laboratories, India) and finasteride (Cipla laboratories, India) were donated. ALLO, progesterone, indomethacin and finasteride were dissolved in 2-hydroxypropyl-B-cyclodextrin (45%, w/v) solution and diluted with 0.9% saline. Except nicotine which was administered in a volume of 3 ml/kg, by subcutaneous (s.c.) route, all other drugs were given intraperitonealy (i.p.) in a volume of 10 ml/kg.

2.3. Nicotine dependence/withdrawal treatment protocol

2.3.1. Effect of neurosteorids ALLO and progesterone

The treatment strategy designed by Isola et al. [12] was adapted with little modification, to establish nicotine dependence. Different groups of mice ($n = 6$) were injected with saline or nicotine base (2 mg/kg, s.c.) 4 times daily at an interval of 4 h starting at 0900 h, for 10 days. To observe the effect on expression of nicotine withdrawal syndrome, ALLO (1 mg/kg, i.p.) or progesterone (15 mg/kg, i.p.) was administered either at 24 h of withdrawal or repeatedly at 0.5 as well as 24 h of withdrawal on 11th day. While, to study the effect of neurosteroids on development of nicotine withdrawal syndrome, vehicle, ALLO (0.5–2 mg/kg, i.p.) or progesterone (5–15 mg/kg, i.p.) were administered on 8th, 9th and 10th day 30 min before the 1st injection of nicotine. Withdrawal testing was initiated on day 11 (at 0900 h) and animals were evaluated at 0.5, 4, 8, 12, 24 and 48 h post withdrawal.

Animals were subjected to evaluation of somatic signs for 30 min at the time period mentioned above. Since the peak effects of somatic signs were noticed during 12–24 h post withdrawal, the subsequent parameters like anxiety and locomotion were recorded in separate groups of mice at 24 h only.

2.3.2. Modulation of progesterone effect by neurosteroid biosynthesis inhibitors, indomethacin and finasteride

These experiments were conducted to assess the effect of exogenously administered progesterone in the presence of the neurosteroid biosynthesis inhibitors. Indomethacin (5 mg/kg, i.p.), a 3,alpha-HSOR inhibitor was injected 30 min before or finasteride (50×2 mg/kg, s.c.), a 5alpha-reductase type I and II inhibitor was injected at 4 and 1.5 h before progesterone (15 mg/kg, i.p.) on 8th, 9th and 10th day. After discontinuation of nicotine on 11th day, withdrawal signs were measured at 24 h as mentioned in Section 2.3.1. Indomethacin at a dose of 5 mg/kg, i.p. has been shown to inhibit the conversion of 5,alpha-dihydroprogesterone (5 alpha-DHP) to ALLO in rats [2], whereas 50 mg/kg, s.c. dose of finasteride found to decrease 5alpha-reductase enzyme activity by 60–80% [16].

2.4. Behavioral studies

2.4.1. Withdrawal somatic signs

The nicotine withdrawal scale scored the frequency of the behavioral signs such as; rearing, jumping, body shakes, head shakes, grooming, scratching, chewing, genital licking, tail licking during 30 min of observation in the mice [12]. The total withdrawal score is the sum of scores of all the significant withdrawal signs. The scoring was completed by an individual 'blind' to the treatment conditions.

2.4.2. Anxiety test

Mice were tested on an elevated plus maze consisted of two plexiglass (painted black) opposite facing open arms (23 cm × 6 cm, $L \times W$) and two enclosed arms (23 cm × 6 cm × 15 cm, $L \times W \times H$) connected by a central platform (5.5 cm × 5.5 cm, $L \times W$) (V J Instruments, Karanja, India). The whole maze was raised 60 cm above the floor and illuminated by 100-W lamp fixed 2 m above the maze floor. On the day of the experiment the animals were transferred to the behavioral room one hour prior testing to facilitate adaptation. Nicotine dependence was induced as previously mentioned in Section 2.3.1. Saline or nicotine treated mice were placed singly in the center square of the maze, with their head facing one of the open arm and were allowed to explore the maze for 5 min [21]. The number of entries into and time spent in each arm was recorded by an observer 'blind' to the treatment conditions. Entry of mice in the open or closed arm was considered only when all four paws of mice were placed inside the arm excluding central platform. The maze was wiped clean with a damp cotton and dried after testing each mouse.

Anxiolytic effects were assessed based on the frequency of entries as well as the time spent into the open arms at 24 h post nicotine withdrawal as mentioned in Section 2.3.1. An increase in the time spent as well as the frequency of entries in the open arms relative to control animals was considered as anxiolytic behavior. Separate groups of animals were used for each treatment and each subject evaluated was given a single 5 min trial or tested once only to avoid 'one trial tolerance' to drug effect [24].

2.4.3. Locomotor activity measurement

Locomotor activity was measured using actophotometer (20 cm × 20 cm × 10 cm) (V J Instruments, Karanja, India) equipped with six infrared photo sensors, 2.5 cm apart from each other. This activity was monitored in the same group of animals immediately following the elevated plus maze test. Mice were habituated to

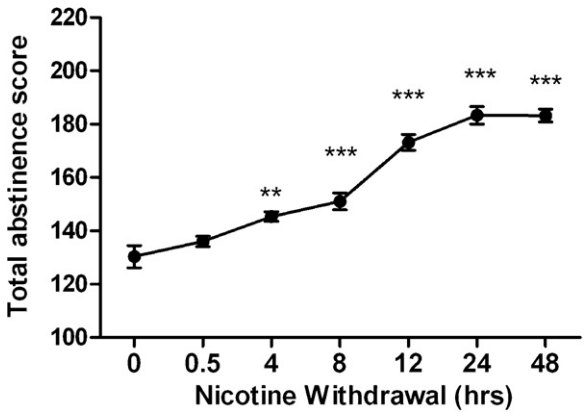

Fig. 1. Mice were treated with saline or nicotine (2 mg/kg, s.c.) four times daily for 10 days. Frequency of somatic signs where the total withdrawal score is the sum of all the significant signs like: rearing, grooming, scratching, genital licking, leg licking, and jumping at 0.5, 4, 8, 12, 24 and 48 h post nicotine withdrawal were measured. Values are expressed as the mean ± S.E.M. ($n = 6$). The data were analyzed by one-way ANOVA followed by post hoc Dunnetts test. *$P < 0.001$, **$P < 0.0001$ vs. vehicle treated control group.

the actophotometer chamber for 15 min prior to behavioral evaluations. The locomotor activity of individual mice from all treatment groups was then recorded for 20 min (as a total count of ambulatory, horizontal and vertical activity) at 24 h post nicotine withdrawal.

2.5. Data analysis

The results were presented as mean ± S.E.M. Effect of ALLO, indomethacin and finasteride on nicotine withdrawal somatic signs, anxiety and locomotor activity were statistically analyzed by one-way analysis of variance followed by post hoc Dunnett's or Bonferroni's test. A value of $P < 0.05$ was considered to be statistically significant in all the cases.

3. Results

3.1. Time dependent somatic signs of nicotine withdrawal

Nicotine withdrawal following chronic administration (2 mg/kg, four times daily, 10 days) resulted in a time-dependent increase in somatic observations (Fig. 1). One-way ANOVA indicated a significant interaction of withdrawal on somatic observations [$F(6, 41) = 58.32$, $P < 0.0001$; $n = 6$] and time factor. Post hoc Dunnett's test shows no significant effect on somatic signs at 0.5 h ($P > 0.05$), however significant ($P < 0.05$) changes were observed at the remaining time points where the peak nicotine withdrawal effects were clearly evident at 24-h. Therefore, the effect of ALLO and progesterone administration were observed at 24-h in subsequent experiments.

3.2. Allo and progesterone inhibited development of nicotine withdrawal syndrome

We found significant induction of nicotine withdrawal syndrome after chronic nicotine administration (2 mg/kg, four times daily, 10 days). Nicotine withdrawn group showed significant increase in the total abstinence score [$F(5, 35) = 71.92$, $P < 0.0001$; $n = 6$), decreased percent time spent in the open arms [$F(5, 35) = 54.42$, $P < 0.0001$; $n = 6$), and decrease [$F(5, 35) = 7.12$, $P < 0.0001$; $n = 6$) in locomotion as compared to the saline treated animals. However, single or repeated administration of ALLO (1 mg/kg) or progesterone (15 mg/kg) on nicotine withdrawn day (11th day) did not affected nicotine withdrawal syndrome. Mice treated with ALLO or progesterone (single or repeat) injections did not show any changes on the total abstinence score ($P > 0.05$), percent time spent in the open arms ($P > 0.05$) or locomotor activity ($P > 0.05$) as compared to the nicotine withdrawn group (Table 1).

As shown in Figs. 2 and 3 mice withdrawn from nicotine showed withdrawal signs such as somatic signs, anxiety and declined locomotion. However, these effects of nicotine withdrawal were not evident in mice that received simultaneously, ALLO (Fig. 2) or progesterone (Fig. 3) during its chronic administration. One-way ANOVA revealed a significant effect of ALLO [$F(3, 23) = 261.8$, $P < 0.0001$; $n = 6$) and progesterone [$F(3, 23) = 51.43$, $P < 0.0001$; $n = 6$) on withdrawal somatic signs (Figs. 2A and 3A). Similarly, one-way ANOVA also revealed that ALLO [$F(3, 23) = 18.52$, $P < 0.0001$; $n = 6$); and progesterone [$F(3, 23) = 35.99$, $P < 0.0001$; $n = 6$) significantly increased the percentage of time spent in the open arms (Figs. 2B and 3B) indicating a reduction in nicotine withdrawal anxiety. Further, these neurosteroids significantly attenuated the nicotine withdrawal induced reduction in locomotor activity in mice (Figs. 2C and 3C) where one-way ANOVA revealed a significant effect of ALLO [$F(3, 23) = 3.44$, $P < 0.05$; $n = 6$] and progesterone [$F(3, 23) = 18.02$, $P < 0.0001$); $n = 6$] on motor activity during nicotine withdrawal.

3.3. Indomethacin, and finasteride attenuates effect of progesterone on nicotine withdrawal syndrome

Progesterone treatment during nicotine administration as described above attenuated the development of nicotine withdrawal behavior. However as depicted in Fig. 4, this protective effect of progesterone was reversed by indomethacin (5 mg/kg, i.p.) or finasteride (50 × 2 mg/kg, s.c.) pre-treatment. One way ANOVA revealed these significant changes on the somatic signs [$F(3, 23) = 42.12$, $P < 0.0001$), Fig. 4A], percent time spent in the open

Table 1
Effect of neurosteroid ALLO and progesterone on expression of nicotine withdrawal syndrome in mice.

Treatment	Total abstinence score	% time spent in open arms	Locomotor activity
Saline (without nicotine)	149.5 ± 8.33	11.81 ± 0.86	78.00 ± 3.45
Nicotine withdrawn			
Vehicle	386.2 ± 7.26*	2.30 ± 0.27*	44.00 ± 3.85*
ALLO (1 mg/kg, at 24 h withdrawal)	366.8 ± 14.51	4.44 ± 0.36	48.67 ± 3.92
ALLO (1 mg/kg, at 0.5 and 24 h withdrawal)	378.3 ± 5.30	2.45 ± 0.32	46.33 ± 4.31
Progesterone (15 mg/kg, at 0.5 and 24 h withdrawal)	352.7 ± 13.03	2.85 ± 0.34	52.67 ± 6.58
Progesterone (15 mg/kg, at 0.5 and 24 h withdrawal)	344.8 ± 11.81	3.23 ± 0.53	56.83 ± 5.08

Mice were treated with nicotine 2 mg/kg, s.c. four times daily for 10 days. ALLO (1.0 mg/kg, i.p.) or progesterone (15 mg/kg, i.p.) was given in single injection (at 24 h withdrawal) or repeatedly (at 0.5 and 24 h withdrawal) on 11th day and nicotine withdrawal symptoms such as (A) somatic signs, (B) % time spent in open arms and (C) locomotor activity were observed 24 h post nicotine withdrawal. The total withdrawal score, % time spent in open arms and locomotor activity is expressed as the mean ± S.E.M. ($n = 6$). The data were analyzed by one-way ANOVA followed by post hoc Dunnetts test.

* $P < 0.0001$ vs. respective saline treated control group.

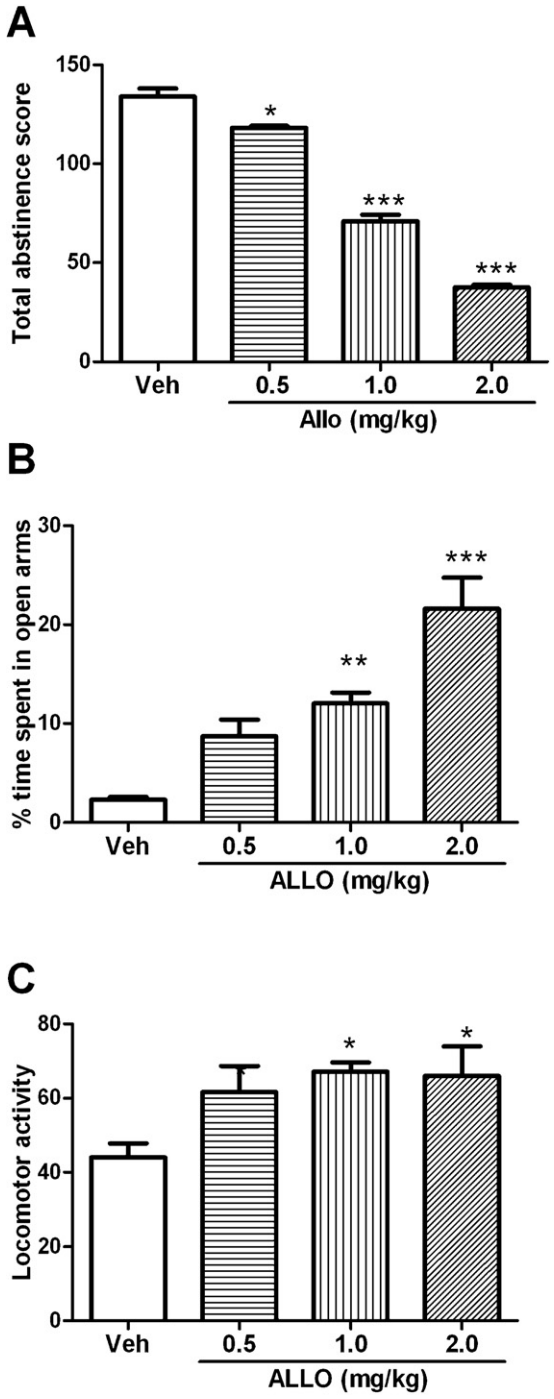

Fig. 2. Effect of ALLO (0.5, 1.0, 2.0 mg/kg, i.p.) on (A) somatic signs, (B) % time spent in open arms and (C) locomotor activity 24 h post nicotine withdrawal. The total withdrawal score, % time spent in open arms and locomotor activity is expressed as the mean ± S.E.M. ($n = 6$). The data were analyzed by one-way ANOVA followed by post hoc Dunnetts test. $^{*}P < 0.05$,$^{**}P < 0.001$, $^{***}P < 0.0001$ vs. respective vehicle treated control group.

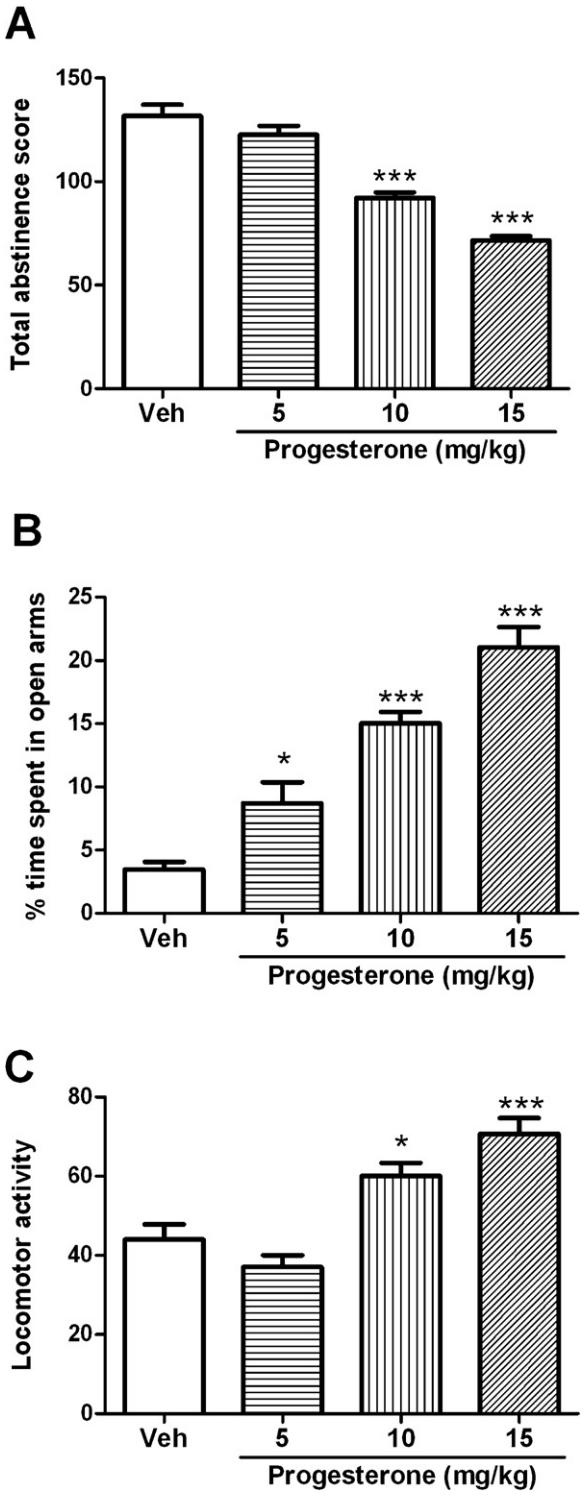

Fig. 3. Effect of progesterone (5–15 mg/kg, i.p.) on (A) somatic signs, (B) % time spent in open arms and (C) locomotor activity 24 h post nicotine withdrawal. The total withdrawal score, % time spent in open arms and locomotor activity is expressed as the mean ± S.E.M. ($n = 6$). The data were analyzed by one-way ANOVA followed by post hoc Dunnetts test. $^{*}P < 0.05$,$^{**}P < 0.001$, $^{***}P < 0.0001$ vs. respective vehicle treated control group.

arms [$F(3, 23) = 55.93$, $P < 0.0001$; $n = 6$ Fig. 4B) and on locomotion [$F(3, 23) = 9.21$, $P < 0.001$; $n = 6$, Fig. 4C].

4. Discussion

The present study was designed to elucidate the relationship between the development of nicotine withdrawal syndrome and neurosteroid ALLO treatment in mice. Mice withdrawn from repeated nicotine administration (for 10 consecutive days) exhibited marked increase in somatic signs (rearing, jumping, body shakes, head shakes, grooming, scratching, chewing, genital licking, tail licking), anxiety like behavior as evident from a decrease in the time spent in the EPM test and decline in the locomotor activity

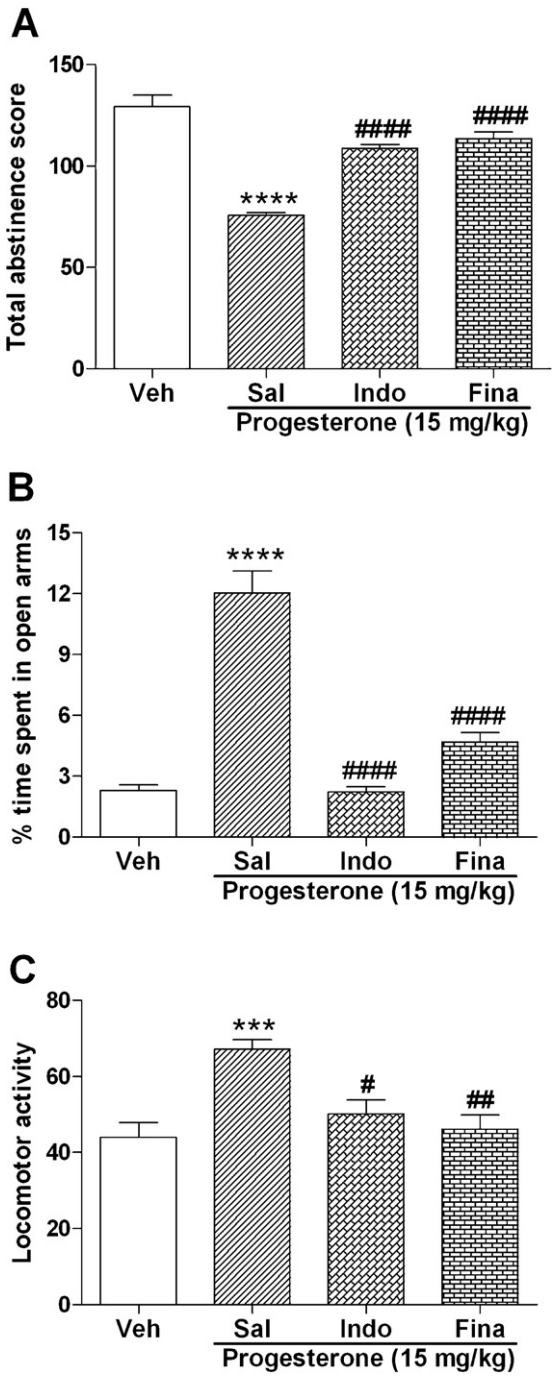

Fig. 4. Effect of neurosteroid biosynthesis inhibitors indomethacin and finasteride on progesterone (15 mg/kg) induced alteration of (A) somatic signs, (B) % time spent in open arms and (C) locomotor activity 24 h post nicotine withdrawal. The total withdrawal score, % time spent in open arms and locomotor activity is expressed as the mean ± S.E.M. ($n = 6$). The data were analyzed by one-way ANOVA followed by post hoc Bonferroni test. $^{*}P < 0.001$ vs. vehicle treated group; $^{\#}P < 0.05$, $^{\#\#}P < 0.001$, $^{\#\#\#}P < 0.0001$ vs. progesterone (15 mg/kg) + vehicle treated group.

(Table 1) with peak effect of the somatic signs between 12 and 24 h (Fig. 1).

However, none of these withdrawal signs were evident in animals receiving ALLO or its precursor progesterone during chronic nicotine treatment (on day 8th, 9th and 10th day, 30 min before the first daily 1st injection of nicotine). Interestingly, ALLO or progesterone were effective only if administered during development phase (on 8th, 9th, and 10th day of nicotine injection) and no effect was observed on expression phase i.e. when injected during withdrawal period (Table 1). These results suggest that ALLO and its precursor progesterone attenuate the process of development but not expression of nicotine dependence, at least in current doses.

Furthermore, biosynthesis inhibitors of ALLO, like finasteride and indomethacin prevented the protective effect of progesterone (as stated in Section 2.3.2). Finasteride, an inhibitor of 5α-reductase enzyme suppresses the conversion [16] of progesterone to 5α-DHP (a precursor of ALLO) and indomethacin, an inhibitor of cytosolic isoform of enzyme 3α-HSOR reduces the conversion of 5α-DHP to ALLO [2]. This supports our assumption that ALLO but not progesterone is important to produce the protective effect on development of nicotine dependence.

There is limited data concerning the effects of ALLO on nicotine withdrawal syndrome. However, few studies have indicated increased brain and plasma levels of pregnanolone, progesterone and ALLO in animals withdrawn from prolonged nicotine exposure [5,22]. It has been hypothesized that decrease in ALLO could contribute to negative effects such as depressive, and anxiety symptoms during nicotine withdrawal [19]. These effects appear to be linked with a direct facilitation of chloride channel opening of $GABA_A$ receptor [18]. Moreover, it has been proposed that compounds that increase the GABAergic neurotransmission decrease both the reinforcing effects of nicotine and reinstatement of cue-induced nicotine-seeking behavior in rats [6]. Alternatively consistent with the notion that cortical ALLO levels play a role in stress regulation [23], it may reduce nicotine withdrawal induced stress by suppressing HPA axis, thereby inhibiting development of withdrawal syndrome. Thus the results of the present experiments strongly support the view of a significant correlation of endogenous ALLO levels with the pathophysiology of nicotine addiction. However, further studies will be required to explain the ability of ALLO to inhibit the process of development of nicotine withdrawal syndrome.

5. Conclusions

The evidence presented here indicates that the endogenous neurosteroid ALLO may be involved in the acquisition of nicotine dependence and may represent a logical candidate for future investigations as a potential pharmacological target for the prevention of relapse.

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