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Effects of nitric oxide synthase inhibitors in attenuating nicotine withdrawal in rats

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Abstract

This study evaluates the effects of three nitric oxide synthase (NOS) inhibitors (L-NNA, L-NAME, L-NMMA) in attenuating the precipitated nicotine withdrawal syndrome in rats. Male albino Wistar rats were made dependent on nicotine by subcutaneous infusion of nicotine (9.0 mg/kg/day) via a 7 day osmotic pump, whereas control rats received saline via osmotic pumps. Test doses of each NOS inhibitor were administered 30 min prior to mecamylamine (1 mg/kg) challenge in control and test rats on the 7th day. Somatic signs of withdrawal were scored for 15 min by using the global Gellert–Holtzman rating scale followed by a measurement of motor activity. A comparison of NOS inhibitors treated rats with the mecamylamine-precipitated nicotine rats showed that at highest dose L-NNA appears to produce a more complete attenuation of all aspects of withdrawal syndrome. On the other hand, L-NAME appears to do so both at moderate and highest doses. This could be due to an incomplete reversal of some signs of withdrawals by L-NMMA. However, motor activity increased in nicotine dependent rats with the administration of NOS inhibitors. This study demonstrates that NO plays an important role in the expression of behavioral signs of nicotine withdrawal syndrome and suggests a potential use of NOS inhibitors as an aid in tobacco smoking cessation.

Keywords: Nicotine; NOS inhibitors; Withdrawal; Dependence

1. Introduction

Nicotine produces a wide range of behavioral effects that are mostly mediated by the central nervous system. Chronic consumption of nicotine has been shown to produce both tolerance and dependence in humans (USDHHS, 1988). An abstinence from nicotine results in a withdrawal syndrome which reaches peak intensity within 24 h (Shiffman and Jarvik, 1976; Hatsukami et al., 1984; Hughes et al., 1991; Kenny and Markou, 2001). This syndrome is characterized by a variety of symptoms notably irritability, anxiety, difficulty in concentrating, restlessness, impatience, excessive hunger, insomnia, drowsiness, and craving for nicotine. In rats, withdrawal reactions from nicotine can be elicited either by a termination of chronic administration of the drug or by an acute challenge with a nicotinic receptor antagonist mecamylamine (Malin et al., 1994; Carboni et al., 1996; Hildebrand et al., 1997).

Evidence suggests that rodent models of nicotine abstinence syndrome are potentially useful for research in understanding the mechanisms of nicotine dependence and in screening proposed interventions to aid in smoking cessation. A few rat models that have been developed rely upon changes in conditioned behavioral responses (Carroll et al., 1989; Corrigall et al., 1989; Helton et al., 1991) or changes in body weight on food consumption (Levin et al., 1987) to measure withdrawal intensity. However, the behavioral responses of rodents to nicotine using these models are complex and varied. For example, acute injections of nicotine can depress locomotor activity (Stolerman et al., 1973; Clarke and Kumar, 1983), while a chronic administration can increase locomotor activity (Stolerman et al., 1973; Cronan et al., 1985).

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A rodent model of nicotine physical dependence and abstinence has also been introduced and validated (Malin, 2001; Malin et al., 1992, 1994). In this model, the dependence is induced by a continuous subcutaneous infusion of nicotine bitartrate via an Alzet osmotic mini-pump and, abstinence is initiated by a removal of the pump or by rapidly precipitating with an injection of nicotinic antagonists such as mecamylamine (Malin et al., 1994). The severity of the abstinence syndrome is assessed by counting occurrences of behavioral abstinence signs on a standard checklist. The prominent signs of this rodent withdrawal syndrome include abdominal constrictions (writhes), facial fasciculations, eye blinks, ptosis and gasps along with miscellaneous other signs including escape attempts, foot licks, genital grooming, shakes, scratches and yawns (Hildebrand et al., 1997; Malin et al., 1998; Watkins et al., 2000; Kenny and Markou, 2001; Malin, 2001). This model is similar to one widely used rat model of the opiate abstinence syndrome (Gianutsos et al., 1975; Malin et al., 1988) and is analogous to methods used in quantifying the nicotine abstinence in humans (Shiffman and Jarvik et al., 1976; Hatsukami et al., 1984).

Findings suggest that some common neurochemical mechanisms underlie opiate and nicotine dependence (Malin et al., 1992, 1993, 1996, 1998). A nicotine receptor stimulation induces a release of endogenous opioid peptides (Pomerleau et al., 1983; Rosecrans et al., 1985; Hexum and Russet, 1987; Jensen et al., 1990; Gilbert et al., 1992; Suh et al., 1995). The nicotine abstinence syndrome in rats involves behavioral signs similar to those in morphine abstinence syndrome (Malin et al., 1988, 1992, 1993, 1996; Malin, 2001) and is potently morphine reversible. Moreover, nicotine abstinence signs can be precipitated by subcutaneous (SC) naloxone injection (Malin et al., 1993). Naloxone also prevents nicotine alleviation of nicotine abstinence (Malin et al., 1996).

In the last few years, attempts have been made to implicate nitric oxide (NO), a prominent neuronal messenger (Monacada et al., 1991) in the expression of withdrawal symptoms in morphine dependent animals. Studies suggest that inhibitors of nitric oxide synthase (NOS) are completely and selectively inhibited by a number of L-arginine analogues (Knowles et al., 1990). The role of NO in the expression of morphine withdrawal symptoms has been studied by utilizing NOS inhibitors. Thus various inhibitors of NOS such as N^G-monomethyl-L-arginine (NMMA), N^G-nitro-L-arginine (LNNA), N^G-nitro-L-arginine-methyl ester (L-NAME) and 7-nitroindazole (7NI) have been shown to attenuate the symptoms of naloxone or naltrexone induced withdrawal in morphine dependent rodents (Kolesnikov et al., 1992; Adams et al., 1993; Kimes et al., 1993; Majeed et al., 1994; Thorat et al., 1994; Cappendijk et al., 1993, 1995; Vaupel et al., 1995; Dunbar and Yaksh, 1996; Malin et al., 1998; Medvedev et al., 1999; Zarrindast et al., 2002; Ozek et al., 2003). Recent research reports on animals suggest the potential role of NO in the suppression of nicotine withdrawal (Adams and Cicero, 1998).

Since nicotine dependence and abstinence syndrome in rats appeared to involve opiate mechanism, it was postulated that the nitric oxide synthase (NOS) activity might be essential for the expression of nicotine abstinence syndrome. The present study compares the effects of different NOS inhibitors viz. L-N^G-monomethyl-arginine (L-NMMA), L-N^G-nitro-arginine (L-NNA), and L-N^G-nitroarginine methyl ester (L-NAME), in alleviating the mecamylamine-precipitated nicotine withdrawal.

2. Methods

2.1. Subjects

Male adult Albino Wistar strains (60–90 days old, 150–175 g, $n=144$) were used in the study. The animals were housed in groups of four per plastic cage in a light and temperature (23 ± 1 °C) controlled environment with a 12 h light and dark cycle (light on 7:00 am to 7:00 pm). They were acclimatized to vicariate conditions for a week and allowed ad-lib access to food and water. The experimental protocol was approved by an Institutional Ethics Committee and was in compliance with the National Institute of Health Guide for Care and Use of Laboratory animals.

2.2. Induction of physical dependence

Under ketamine anesthesia, one Alzet 2ML1 osmotic mini-pump (Alza Scientific Products, Palo, Alto, CA, USA) filled with nicotine hydrochloride in saline was implanted subcutaneously (SC) in the scapular region of each rat. The concentration of nicotine was adjusted for the differences in subject weights. This resulted in a continuous SC infusion at the rate of 9 mg/kg/day (corresponding to 3.15/kg/day nicotine base) for 7 days. Before implantation, each pump was primed for 4 h in physiological saline. This method of infusion has been reported to induce a strong nicotine dependence, as indicated by a vigorous nicotine abstinence syndrome (Malin et al., 1992, 1994).

2.3. Drug treatment

Four experiments were carried out to assess the effect of NOS inhibitors on the development of nicotine dependence at various doses.

In Experiment 1, animals were divided into two groups each containing eight rats. The rats of Group I were implanted with nicotine filled Alzet mini-osmotic pumps whereas rats of Group II were implanted with saline filled Alzet mini-osmotic pumps. On the 7th day prior to the removal of the pump, rats from both groups were challenged with subcutaneous injection of nicotine antagonist mecamylamine (1 mg/kg). This dose of mecamylamine has been shown to precipitate robust nicotine abstinence (Malin et al., 1994). This experiment served as a control experiment.

In Experiment 2, animals were divided into two groups (I and II) each containing 32 rats. The rats in Group I were implanted with nicotine pumps whereas rats in Group II were implanted with saline pumps. Rats in both groups were further divided into four subgroups (I, II, III, and IV) each containing eight rats. The subgroup of each group received intraperitoneally test doses of 1, 3, 10 and 30 mg/kg of L-NAME respectively, 30 min prior to the

challenge of subcutaneous injection of nicotine antagonist mecamylamine (1 mg/kg) on the 7th day of infusion of pumps.

In Experiments 3 and 4, a similar protocol as described in Experiment 2 was followed. Thus, each experiment comprised of 32 rats. Experiment 3 was carried out for testing L-NNA whereas Experiment 4 was carried out for testing L-NMMA. Test doses were administered intraperitoneally in the subgroups (1, 3, 10 and 30 mg/kg respectively), 30 min prior to the challenge of subcutaneous injection of nicotine antagonist mecamylamine (1 mg/kg) on the 7th day of infusion for L-NNA and L-NMMA.

2.4. Behavioral observation

2.4.1. Measurements of precipitated withdrawals

On day 7 of nicotine infusion (168 h) after the pump's implantation, each rat (nicotine treated/saline treated) was weighed and challenged by a subcutaneous injection of mecamylamine (1 mg/kg) (Malin et al., 1994). As discussed in Experiments 2–4, test drugs were administered intraperitoneally (i.p.) prior to a mecamylamine induced withdrawal. Test drug solutions were blind coded and the experimenter was unaware of the treatment. Immediately after mecamylamine injection, animals were placed into an activity cage and visually observed for 15 min. They remained in the activity cage for an additional 10 min.

The somatic signs of withdrawal were scored using the global Gellert–Holtzman rating scale (Gellert and Holtzman, 1978). The experimenter was blind to the treatment. The global GH rating scale has overlapping, graded and checked, behavioral signs used by Malin et al. (1992, 1993). This rating scale assesses an overall index of withdrawal intensity. It consists of graded signs of weight loss, number of escape attempts, number of wet dog shakes, instances of abdominal constrictions, and checked signs (simply scored as present or absent) including diarrhea, facial fasciculations/teeth chattering, swallowing movements, profuse salivation, chromodacryorrhea, ptosis, abnormal posture, penile grooming/erection/ejaculation, and irritability upon handling. On the global Gellert–Holtzman rating scale, graded signs, with the exception of weight loss, are assigned a weighting factor from 1 to 4 based on the frequency of appearance. The checked signs receive values of 2–7 depending upon the withdrawal signs noted, but regardless of the frequency of appearance. With the exception of weight loss, somatic signs were observed for the first 15 min after the injection. The weight loss was calculated as the difference between the weight determined immediately prior to mecamylamine administration, and, the second determination made 60 min after the mecamylamine injection. No food was made available to the rats during this interval. One point was assigned to each 1% of body weight lost (Espejo et al., 1995; Schulteis et al., 1997). Thus, the scores of precipitated withdrawals were observed for the first 15 min along with the gross activities.

2.4.2. Assessment of motor activity

Motor activity was measured by a video path activity analyzer system (Coulbourn Instruments, Inc. USA). The activity was recorded for 25 min at a 5 min interval on the 2nd, 4th and 6th days after the implantation of the pump. The animals were weighed on the same days prior to measurements of motor activity. Rats were also weighed prior to and 2 h following the mecamylamine challenge.

2.5. Drugs

Nicotine hydrogen tartrate and mecamylamine were purchased from Sigma-Aldrich Gmbh, Steinheim, Germany. L-NAME and L-NNA were obtained from Research Biochemicals Inc., Natick, MA, USA. Ketamine hydrochloride was procured from Neon Laboratory, Ltd, Mumbai, India. All drugs were dissolved in physiological saline. Mecamylamine was administered subcutaneously and ketamine and test doses of NOS inhibitors were applied intraperitoneally. All drugs were administered in a volume of 1 ml/kg body weight with doses expressed as the free base.

2.6. Data analysis

The data were analyzed with the statistical package Graph Pad Prism Version 4 and Stata 9.1. Statistical analysis was done by one-way ANOVA with post hoc analysis using Dunnett's test for motor activity and GH score. For weight loss, Kruskal–Wallis one-way ANOVA statistics with Bonferroni correction for multiple comparisons was applied. The checked signs and escape attempts were analyzed by using Fisher's Exact test. Each dose group was compared with the control group and the Bonferroni correction was applied for multiple comparisons. The results were considered significant when the probability level was less than 0.05.

3. Results

3.1. Comparison of GH scores of three NOS inhibitors

Fig. 1A–C represents comparisons of global rating score (GH score) of mecamylamine-precipitated withdrawals in nicotine dependent control rats and saline treated rats (control rats) with nicotine dependent rats and saline rats treated with different doses of all three NOS inhibitors. All groups administered with NOS inhibitors showed a decrease in global withdrawal scores as compared to control rats in mecamylamine-precipitated nicotine treated group. L-NAME showed the maximum suppression of GH scores at the doses tested. The dose of 10 mg/kg was found to be the most potent dose for all NOS inhibitors.

3.2. Attenuation of nicotine withdrawal by L-NMMA

Table 1 shows the results of GH score and individual behavioral signs (% response by the subjects for escape attempts and checked signs) in mecamylamine-precipitated nicotine dependent rats (control rats) and nicotine dependent rats administered with different doses of L-NMMA. The GH score of L-NMMA treated rats was found to be significantly less at all doses as

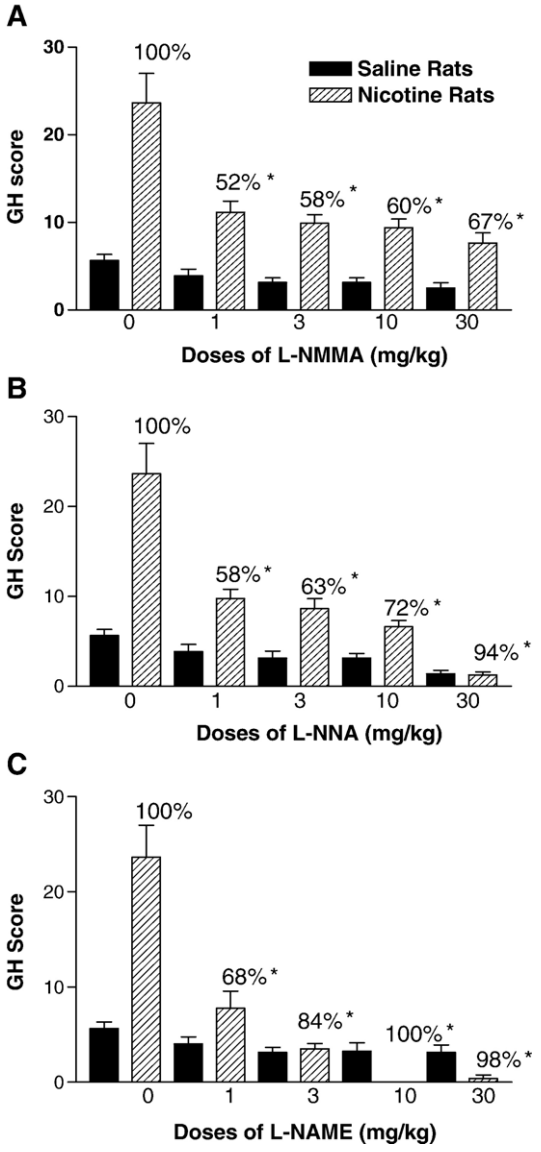

Fig. 1. A–C: represent the effects of three NOS inhibitors (L-NMMA, L-NNA, L-NAME) on Gellert and Holtzman rating score (GH Score) of mecamylamine-precipitated withdrawals in nicotine and saline treated rats. The comparison of the GH scores of mecamylamine-precipitated nicotine withdrawal rats, tested at different test doses of NOS inhibitor was done with mecamylamine-precipitated nicotine treated rats without any NOS inhibitor. *X*-axis represents the doses of each NOS inhibitor; *Y*-axis represents the GH score. One-way ANOVA indicates that at doses 1, 3, 10 and 30 mg/kg of three NOS inhibitors, the percentage decrease in GH score was 52%, 57.5%, 60%, and 67% respectively (L-NMMA); 58%, 63%, 72%, and 95% respectively (L-NNA); 68.0%, 84.6%, 100%, and 98.0% respectively (L-NAME) as compared to 100% GH score in mecamylamine-precipitated nicotine treated control rats. *Asterisks* indicate a significant decrease ($p<0.05$) in GH score, compared to 100% GH score in mecamylamine-precipitated nicotine treated control rats. ■ Saline rats, ▨ nicotine rats.

compared to the score of nicotine control rats [$F(4,35)=11.0$, $p<0.001$]. There was a declining trend in the mean GH score from the control group to L-NMMA pre-treated experimental rats. The highest percentage weight loss was seen in nicotine control rats. No weight loss was observed at different doses of L-NMMA in mecamylamine-precipitated nicotine dependent rats. The difference in weight loss between the control group and each dose group pre-treated with L-NMMA was found to be significant ($p<0.01$).

The rats pre-treated with different doses of L-NMMA prior to mecamylamine challenge displayed a complete suppression of escape attempts at all the test doses of L-NMMA NOS inhibitor as compared to control rats ($p<0.01$).

Except for the facial fasciculations, there was a declining trend in the incidence of checked signs (diarrhea, abnormal posture, erection/ejaculation/genital grooming, irritability) from control (nicotine dependent) rats to experimental rats in each group from low to high test doses (1–30 mg/kg) of L-NMMA NOS inhibitor. However, among the checked signs, the significance was only observed at a dose of 30 mg/kg of L-NMMA for ptosis ($p<0.01$).

To assess the effect of L-NMMA in saline treated rats, signs like weight loss, escape attempts, diarrhea and ptosis did not take place at any dose. However, abnormal posture and erection could be seen at low doses. Other signs like facial fasciculations and irritability were observed at all doses of L-NMMA tested.

3.3. Attenuation of nicotine withdrawal by L-NNA

The results of GH score and individual behavioral signs (% response by the subjects for escape attempts and checked signs) in mecamylamine-precipitated nicotine dependent rats (control rats) and nicotine dependent rats administered with different

Table 1
Effect of L-NMMA NOS inhibitor on mecamylamine-precipitated nicotine withdrawals in nicotine dependent rats ($n=8$, in each group)

Individual behavioral signs	Dose (mg/kg)				
	0	1	3	10	30
l-NMMA					
GH score [a]	23.64	11.12***	9.87***	9.37***	7.62***
Graded signs					
% Weight loss in 60 min [a]	8.82	0.00*	0.00*	0.00*	0.00*
Number of escape attempts [b]					
0–1	2 (25%)	8 (100%)*	8 (100%)*	8 (100%)*	8 (100%)*
2–4	6 (75%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Checked signs [b]					
Diarrhea	5 (62%)	3 (37%)	2 (25%)	0 (0%)	0 (0%)
Facial fasciculations	8 (100%)	8 (100%)	8 (100%)	8 (100%)	8 (100%)
Ptosis	8 (100%)	5 (62%)	5 (62%)	4 (50%)	0 (0%)*
Abnormal posture	8 (100%)	6 (75%)	6 (75%)	5 (62%)	4 (50%)
Erection/ejaculations/genital grooming	8 (100%)	6 (75%)	6 (75%)	6 (75%)	5 (75%)
Irritability	8 (100%)	7 (87%)	6 (75%)	6 (75%)	6 (75%)

The results were considered significant when the probability level was less than 0.05 [*($p<0.01$) ($p<0.001$), ***($p<0.0001$)].

[a] Based on the weight scale of Gellert and Holtzman (1978). Data reflect mean values for eight rats in each group ($n=8$ rats).

[b] Data reflect % of subjects showing individual signs in each group in a 15 min test.

doses of L-NNA are shown in Table 2. As the dose of L-NNA increased a gradual decline in the mean GH was observed at all doses as compared to the GH score of nicotine control rats [$F(4,35)=23.52$, $p<0.0001$].

The percentage weight loss was maximum in the nicotine dependent control rats. No weight loss was observed at all the test doses of L-NNA NOS inhibitor. Using Kruskal–Wallis (non-parametric test) one-way ANOVA with Bonferroni correction for multiple comparisons revealed a difference in weight for all doses of L-NNA with control (nicotine dependent) rats and was found to be statistically significant [$F(4,35)=15.70$, $p<0.0001$]. Escape attempts were completely attenuated at all doses in L-NNA treated nicotine dependent rats ($p<0.01$).

Diarrhea was completely inhibited at higher doses of L-NNA in nicotine dependent rats, whereas 25% rats showed a reduction in diarrhea at low doses (1 and 3 mg/kg) of L-NNA. In contrast, the sign of facial fasciculations was not altered at 1, 10, and 30 mg/kg of the test doses of L-NNA in nicotine dependent rats.

The signs of ptosis and abnormal posture were effectively suppressed at 10 mg/kg ($p<0.03$) and 30 mg/kg ($p<0.01$) doses of L-NNA. At low doses, 62% rats showed a suppression for ptosis. Similarly 62% and 50% rats elicited a suppression for the sign of abnormal posture at doses 1 and 3 mg/kg of L-NNA respectively. As the dose of L-NNA increased a gradual suppression of erection and irritability behavioral signs were observed as compared to control rats. However, a pre-treatment with 30 mg/kg L-NNA showed a complete suppression in erection and the signs of irritability ($p<0.01$).

Table 2
Effect of L-NNA NOS inhibitor on mecamylamine-precipitated nicotine withdrawals in nicotine dependent rats ($n=8$, in each group)

Individual behavioral signs	Dose (mg/kg)				
	0	1	3	10	30
l-NNA					
Mean GH score [a]	23.64	9.75***	8.63***	6.62***	1.25***
Graded signs					
% Weight loss in 60 min [a]	8.82	0.00*	0.00*	0.00*	0.00*
Number of escape attempts [b]					
0–1	2 (25%)	8 (100%)*	8 (100%)*	8 (100%)*	8 (100%)*
2–4	6 (75%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Checked signs [b]					
Diarrhea	5 (62%)	2 (25%)	2 (25%)	0 (0%)	0 (0%)
Facial fasciculations	8 (100%)	8 (100%)	8 (100%)	8 (100%)	6 (75%)
Ptosis	8 (100%)	5 (62%)	5 (62%)	2 (25%)*	0 (0%)*
Abnormal posture	8 (100%)	5 (62%)	4 (50%)	2 (25%)*	0 (0%)*
Erection/ejaculations/ genital grooming	8 (100%)	7 (87%)	4 (50%)	3 (37%)	0 (0%)*
Irritability	8 (100%)	7 (87%)	6 (75%)	6 (75%)	0 (0%)*

The results were considered significant when the probability level was less than 0.05 [*($p<0.01$) ($p<0.001$), ***($p<0.0001$)].

[a] Based on the weight scale of Gellert and Holtzman (1978). Data reflect mean values for eight rats in each group ($n=8$ rats).

[b] Data reflect % of subjects showing individual signs in each group in a 15 min test.

Table 3
Effect of L-NAME NOS inhibitor on mecamylamine-precipitated nicotine withdrawals in nicotine dependent rats ($n=8$, in each group)

Individual behavioral signs	Dose (mg/kg)				
	0	1	3	10	30
l-NAME					
GH score [a]	23.64	7.75***	3.50***	0.00***	1.37***
Graded signs					
% Weight loss in 60 min [a]	8.82	1.25*	0.00*	0.00*	0.00*
Number of escape attempts [b]					
0–1	2 (25%)	8 (100%)*	8 (100%)*	8 (100%)*	8 (100%)*
2–4	6 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Checked signs [b]					
Diarrhea	5 (62%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Facial fasciculations	8 (100%)	8 (100%)	8 (100%)	0 (0%)*	0 (0%)*
Ptosis	8 (100%)	3 (37%)	0 (0%)*	0 (0%)*	0 (0%)*
Abnormal posture	8 (100%)	1 (12%)*	0 (0%)*	0 (0%)*	0 (0%)*
Erection/ejaculations/ genital grooming	8 (100%)	2 (0%)*	0 (0%)*	0 (0%)*	0 (0%)*
Irritability	8 (100%)	6 (75%)	4 (50%)	0 (0%)*	0 (0%)*

The results were considered significant when the probability level was less than 0.05 [*($p<0.01$) ($p<0.001$), ***($p<0.0001$)].

[a] Based on the weight scale of Gellert and Holtzman (1978). Data reflect mean values for eight rats in each group ($n=8$ rats).

[b] Data reflect % of subjects showing individual signs in each group in a 15 min test.

On comparing the effect of L-NNA in saline treated rats, signs like weight loss, escape attempts, diarrhea and ptosis were not observed at any dose. Abnormal posture and erections could be seen only at low doses. Other signs like facial fasciculations and irritability were not altered for all doses.

3.4. Attenuation of nicotine withdrawal by L-NAME

Table 3 shows GH scores and individual behavioral signs (% response by the subjects for escape attempts and checked signs) in mecamylamine-precipitated nicotine dependent rats (control rats) and nicotine dependent rats administered with different doses of L-NAME. The GH score of L-NAME treated rats was found to be significantly lesser at all doses than the score of nicotine control rats [$F(4,35)=31.56$, $p<0.0001$]. No significant effect on the GH score was observed at any dose of NOS inhibitor in saline treated rats.

The percentage weight loss was maximum in nicotine dependent control rats. No weight loss was observed in mecamylamine-precipitated nicotine dependent rats at any dose of L-NAME except at the 1 mg/kg dose. Analysis revealed a difference in weight for all doses of L-NAME with control rats. The difference was observed to be significantly less ($p<0.01$). No effect was seen in saline treated rats.

The nicotine rats pre-treated with L-NAME prior to mecamylamine challenge displayed a complete suppression of escape attempts at all doses when compared to nicotine control rats without NOS inhibitors and was found to be statistically

significant ($p<0.01$). No escape attempts were seen in the saline control rats treated with NOS inhibitors.

Diarrhea was completely inhibited in L-NAME pre-treated experimental rats ($p<0.01$) at all doses of L-NAME. This sign was not observed in saline control rats treated with different doses of NOS inhibitors.

The sign of facial fasciculations of L-NAME was suppressed only at higher doses (10 and 30 mg/kg) of L-NAME ($p<0.01$). The sign of facial fasciculations was also seen in saline control rats treated with all doses of NOS inhibitors.

Ptosis was completely suppressed in nicotine dependent rats pre-treated with L-NAME, prior to mecamylamine-precipitated withdrawals at 3, 10, 30 mg/kg doses ($p<0.01$), as compared to nicotine control rats. No signs of ptosis could be observed in saline treated rats.

Abnormal postures and erection/ejaculation/genital grooming behavioral signs showed a statistically significant decrease for all doses of L-NAME in the nicotine treated rats as compared to control nicotine treated rats ($p<0.01$). Abnormal postures were observed only at 1 mg/kg dose of L-NAME in saline treated rats. On an average, 20% of subjects elicited a reduction in erection/ejaculation or genital grooming at all doses of L-NAME in saline control rats.

A pre-treatment of low to moderate doses of L-NAME (10 and 30 mg/kg) showed a significant suppression in irritability ($p<0.01$). However, 75% and 50% subjects showed a reduction in irritability when compared with control rats at doses of 1 and 3 mg/kg of L-NAME respectively. Saline treated rats also showed irritability at all the doses of L-NAME tested.

3.5. Effect of NOS inhibitors on motor activity in nicotine dependent rats

The comparative data of the effects of all three NOS inhibitors on motor activity in nicotine dependent rats is shown in Table 4. It was observed that the motor activity decreased during mecamylamine-precipitated nicotine withdrawals. There was an increase in motor activity after an administration of test doses of NOS inhibitors prior to mecamylamine challenge in nicotine treated rats. ANOVA with post hoc analysis revealed that motor activity increased in a dose dependent manner at 3 and 10 mg/kg test doses of L-NAME in nicotine dependent rats when compared to nicotine control rats without NOS inhibitor [$F(4,35)=31.56$, $p<0.0001$]. A significant difference in motor activity could be observed only at 30 mg/kg of L-NNA [$F(4,35)=7.37$ $p<0.001$]. An increase in motor activity was seen after a mecamylamine challenge in nicotine dependent rats pre-treated with L-NMMA at doses 3, 10 and 30 mg/kg at a trend level [$F(4,35)=1.8$, $p>0.05$]. The motor activity was not much affected with all NOS inhibitors at any test dose in saline treated rats. The data has not been included in Table 4.

Table 4
Effect of three NOS inhibitors on motor activity in mecamylamine-precipitated nicotine withdrawals in rats

	0	1	3	10	30
L-NMMA	56.25	46.50	56.87	66.62	73.75
L-NNA	56.25	58.25	70.12	117.37	168.37***
L-NAME	56.25	65.5	194.37***	193.37**	149.50***

Values represent mean comparison of the motor activity of NOS inhibitors in nicotine dependent rats and nicotine dependent control rats ($n=8$) of Experiment 1. The results were considered significant when the probability level was less than 0.05 [*($p<0.01$), **($p<0.001$), ***($p<0.001$)].

4. Discussion

There are several reports which show that a chronic administration of nicotine produces physical dependence in animals. The animals also show various withdrawal signs when nicotine is withdrawn (Malin et al., 1992, 1993; Malin, 2001). In addition, an administration of mecamylamine to rats, who have been chronically treated with nicotine using an osmotic mini-pump, induced various withdrawal signs such as teeth chattering, chews, abdominal writhes, gasps, ptosis, wet shakes, and tremors (Malin et al., 1994). In the present study, nicotine was infused subcutaneously with an osmotic mini-pump according to the method of Malin et al. (1993, 1994). The nicotine antagonist mecamylamine promptly precipitates nicotine abstinence signs at a dose, which does not affect nicotine naïve rats. Pilot studies also suggest that the symptoms of nicotine abstinence in rats are remarkably similar to those observed during opiate abstinence syndrome (Gianutsos et al., 1975; Malin et al., 1988).

This study employed the global Gellert–Holtzman (GH) rating scale (Gellert and Holtzman, 1978) for measuring the mecamylamine-precipitated nicotine withdrawal signs. Other investigators have used a different scale for measuring nicotine withdrawals (Hildebrand et al., 1997; Malin et al., 1992; 1993, 1996, 1998; Watkins et al., 2000). This was based on a suggestion that common mechanisms underlie opiate and nicotine dependence (Malin et al., 1992, 1993, 1996). The global Gellert–Holtzman rating scale measures the overall index of withdrawal intensity. The scale has been validated for assessing opioid withdrawal signs (Espejo et al., 1995; Schulteis et al., 1997). The global GH scale has overlapping graded and checked behavioral signs used by Malin and other investigators. In the present study, the withdrawal signs observed were weight loss, escape attempts, diarrhea, facial fasciculations/teeth chattering, abnormal posture, irritability, ptosis, erection/ejaculation or genital grooming. By and large, the observations are in agreement with those of Malin et al. (1992, 1993, 1994). It is important to highlight that the GH rating scale measures other behavioral signs like swallowing movements, profuse salivation, and chromodacryorrhea which are not used in Malin's scale for measuring nicotine withdrawals. However, the GH score does not measure the signs of chews, tremors and gasps used in Malin's scale. The opioid withdrawal global GH rating scale, used in the current study appeared to be satisfactory for measuring nicotine withdrawals in rats.

Behavioral signs like writhes (abdominal constriction), gasps, wet shakes (wet dog shakes), chews, and tremors were not seen in the present study. This is in contrast to the findings of Malin et al. (1992, 1993, 1994). The difference could be on

account of the different rat strains (Sprague–Dawley) used in Malin's experiments. Some autonomic symptoms such as lacrimation, salivation and rhinorrhoea which are frequently seen in opiate-withdrawn rats, especially after the administration of naloxone, were also not observed in the present study. This is in accordance with a previous published report (Hildebrand et al., 1997). The dose of nicotine delivered 24 h a day by the mini-pump was in the same range as previously used in several other studies of nicotine withdrawal reactions in rats (Malin et al., 1992, 1993, 1994) and corresponded to the ingested daily dose of nicotine by a heavy smoker.

The effects of three NOS inhibitors, L-NAME, L-NNA and L-NMMA at four different doses in alleviating the nicotine withdrawals were also examined in the current study. The GH score of mecamylamine-precipitated nicotine dependent rats was higher than the GH score of saline treated rats challenged with mecamylamine. The GH score was significantly reduced at all doses of the three NOS inhibitors. Similarly, the weight loss was suppressed at all doses of the three NOS inhibitors, which shows that all three inhibitors are equipotent in suppressing a weight loss. Escape attempts were completely attenuated by all three NOS inhibitors. Except for the facial fasciculations, there was a declining trend in the incidence of checked signs (diarrhea, abnormal posture, erection/ejaculation/genital grooming and irritability) from control (nicotine dependent) rats to experimental rats in each group from low to high test doses (1–30 mg/kg) of L-NMMA, L-NNA and L-NAME. The diarrhea was completely suppressed by all doses of L-NAME. Facial fasciculations were suppressed in rats pre-treated at moderate and higher doses of L-NAME whereas a suppression in facial fasciculations was seen only at 30 mg/kg dose of L-NNA. Ptosis, abnormal posture and erection were also significantly suppressed at moderate and high doses of L-NAME and L-NNA. Irritability was suppressed at moderate and higher doses of L-NAME (10 and 30 mg/kg) whereas it was suppressed only at a higher dose (30 mg/kg) of L-NNA.

In the current study, a comparison of NOS inhibitors treated rats with the mecamylamine-precipitated nicotine control rats showed that at highest doses L-NNA appears to produce a more complete attenuation of all aspects of the withdrawal syndrome. On the other hand, L-NAME appears to do so both at moderate and highest doses. This may be due to an incomplete reversal of some signs of withdrawal by L-NMMA. Moreover, in the present study, the effect of L-NAME was examined at low and moderate doses (1, 3, 10 and 30 mg/kg) as compared to only one high dose (100 mg/kg) used by Adams and Cicero (1998) which showed a significant suppression in behavioral signs. In addition, Adams and Cicero (1998) used 3 mg/kg dose of mecamylamine instead of 1 mg/kg of mecamylamine for nicotine challenge used in the present study. Furthermore, L-NNA has also been shown to attenuate somatic behavioral signs of nicotine withdrawal at 18 and 30 mg/kg doses (Malin et al., 1998). The body weight changes are understandable in light of the fact that a nicotine abstinence increases body weight in humans and animals due to metabolic and appetite effects (Benowitz, 1996; Malin et al., 1992).

The current study also showed a decrease in motor activity during the mecamylamine-precipitated nicotine challenge. Similar findings are seen in earlier studies (Malin et al., 1994; Hildebrand et al., 1997). A pre-treatment of NOS inhibitors showed an increase in motor activity in mecamylamine-precipitated nicotine withdrawals suggesting an involvement of NO in this phenomenon. Importantly, no adverse effects of NOS inhibitors were observed at all test doses used in the present study.

The results indicate that NO inhibition can alleviate nicotine withdrawal symptoms in rats that are physically dependent on nicotine. This suggests that NO mediates important aspects of nicotine abstinence syndrome. The mechanism by which NOS inhibitors attenuate withdrawals is unclear. It has been suggested that multiple mechanisms operate in these processes (Kenny and Markou, 2001; Malin, 2001). It is not known to what extent these drugs prevent the development of nicotine dependence or reverse existing dependence, or block the expression of withdrawal.

Recently it has been reported that the *N*-methyl-D-aspartate (NMDA) receptor antagonist MK-801 attenuates the development of opiate tolerance and dependence in rats (Trujillo and Akil, 1991; Marks et al., 1991). It has also been observed that some central effects of NMDA are likely to be mediated via the activation of NO synthase, with a subsequent release of NO (Bredt and Snyder, 1992; Snyder and Bredt, 1991). These findings suggest a possibility that L-NAME blocks the NO synthase activity induced by the activation of the NMDA receptor, which modulates the effect of morphine. Since opioids and nicotine share some common neurochemical processes (Corrigall et al., 1989), it is possible that nicotine also acts in a similar manner (Shim et al., 2002). An additional point of similarity between nicotine and opioid withdrawals could be an involvement of an endogenous opioid peptide component in nicotine dependence (Malin et al., 1998).

The overall pattern of findings suggests that NOS inhibitors suppress nicotine abstinence signs at higher doses. Moreover, nitric oxide systems mediate important aspects of the expression of nicotine physical dependence, and may play a potential role in ameliorating some of the discomforts experienced during nicotine cessation.

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