

Varenicline and Cytisine Diminish the Dysphoric-Like State Associated with Spontaneous Nicotine Withdrawal in Rats

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Tobacco addiction is characterized by a negative mood state upon smoking cessation and relapse after periods of abstinence. Clinical studies indicate that negative mood states lead to craving and relapse. The partial $\alpha 4/\alpha 6/\beta 2^*$ nicotinic acetylcholine receptor (nAChR) agonists varenicline and cytisine are widely used as smoking cessation treatments. Varenicline has been approved in the United States for smoking cessation and cytisine is used in Eastern European countries. Despite the widespread use of these compounds, very little is known about their effects on mood states. These studies investigated the effects of varenicline, cytisine, and the cytisine-derivative 3-(pyridin-3'-yl)-cytisine (3-pyr-Cyt) on brain reward function in nicotine-naïve and nicotine-withdrawing rats. The cytisine-derivative 3-pyr-Cyt is a very weak $\alpha 4\beta 2^*$ nAChR partial agonist and like cytisine and varenicline has antidepressant-like effects in animal models. The intracranial self-stimulation (ICSS) procedure was used to investigate the effects of these compounds on brain reward function. Elevations in ICSS thresholds reflect a dysphoric state and a lowering of thresholds is indicative of a potentiation of brain reward function. It was shown that acute administration of nicotine and varenicline lowered ICSS thresholds. Acute administration of cytisine or 3-pyr-Cyt did not affect ICSS thresholds. Discontinuation of chronic, 14 days, nicotine administration led to elevations in ICSS thresholds that lasted for about 2 days. Varenicline and cytisine, but not 3-pyr-Cyt, diminished the nicotine withdrawal-induced elevations in ICSS thresholds. In conclusion, these studies indicate that varenicline and cytisine diminish the dysphoric-like state associated with nicotine withdrawal and may thereby prevent relapse to smoking in humans.

Neuropsychopharmacology (2014) **39**, 445–455; doi:10.1038/npp.2013.216; published online 25 September 2013

Keywords: nicotine; varenicline; cytisine; withdrawal; ICSS; rats

INTRODUCTION

Tobacco products are highly addictive and smoking cessation leads to dysphoria, craving, and impaired cognitive function (American Psychiatric Association, 2000; Bruijnzeel, 2012). Extensive evidence indicates that nicotine is the main psychoactive component of tobacco that leads to addiction (Stolerman and Jarvis, 1995). Nicotine mediates its psychoactive effects by stimulating nicotinic acetylcholine receptors (nAChRs) in the brain. The nAChRs are pentameric ligand-gated ion channels. In the mammalian brain, the heteromeric nAChRs consist of $\alpha 2$ –6 and $\beta 2$ –4 subunits and the homomeric nAChRs consist of $\alpha 7$ subunits (Dani and Bertrand, 2007; Leslie *et al*, 2013). Nicotine mediates its rewarding effects at least partly by activating $\alpha 4/\alpha 6/\beta 2^*$, $\alpha 3\beta 4^*$, and $\alpha 7$ nAChRs (Liu *et al*, 2012; Picciotto and Kenny, 2013; Jackson *et al*, 2013; Toll *et al*, 2012; Markou and Paterson, 2001). Cessation of nicotine administration leads to negative affective (dyspho-

ric- and anxiety-like behavior) and somatic withdrawal signs (Epping-Jordan *et al*, 1998). Clinical and preclinical studies suggest that relapse to smoking is mainly due to craving and negative affective withdrawal signs (Bruijnzeel, 2012; Koob and Volkow, 2010). There is extensive evidence for a role of $\alpha 4\beta 2^*$ nAChRs in dysphoric- and anxiety-like behavior associated with nicotine withdrawal (Picciotto and Kenny, 2013; Changeux, 2010).

During the past decade, a great deal of progress has been made towards the development of treatments for tobacco addiction. Smoking cessation treatments target non-cholinergic receptors (eg, clonidine), a combination of cholinergic and non-cholinergic receptors (eg, bupropion), or only cholinergic receptors (eg, varenicline and cytisine) (Gourlay *et al*, 2004; Slemmer *et al*, 2000; Jorenby *et al*, 2006). In the present studies, we evaluated the effects of three structurally related cholinergic receptor agonists (cytisine, varenicline, and 3-(pyridin-3'-yl)-cytisine (3-pyr-Cyt)) on nicotine withdrawal in rats. Both varenicline and 3-pyr-Cyt were developed by modifying the chemical structure of cytisine. Varenicline was discovered while searching for more potent and efficacious $\alpha 4\beta 2^*$ nAChR partial agonists and 3-pyr-Cyt was discovered during a search for more selective $\alpha 4\beta 2^*$ nAChR partial agonists (Coe *et al*, 2005; Mineur *et al*, 2009). Cytisine has been used as a smoking cessation aid in Eastern European countries since the 1960s (Etter, 2006;

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Received 27 June 2013; revised 7 August 2013; accepted 11 August 2013; accepted article preview online 21 August 2013

West *et al*, 2011). Cytisine is a partial agonist at $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs and a full agonist at $\alpha 3\beta 4^*$ and $\alpha 7$ nAChRs (Salminen *et al*, 2004; Grady *et al*, 2010). The cytisine derivative varenicline is an US FDA-approved smoking cessation treatment and activates the same nAChRs as cytisine (Grady *et al*, 2010; Mihalak *et al*, 2006). Cytisine and varenicline have a similar efficacy at $\alpha 4\beta 2^*$, $\alpha 3\beta 4^*$, and $\alpha 7$ rodent nAChRs, but cytisine is more efficacious at $\alpha 6\beta 2^*$ nAChRs (Grady *et al*, 2010; Salminen *et al*, 2004).

Some preclinical studies suggest that cytisine has rewarding effects. Cytisine is self-administered by mice, induces conditioned place preference (CPP), and repeated administration of cytisine leads to a sensitized locomotor response in rats (Museo and Wise, 1994a, b; Rasmussen and Swedberg, 1998). Drug discrimination studies indicate that cytisine partially generalizes (ie, substitutes) for nicotine (Stolerman *et al*, 1984; LeSage *et al*, 2009). Few studies have investigated the rewarding effects of varenicline. One study showed that low, but not high, doses of varenicline induce a leftward shift in a rate–frequency curve-shift intracranial self-stimulation (ICSS) procedure, which suggests that varenicline has rewarding effects (Spiller *et al*, 2009). In contrast, varenicline does not induce CPP, which would suggest that varenicline does not have rewarding properties (Biala *et al*, 2010).

Despite the fact that cytisine and varenicline are widely used as treatments for smoking cessation, it has not been investigated if varenicline and cytisine diminish the dysphoria associated with nicotine withdrawal. The aim of these studies was to investigate the effects of cytisine and varenicline on brain reward function in nicotine-naïve and nicotine-withdrawing rats by using a discrete-trial ICSS procedure. The acute administration of drugs of abuse lowers ICSS thresholds, which is indicative of a potentiation of brain reward function. In contrast, drug withdrawal leads to elevations in ICSS thresholds, which reflects a dysphoric-like state (Barr *et al*, 2002). In addition to the effects of cytisine and varenicline, the effects of the cytisine derivative 3-pyr-Cyt on brain reward function was evaluated. This compound is a very weak partial agonist at $\alpha 4\beta 2^*$ nAChRs and inhibits acetylcholine-mediated responses on $\alpha 4\beta 2^*$ nAChRs expressed in oocytes (Mineur *et al*, 2009; Papke *et al*, 2010). In contrast to varenicline and cytisine, 3-pyr-Cyt does not bind to $\alpha 3\beta 4^*$ or $\alpha 7$ nAChRs and might therefore have a better side-effect profile (Mineur *et al*, 2009). Like varenicline and cytisine, 3-pyr-Cyt has antidepressant-like effects in a variety of mouse models (Mineur *et al*, 2009).

MATERIALS AND METHODS

Subjects

Male wistar rats (200–225 g; Charles River, Raleigh, NC) were used for the experiments. The animals were housed (2 per cage) in a temperature- and humidity-controlled vivarium and maintained on a 12-h light–dark cycle (light off at 0800 hours). All experiments were conducted during the dark phase. All subjects were treated in accordance with the National Institute of Health guidelines regarding the principles of animal care. Animal facilities and experimental protocols were in accordance with the Association for the

Assessment and Accreditation of Laboratory Animal Care (AAALAC) and approved by the University of Florida Institutional Animal Care and Use Committee.

Drugs

Nicotine and mecamylamine were purchased from Sigma-Aldrich (St Louis, MO). Varenicline, cytisine, and 3-pyr-Cyt were purchased from Tocris (Ellisville, MO). All drugs were dissolved in sterile saline. The pH of the nicotine solution was adjusted to 7.0 immediately before the subcutaneous injections. The drug 3-pyr-Cyt was dissolved by heating the mixture to 40 °C. All drugs were injected (subcutaneously) in a volume of 1 ml/kg of body weight. Nicotine doses are expressed as free base and other drug doses are expressed as salt.

Surgical Procedures

The surgical procedures were conducted as described previously by our group (Bruijnzeel *et al*, 2009, 2012; Marcinkiewicz *et al*, 2009).

Electrode implantations. The rats were anesthetized with isoflurane and prepared with electrodes (Plastic One, Roanoke, VA) in the medial forebrain bundle (anterior–posterior, –0.5 mm; medial lateral, ± 1.7 mm; dorsal–ventral, –8.3 mm from dura).

Osmotic minipump implantations and removal. Anesthetized rats were prepared with minipumps (28 days; Durect Corporation, Cupertino, CA) that were filled with either saline or nicotine. The nicotine concentration was adjusted for body weight and to deliver 3.16 mg/kg of nicotine base per day.

ICSS Procedure

Rats were trained on a modified discrete-trial ICSS procedure (Kornetsky and Esposito, 1979), as described previously (Markou and Koob, 1992; Bruijnzeel *et al*, 2007). The operant conditioning chambers were housed in sound-attenuating chambers (Med Associates, Georgia, VT). The operant conditioning chambers had a response wheel centered on a sidewall and a photobeam detector recorded the rotations. Brain stimulation was delivered by constant current stimulators (Stimtek, Acton, MA). After the rats were trained, each test session provided an ICSS threshold and response latency. The ICSS threshold was defined as the midpoint between stimulation intensities that supported responding and current intensities that failed to support responding. The response latency was defined as the time interval between the beginning of the non-contingent stimulus and a positive response.

Experimental Design

Experiments 1.1–1.4: Acute effect of nicotine (1.1), varenicline (1.2), cytisine (1.3), and 3-pyr-Cyt (1.4) on brain reward function. Drug-naïve rats were used for all experiments. Rats were prepared with electrodes and trained on the ICSS procedure. When the ICSS thresholds

were stable (<10% variation over 5-day period), the rats received nicotine (0, 0.03, 0.1, 0.3, and 0.6 mg/kg, $n = 12$), varenicline (0, 0.1, 0.3, 1, and 3 mg/kg, $n = 9$), cytisine (0, 0.1, 0.3, 1, 3, and 5 mg/kg, $n = 12$), or 3-pyr-Cyt (0, 0.3, 0.6, and 0.9 mg/kg, $n = 13$). Varenicline and 3-pyr-Cyt were administered 30 min before testing and cytisine and nicotine 15 min before testing. The drugs were administered according to a Latin-square design. One dose of cytisine (5 mg/kg) and one dose of varenicline (0.1 mg/kg) were added after the Latin-square to complete the dose–response curves. It was also investigated if the effects of varenicline on ICSS thresholds were mediated by the activation of nAChRs. Therefore, the nAChR antagonist mecamylamine (3 mg/kg) was injected 15 min before the administration of 0.3 mg/kg of varenicline. In all the experiments, the minimum time interval between the drug injections was at least 72 h. All the tested drugs have short half-lives in rats (nicotine $t_{1/2} = 1.3$ h; varenicline $t_{1/2} = 4$ h; cytisine $t_{1/2} = 1.5$ h; mecamylamine $t_{1/2} = 1.2$ h) (Kyerematen *et al*, 1988; Rollema *et al*, 2010; Obach *et al*, 2006; Debruyne *et al*, 2003). The half-life of 3-pyr-Cyt has not been reported. However, in previous studies a wash-out period of 48 h (>72 h in the present study) was used between injections (Mineur *et al*, 2009).

Experiments 2.1–2.3: Effect of varenicline (2.1), cytisine (2.2), and 3-pyr-Cyt (2.3) on brain reward function in nicotine-withdrawing rats. The rats were trained on the ICSS procedure and when the ICSS thresholds were stable, the rats were prepared with nicotine or saline pumps. The minipumps were removed after 14 days. The ICSS thresholds were assessed 6, 12, 24, 36, 48, 72, 83, 96, 120, 144, and 168 h after minipump removal. Varenicline (0.3 mg/kg, subcutaneously; saline-pump/varenicline-injection (inj), $n = 9$; nicotine-pump/varenicline-inj, $n = 13$), 3-pyr-Cyt (0.3 mg/kg, subcutaneously; saline-pump/3-pyr-Cyt-inj, $n = 9$; nicotine-pump/3-pyr-Cyt-inj, $n = 12$), or saline (saline-pump/saline-inj, $n = 9$; nicotine-pump/saline-inj, $n = 14$) were injected 30 min before ICSS testing at the 12–72 h time points. Cytisine (3 mg/kg, subcutaneously; saline-pump/cytisine-inj, $n = 10$; nicotine-pump/cytisine-inj, $n = 12$) was administered 15 min before ICSS testing. The same saline-pump/saline-inj and nicotine-pump/saline-inj groups served as control for Experiments 2.1–2.3. The ICSS thresholds were assessed for an additional 4 days after discontinuing varenicline, cytisine, or 3-pyr-Cyt administration.

Statistical Analyses

The ICSS parameters were expressed as a percentage of the 3-day baseline before the first drug injection (Exp. 1) or before pump removal (Exp. 2). For Experiment 1, one-way repeated-measures analyses of variance (ANOVA) were used to analyze the effects of nicotine, varenicline, cytisine, and 3-pyr-Cyt on ICSS parameters. When the ANOVAs revealed significant effects, then Newman–Keuls *post hoc* tests were conducted. For Experiment 2, paired *t*-tests were conducted to investigate the effect of chronic (14 days) administration of nicotine or saline on ICSS parameters (prepump implantation *vs* prepump removal). Bonferroni corrected *t*-tests were conducted to compare ICSS para-

Table 1 Absolute Baseline ICSS Thresholds and Response Latencies Before First Drug Injection

Compound	N	Thresholds (μ A)	Latencies (s)
Nicotine (Exp. 1.1)	12	91.1 \pm 4.9	3.1 \pm 0.1
Varenicline (Exp. 1.2)	9	124.1 \pm 13.2	3.4 \pm 0.1
Cytisine (Exp. 1.3)	12	126.8 \pm 8.4	3.3 \pm 0.1
3-pyr-Cyt (Exp. 1.4)	13	102.1 \pm 5.4	3.4 \pm 0.1

The baselines are averages of thresholds and latencies that were obtained on three consecutive days before the first drug injection.

eters between groups. Three-way ANOVAs (6–168 h time points) were conducted to investigate the effects of varenicline, cytisine, and 3-pyr-Cyt on spontaneous nicotine withdrawal with time as within-subjects factor and pump content and drug as between-subjects factors. To investigate the effects of the drugs on nicotine-withdrawing rats or control rats (12–72 h) additional two-way ANOVAs were conducted. Area under the curves (AUCs) were analyzed with a two-way ANOVA with pump content and drug as between-subjects factors. After the ANOVA, all the saline groups were compared with the saline–saline group and all nicotine groups to the nicotine–nicotine group by using the Dunnett's multiple comparison procedure (Dunnett, 1955). Statistical analyses were performed using IBM SPSS (version 21) for Windows software.

RESULTS

Experiments 1.1–1.4: Effect of Nicotine, Varenicline, Cytisine, and 3-Pyr-Cyt on ICSS Thresholds

The mean (\pm SEM) absolute ICSS thresholds and response latencies (3-day averages) before the administration of nicotine, varenicline, cytisine, and 3-pyr-Cyt are reported in Table 1. Repeated administration of the drugs did not alter the pretest day ICSS thresholds or response latencies (data not shown).

Nicotine (Exp. 1.1). There was a main effect of nicotine on ICSS thresholds ($F_{4,44} = 15.14$, $p < 0.001$, Figure 1a) and response latencies ($F_{4,44} = 2.91$, $p < 0.05$; Figure 1b). *Post hoc* analyses indicated that the effect of nicotine on ICSS thresholds is bidirectional, low doses of nicotine (0.1 and 0.3 mg/kg) lowered ICSS thresholds and high doses of nicotine (0.6 mg/kg) elevated ICSS thresholds. In addition, nicotine (0.3 mg/kg) decreased the response latencies, which is indicative of a stimulant-like effect.

Varenicline (Exp. 1.2). The ANOVA analysis indicated that there was a main effect of varenicline on ICSS thresholds ($F_{4,32} = 3.60$, $p < 0.05$; Figure 2a) and response latencies ($F_{4,32} = 3.38$, $p < 0.05$; Figure 2b). The *post hoc* comparisons revealed that low doses of varenicline lowered ICSS thresholds (0.1, 0.3, and 1 mg/kg) and decreased the response latencies (0.1 and 0.3 mg/kg). Pretreatment with the nAChR antagonist mecamylamine prevented the varenicline-induced lowering of ICSS thresholds and the decrease in response latencies.

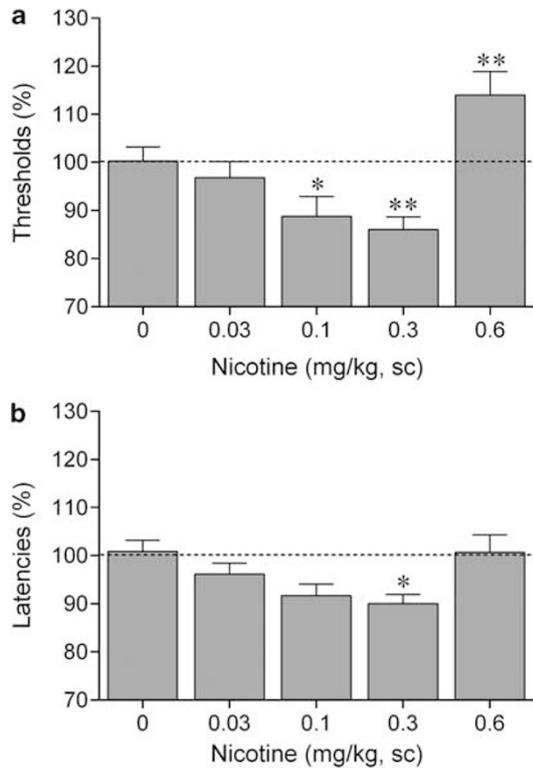


Figure 1 Nicotine potentiates brain reward function. (a) Acute nicotine lowers intracranial self-stimulation (ICSS) thresholds and (b) decreases response latencies. Asterisks (* $p < 0.05$, ** $p < 0.01$) indicate lower ICSS thresholds or shorter response latencies compared with saline-treated rats. Data are expressed as means \pm SEM ($n = 12$ per group). Sc, subcutaneous.

Cytisine (Exp. 1.3). Cytisine did not affect ICSS thresholds ($F_{5, 55} = 1.80$, NS; Table 2) or response latencies ($F_{5, 55} = 0.91$, NS; Table 2).

3-Pyr-Cyt (Exp. 1.4). The cytisine derivative 3-pyr-Cyt did not affect ICSS thresholds ($F_{3, 36} = 1.69$, NS; Table 2) or response latencies ($F_{3, 36} = 1.44$, NS; Table 2).

Experiments 2.1–2.3: Effect of Varenicline, Cytisine, and 3-Pyr-Cyt on Brain Reward Function in Nicotine-Withdrawing Rats

Mean (\pm) absolute ICSS thresholds and response latencies before minipump implantation and before minipump removal are shown in Table 3. Chronic administration of nicotine ($n = 51$) or saline ($n = 37$) did not affect ICSS thresholds. Chronic administration of nicotine ($n = 51$, all groups combined) led to a small, but significant, decrease in the response latencies (prepump implantation vs prepump removal; $t(50) = 2.03$, $p < 0.05$). There were no significant differences in ICSS thresholds or latencies between the various nicotine and saline groups before minipump removal (Table 3).

Experiment 2.1: Effect of Varenicline on Brain Reward Function in Nicotine-Withdrawing Rats

The removal of the nicotine pumps, but not saline pumps, led to elevations in ICSS thresholds (Pump: $F_{1, 41} = 41.62$,

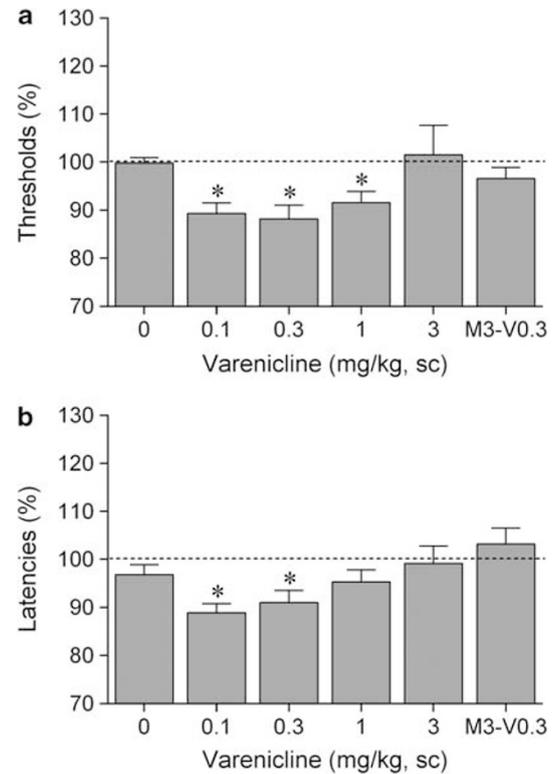


Figure 2 Varenicline potentiates brain reward function. (a) Acute varenicline lowers intracranial self-stimulation (ICSS) thresholds and (b) decreases response latencies. Asterisks (* $p < 0.05$) indicate lower ICSS thresholds or shorter response latencies compared with saline. Data are expressed as means \pm SEM ($n = 9$ per group). M3, mecamylamine, 3 mg/kg; sc, subcutaneous; V0.3, varenicline 0.3 mg/kg.

$p < 0.001$; Pump \times Time: $F_{10, 410} = 3.47$, $p < 0.001$; Figure 3a). Varenicline lowered ICSS thresholds and this effect was dependent on pump content (nicotine or saline) (Drug \times Time: $F_{10, 410} = 5.72$, $p < 0.001$; Drug \times Pump \times Time: $F_{10, 410} = 1.99$, $p < 0.05$). Additional two-way ANOVAs were conducted for the varenicline treatment period (12–72 h). These analyses showed that varenicline lowered the ICSS thresholds of the nicotine-withdrawing rats (Drug: $F_{1, 25} = 14.97$; $p < 0.001$). There was a trend toward a decrease in ICSS thresholds in the saline-control rats (after saline-pump removal) treated with varenicline, but this effect did not reach statistical significance (Drug: $F_{1, 16} = 4.31$, $p = 0.054$).

Post hoc analyses indicated that in the nicotine-withdrawing rats treated with saline, the ICSS thresholds were elevated from 6 to 48 h after pump removal. In the nicotine-withdrawing rats treated with varenicline, the ICSS thresholds were only elevated at one time point (36 h). Varenicline diminished the elevations in ICSS thresholds associated with nicotine withdrawal. At the 12, 24, and 36 h time points, the ICSS thresholds were lower in the nicotine-withdrawing rats treated with varenicline than in the nicotine-withdrawing rats treated with saline. After the cessation of varenicline administration, there was an increase in the ICSS thresholds in the rats that had been exposed to the nicotine pumps (96, 120, and 144 h time points). This suggests that cessation of varenicline administration leads to a dysphoric-like state in animals with a history of nicotine dependence.

Table 2 Acute Effects of Cytisine and 3-pyr-Cyt on ICSS Thresholds and Response Latencies

<i>Cytisine (Exp. 1.3, n = 12)</i>						
Dose (mg/kg)	0	0.1	0.3	1	3	5
Thresh. (μ A)	97.6 \pm 3.0	101.6 \pm 2.5	102.2 \pm 1.3	98.8 \pm 1.6	103.4 \pm 2.9	105.9 \pm 4.3
Latency (s)	97.6 \pm 2.5	102.0 \pm 2.3	98.4 \pm 2.6	98.5 \pm 3.1	96.7 \pm 3.4	99.1 \pm 3.5
<i>3-pyr-Cyt (Exp. 1.4, n = 13)</i>						
Dose (mg/kg)	0	0.3	0.6	0.9		
Thresh. (μ A)	99.4 \pm 2.9	100.0 \pm 2.6	102.2 \pm 3.1	106.5 \pm 4.2		
Latency (s)	101.3 \pm 1.7	98.2 \pm 1.8	100.8 \pm 2.0	96.9 \pm 1.2		

Abbreviation: Thresh., ICSS thresholds.

Drugs were administered subcutaneously and data are expressed as a percentage of three-day baselines before the first drug injection.

Table 3 Absolute Baseline ICSS Thresholds and Response Latencies Before Minipump Implantation and Removal

Pump	Drug	N	Prepump implantation		Prepump removal	
			Threshold (μ A)	Latencies (s)	Threshold (μ A)	Latencies (s)
Saline (all groups)		37	105.4 \pm 3.8	3.3 \pm 0.1	106.7 \pm 3.7	3.3 \pm 0.1
Nicotine (all groups)		51	101.6 \pm 3.5	3.4 \pm 0.0	102.6 \pm 3.5	3.3 \pm 0.1*
Saline	Saline	9	115.8 \pm 11.8	3.4 \pm 0.1	118.8 \pm 12.2	3.3 \pm 0.1
Saline	Varenicline	9	105.3 \pm 3.5	3.2 \pm 0.1	107.0 \pm 2.4	3.3 \pm 0.1
Saline	Cytisine	10	106.1 \pm 7.7	3.4 \pm 0.1	105.6 \pm 6.2	3.3 \pm 0.1*
Saline	3-pyr-Cyt	9	94.3 \pm 4.0	3.3 \pm 0.1	95.6 \pm 3.6	3.3 \pm 0.1
Nicotine	Saline	14	96.7 \pm 5.3	3.2 \pm 0.1	98.9 \pm 5.2	3.2 \pm 0.1
Nicotine	Varenicline	13	106.9 \pm 8.5	3.3 \pm 0.1	107.4 \pm 8.6	3.3 \pm 0.1
Nicotine	Cytisine	12	101.2 \pm 8.0	3.4 \pm 0.1	102.4 \pm 8.4	3.2 \pm 0.1
Nicotine	3-pyr-Cyt	12	102.1 \pm 6.1	3.6 \pm 0.1 ⁺⁺	105.0 \pm 5.5	3.4 \pm 0.1*

The ICSS thresholds and latencies are three-day baselines that were obtained on consecutive days before pump implantation or pump removal. Asterisks (* $p < 0.05$) indicate difference between prepump-implantation and prepump-removal baselines within the same group. Plus signs (⁺ $p < 0.01$) indicate significant difference compared with saline-pump/3-pyr-Cyt-inj group.

There were no main effects of Pump content or Drug treatment on the response latencies (Figure 3a). There was, however, a significant effect of Time ($F_{10, 410} = 4.13$, $p < 0.001$), Pump \times Time interaction ($F_{10, 410} = 3.49$, $p < 0.001$) and Drug \times Time interaction ($F_{10, 410} = 3.31$, $p < 0.001$). The *post hoc* comparisons revealed that varenicline decreased the response latencies in the saline-pump (12 h) and nicotine-pump rats (72 h). Furthermore, the latencies of the nicotine-withdrawing rats (nicotine-pump/saline-inj) were slightly increased ($\sim 10\%$), but this did not reach statistical significance. Previous studies have also shown that cessation of nicotine administration leads to a small increase in response latencies (Bruijnzeel *et al*, 2007).

Experiment 2.2: Effect of Cytisine on Brain Reward Function in Nicotine-Withdrawing Rats

The removal of the nicotine pumps, but not saline pumps, led to elevations in the ICSS thresholds (Pump: $F_{1,41} = 13.75$, $p < 0.001$; Time: $F_{10,410} = 8.13$, $p < 0.001$; Pump \times Time: $F_{10,410} = 2.49$, $p < 0.01$; Figure 4a). The administration of cytisine lowered ICSS thresholds and this effect was dependent on pump content (Pump \times Drug \times Time: $F_{10,410} = 4.95$, $p < 0.001$). The two-way ANOVA (12–72 h period) indicated that cytisine lowered the ICSS

thresholds of the nicotine-withdrawing rats (Drug: $F_{1,24} = 7.50$, $p < 0.05$) but did not affect the ICSS thresholds of the saline-pump control rats (Drug: $F_{1,17} = 3.62$, NS). The *post hoc* analyses indicated that at the 24 h time point, the ICSS thresholds of the nicotine-withdrawing animals treated with cytisine were lower than those of the nicotine-withdrawing rats treated with saline. Furthermore, cytisine reduced the duration of the elevations in ICSS thresholds in the nicotine-withdrawing rats (36 vs 48 h). Nicotine withdrawal or pretreatment with cytisine did not affect the response latencies (Figure 4b). There was, however, a main effect of Time on response latencies (Time: $F_{10,410} = 6.21$, $p < 0.001$). This might have been caused by the removal of the minipumps (surgery effect). This led to a very small increase in response latencies and the latencies gradually returned to baseline levels. The *post hoc* analyses did not detect any differences in response latencies between the experimental groups.

Experiment 2.3: Effect of 3-pyr-Cyt on Brain Reward Function in Nicotine-Withdrawing Rats

Statistical analyses indicated that removal of the nicotine pumps led to an elevation in ICSS thresholds (Pump: $F_{1,40} = 49.00$, $p < 0.001$; Time: $F_{10,400} = 5.17$, $p < 0.001$;

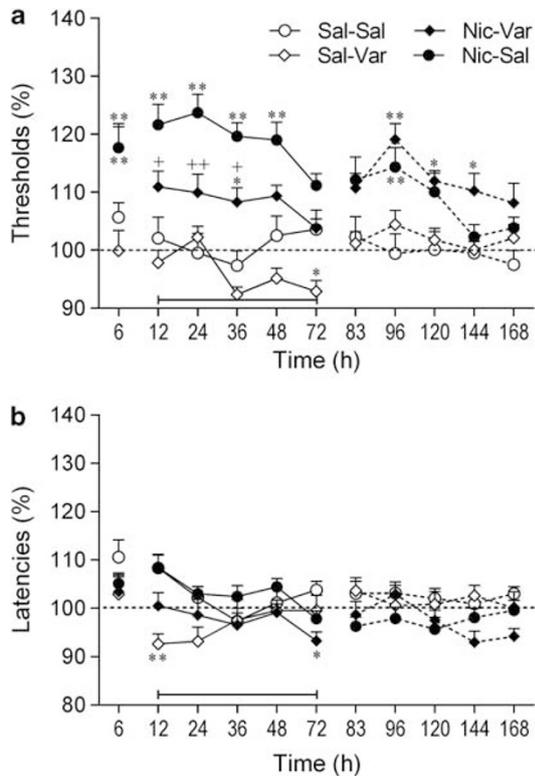


Figure 3 Varenicline diminishes the dysphoric-like state associated with nicotine withdrawal. (a) Effect of varenicline on the elevations in intracranial self-stimulation (ICSS) threshold associated with spontaneous nicotine withdrawal. (b) Effect of varenicline on response latencies. The number of animals per group was: saline–saline, $n=9$; saline–varenicline, $n=9$; nicotine–saline, $n=14$; and nicotine–varenicline, $n=13$. (a) At the 6 h time point, the ICSS thresholds of the nicotine–saline rats and the nicotine–varenicline rats were similar (117% of baseline). Therefore, only one data point is visible for these two groups. Asterisks ($*p<0.05$, $**p<0.01$) indicate elevated ICSS thresholds compared with the saline–saline group. Plus signs ($+p<0.05$, $++p<0.01$) indicate lower ICSS thresholds compared with nicotine–saline group. (b) Asterisks ($*p<0.05$, $**p<0.01$) indicate shorter latencies compared with saline–saline group. Three specific periods are depicted in the figures (before, during, and after varenicline treatment) and these periods are separated by line breaks. The bar at the bottom of each figure indicates the varenicline treatment period. The rats received varenicline or saline before each time point (12–72 h). Data are expressed as means \pm SEM. Nic, nicotine; Sal, saline; Var, varenicline.

Pump \times Time: $F_{10,400}=8.61$, $p<0.001$; Figure 5a). The overall three-way ANOVA indicated that 3-pyr-Cyt did not affect the ICSS thresholds. The three-way ANOVA analyses indicated that the latencies of the nicotine-withdrawing rats were longer than the latencies of the saline control rats (Time: $F_{10,400}=7.77$, $p<0.001$; Pump \times Time: $F_{10,400}=3.58$, $p<0.001$; Figure 5b). The statistical analyses also revealed a trend towards a Pump \times Drug interaction ($F_{1,40}=3.48$, $p=0.07$), which suggests that the effects of 3-pyr-Cyt on the latencies depend on the treatment history (chronic nicotine or saline). The *post hoc* test indicated that 3-pyr-Cyt increased the latencies in the nicotine-withdrawing rats (36 h time point) but not in the control rats.

An additional statistical analysis was conducted to compare the effects of all three drugs (varenicline, cytosine, and 3-pyr-Cyt) on nicotine withdrawal (12–72 h). This study

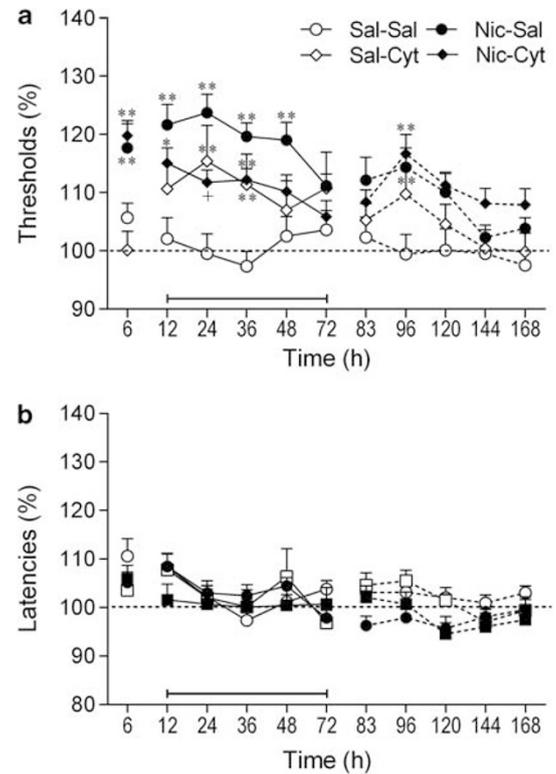


Figure 4 Cytosine diminishes the dysphoric-like state associated with nicotine withdrawal. (a) Effect of cytosine on the elevation in intracranial self-stimulation (ICSS) thresholds associated with spontaneous nicotine withdrawal. (b) Effect of cytosine on response latencies. The number of animals per group was: saline–saline, $n=9$; saline–cytosine, $n=10$; nicotine–saline, $n=14$; and nicotine–cytosine, $n=12$. Asterisks ($*p<0.05$, $**p<0.01$) indicate elevated ICSS thresholds compared with the saline–saline group. Plus sign ($+p<0.05$) indicates lower ICSS thresholds compared with nicotine–saline group. Three specific periods are depicted in the figures (before, during, and after cytosine treatment) and these periods are separated by line breaks. The bar at the bottom of each figure indicates the cytosine treatment period. The rats received cytosine or saline before each time point (12–72 h). Data are expressed as means \pm SEM. Cyt, cytosine; Nic, nicotine; Sal, saline.

showed that the drug treatments affected the ICSS thresholds of the nicotine-withdrawing rats and that the thresholds of these rats returned to baseline levels over time (Drug: $F_{3,47}=14.04$, $p<0.001$; Time: $F_{4,188}=10.05$, $p<0.001$). The *post hoc* comparisons indicated that there was no difference in ICSS thresholds between the nicotine-withdrawing rats treated with varenicline or cytosine (12–72 h). Furthermore, the ICSS thresholds of the nicotine-withdrawing rats treated with varenicline ($p<0.01$) or cytosine ($p<0.05$) were lower than those of the nicotine-withdrawing rats treated with saline. The ICSS thresholds of the nicotine-withdrawing rats treated with varenicline ($p<0.001$) or cytosine ($p<0.001$) were also lower than those of the nicotine-withdrawing rats treated with 3-pyr-Cyt.

An AUC analysis was conducted to compare the cumulative effects of the various treatments on ICSS thresholds during the treatment period (12–72 h). The AUC was calculated using the following formula: $AUC = \left(\sum_{i=1}^{n-1} \frac{(m(i+1)+m_i) \times t_i}{2} \right) - (baseline \times time)$ (Pruessner et al, 2003). In this formula, t_i indicates the

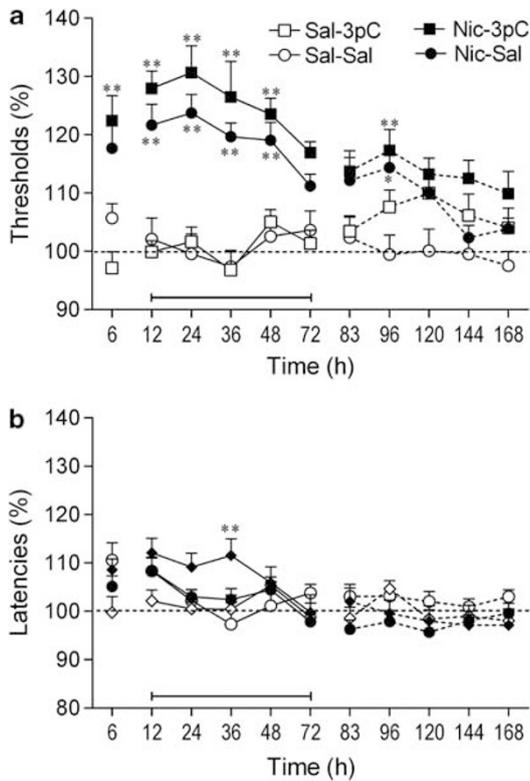


Figure 5 The cytisine derivative 3-pyr-Cyt does not affect the dysphoric-like state associated with nicotine withdrawal. (a) Effect of 3-pyr-Cyt on the elevation in intracranial self-stimulation (ICSS) thresholds associated with spontaneous nicotine withdrawal. (b) Effect of 3-pyr-Cyt on response latencies. The number of animals per group was: saline–saline, $n = 9$; saline–3-pyr-Cyt, $n = 9$; nicotine–saline, $n = 14$; and nicotine–3-pyr-Cyt, $n = 12$. Asterisks ($*p < 0.05$, $**p < 0.01$) indicate elevated ICSS thresholds or increased response latencies compared with the saline–saline group. Three specific periods are depicted in the figures (before, during, and after 3-pyr-Cyt-treatment) and these periods are separated by line breaks. The bar at the bottom of each figure indicates the 3-pyr-Cyt treatment period. The rats received 3-pyr-Cyt or saline before each time point (12–72 h). Data are expressed as means \pm SEM. Nic, nicotine; Sal, saline.

time distance between the points at which ICSS thresholds were assessed, m_i indicates the ICSS thresholds at a specific time point, n the number of time points, the *baseline* is 100 (see Figures 3–5), and *time* is the treatment period (60 h). The ANOVA analysis indicated that ICSS thresholds were elevated in the nicotine-withdrawing rats and that the drug treatments (varenicline, cytisine, and 3-pyr-Cyt) differentially affected the nicotine-withdrawing and the control rats (Pump: $F_{1,80} = 59.50$, $p < 0.001$; Drug: $F_{3,80} = 7.50$, $p < 0.001$; Pump \times Drug interaction: $F_{3, 80} = 8.38$, $p < 0.001$; Figure 6). Dunnett *t*-tests comparisons indicated that varenicline and cytisine lowered the ICSS thresholds of the nicotine-withdrawing rats. Furthermore, cytisine elevated the ICSS thresholds of the saline-treated control rats and 3-pyr-Cyt did not affect the ICSS thresholds of the nicotine-withdrawing rats or the saline-pump control rats.

DISCUSSION

The present studies investigated the effects of varenicline, cytisine, and 3-pyr-Cyt on ICSS thresholds in nicotine-naive

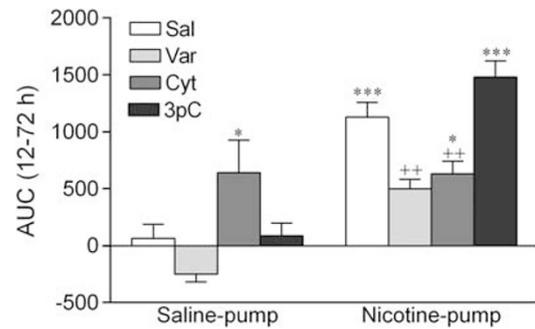


Figure 6 Area under the curve (AUC) analyses for the effects of varenicline, cytisine, and 3-pyr-Cyt on nicotine withdrawal-induced elevations in intracranial self-stimulation (ICSS) thresholds. The treatment period was from 12–72 h after removal of the minipumps. The number of animals per group was: saline–saline, $n = 9$; saline–varenicline, $n = 9$; saline–cytisine, $n = 10$; saline–3-pyr-Cyt, $n = 9$; nicotine–saline, $n = 14$; nicotine–varenicline, $n = 13$; nicotine–cytisine, $n = 12$; and nicotine–3-pyr-Cyt, $n = 12$. Asterisks ($*p < 0.05$, $**p < 0.001$) indicate higher ICSS thresholds compared with saline–saline group. Plus signs ($^{++}p < 0.01$) indicate lower ICSS thresholds compared with nicotine–saline group. Data are expressed as means \pm SEM. Cyt, cytisine; 3pC, 3-pyr-Cyt; Nic, nicotine; Sal, saline; Var, varenicline.

and nicotine-withdrawing rats. The first series of experiments showed that nicotine and varenicline, but not cytisine or 3-pyr-Cyt, lowered ICSS thresholds in nicotine-naive rats. The second series of experiments showed that varenicline and cytisine, but not 3-pyr-Cyt, diminishes the elevations in ICSS thresholds associated with nicotine withdrawal. These findings indicate that varenicline potentiates brain reward function and diminishes the dysphoric-like state associated with nicotine withdrawal. Cytisine does not potentiate brain reward function but, like varenicline, also diminishes the dysphoric-like state associated with nicotine withdrawal.

The first set of experiments showed that the effects of nicotine and varenicline on ICSS thresholds in nicotine-naive rats are somewhat similar. Both nicotine and varenicline produced an inverted U-shaped dose–effect curve. Low doses of nicotine and varenicline did not affect ICSS thresholds and medium doses lowered ICSS thresholds. A high dose of nicotine (0.6 mg/kg nicotine base) elevated the thresholds above baseline levels, whereas a high dose of varenicline (1.8 mg/kg base or 3 mg/kg salt; MW varenicline salt: 361.35 Da, MW varenicline base: 211.27 Da) did not affect ICSS thresholds. The effects of nicotine on ICSS thresholds are in line with a previous study that showed that low, but not high, doses of nicotine lower ICSS thresholds (Kenny *et al*, 2009). In this study by Kenny *et al* (2009), the highest dose of nicotine (0.5 mg/kg base) did not elevate ICSS thresholds above baseline levels. This nicotine dose was slightly lower than the highest nicotine dose in the present study (0.6 mg/kg base) and therefore it is likely that a slightly higher dose would have elevated the ICSS thresholds above baseline levels. Place conditioning studies have also shown that low doses of nicotine are rewarding (ie, place preference) and that high doses of nicotine are aversive (ie, place aversion) (Le Foll and Goldberg 2005; Fudala *et al*, 1985). It should be noted that one study has reported that a high dose of nicotine (0.5 mg/kg base) can lower ICSS thresholds (Harrison *et al*, 2002). This study is

not in line with the present ICSS study (Figure 1a) or another ICSS study by the same research group (Kenny *et al*, 2009). At this point, it is not clear what caused this discrepancy between these studies, but it should be noted that Harrison *et al* (2002) also reported a much greater nicotine-induced decrease in ICSS thresholds. The present study and Kenny *et al* (2009) showed that nicotine lowers the ICSS thresholds by 10–15%, whereas Harrison *et al* (2002) reported a 25–30% decrease in ICSS thresholds. It is unlikely that these differences were due to differences in rat strains or test procedures because male wistar rats were used for all these studies and the same test procedure was used to assess ICSS thresholds.

In the present study, one noteworthy difference between the nicotine and the varenicline dose–effect curves was the dose range that lowered ICSS thresholds. In the nicotine study, low doses of nicotine lowered ICSS thresholds, but the highest dose (0.6 mg/kg of nicotine base) elevated ICSS thresholds. In contrast, low doses of varenicline and a relatively high dose (0.6 mg/kg of varenicline base, equivalent to 1 mg/kg varenicline salt) lowered ICSS thresholds. Thus, 0.6 mg/kg of nicotine base has aversive effects and the same dose of varenicline has rewarding effects. This difference between nicotine and varenicline might be due to the fact that varenicline has a lower efficacy for a variety of nAChRs (Papke *et al*, 2011). The maximal efficacy of varenicline for the $\alpha 4\beta 2$ nAChR receptor is only 24% of that of nicotine (Coe *et al*, 2005). More importantly, a recent study showed that the efficacy of nicotine for the $\alpha 4\beta 2\alpha 5$ nAChR was 35% that of acetylcholine, whereas the efficacy of varenicline was only 9% of that of acetylcholine (Papke *et al*, 2011). The activation of $\alpha 5^*$ nAChRs in the medial habenula has an important role in the aversive effects of nicotine (Fowler *et al*, 2011). Therefore, it could be speculated that the low efficacy of varenicline for the $\alpha 5^*$ nAChR explains the fact that a relatively high dose of varenicline (0.6 mg/kg base) has rewarding effects, whereas a similar dose of nicotine (0.6 mg/kg base) has aversive effects.

In the present study, cytisine did not affect the ICSS thresholds of nicotine-naive rats. This would suggest that cytisine does not potentiate brain reward function. Very few studies have compared the effects of nicotine, cytisine, and varenicline on the brain reward system. Cytisine and varenicline have a similar affinity and efficacy for the nAChRs ($\alpha 4\beta 2^*$, $\alpha 6\beta 2^*$) that mediate the rewarding effects of nicotine (Papke *et al*, 2010; Grady *et al*, 2010; Picciotto and Kenny, 2013). Therefore, it is unlikely that the differences between varenicline and cytisine on ICSS thresholds are due to differences in nAChR binding and efficacy. One study compared the maximum nicotine, cytisine, and varenicline-induced increase in dopamine turnover in the nucleus accumbens (Coe *et al*, 2005). Nicotine (subcutaneously) induced a 177% increase in dopamine turnover in the nucleus accumbens and the effect of cytisine (subcutaneously) and varenicline (subcutaneously) were 40 and 32% of those of nicotine. In a similar study, nicotine (subcutaneously) induced a 180% increase in dopamine turnover in the nucleus accumbens and orally administered varenicline and cytisine induced a 130–140% increase in dopamine turnover (Rollema *et al*, 2010). Importantly, the same study showed that orally administered varenicline and cytisine are rapidly and completely

absorbed into the circulation (Rollema *et al*, 2010). Taken together, these studies suggest that cytisine and varenicline induce a similar increase in dopamine levels, but both these drugs are less efficacious than nicotine. In the same study, it was shown that varenicline was 20 times more potent in increasing dopamine turnover than cytisine. This is most likely because systemic administration of cytisine leads to relatively low levels of this drug in the brain (Rollema *et al*, 2010). Oral administration of cytisine and varenicline leads to similar plasma levels, but the plasma / brain ratio is 3.9 for varenicline and 0.11 for cytisine (Rollema *et al*, 2010). It has been suggested that varenicline and cytisine readily enter the brain, but that cytisine is removed via an active efflux mechanism (Rollema *et al*, 2010). Therefore, it might be possible that acute cytisine does not lower ICSS thresholds in nicotine-naive rats because it is rapidly removed from the brain. In the present study, the cytisine derivative 3-pyr-Cyt did affect ICSS thresholds. Low doses did not affect ICSS thresholds and higher doses tended to elevate the ICSS thresholds. This is mostly likely due to the fact that this compound is a very weak partial agonist at $\alpha 4\beta 2^*$ nAChRs and at high doses diminishes acetylcholine transmission (Mineur *et al*, 2009; Papke *et al*, 2010).

The second set of experiments showed that varenicline and cytisine, but not 3-pyr-Cyt, diminishes the elevations in ICSS thresholds associated with nicotine withdrawal. A close look at the data indicates that the ICSS thresholds in the nicotine-withdrawing rats treated with saline were elevated for 2 days (6–72 h). In the nicotine-withdrawing rats treated with varenicline, the ICSS thresholds were only elevated at one time point (36 h), and in the nicotine-withdrawing rats treated with cytosine, the ICSS thresholds were elevated at two time points (12 and 36 h). This pattern of results would suggest that varenicline is slightly more effective than cytisine in diminishing the dysphoric-like state associated with nicotine withdrawal. However, an additional ANOVA analysis indicated that there was no significant difference in ICSS thresholds between the nicotine-withdrawing rats treated with varenicline or cytisine. These findings suggest that both varenicline and cytisine diminish the dysphoric-like state associated with nicotine withdrawal. The administration of 3-pyr-Cyt did not diminish the elevations in ICSS thresholds associated with nicotine withdrawal. At all time points, the ICSS thresholds of the nicotine-withdrawing rats treated with 3-pyr-Cyt were higher than those of nicotine-withdrawing rats treated with saline. There were, however, no significant differences between the nicotine-withdrawing rats treated with saline or 3-pyr-Cyt. It has been reported that 3-pyr-Cyt is a very weak partial agonist at $\alpha 4\beta 2^*$ nAChRs (Mineur *et al*, 2009; Papke *et al*, 2011). The present data suggest that a very weak partial agonist at $\alpha 4\beta 2^*$ nAChRs does not attenuate the elevations in ICSS thresholds associated with nicotine withdrawal and might therefore not diminish the negative mood state associated with smoking cessation.

After the cessation of varenicline, cytisine, and 3-pyr-Cyt administration, the ICSS thresholds were assessed for an additional 4 days. The ICSS thresholds of the nicotine-withdrawing rats treated with saline, cytisine, or 3-pyr-Cyt were elevated at one time point (96 h). However, the ICSS thresholds of the nicotine-withdrawing rats treated with varenicline were elevated for an additional 2 days

(96–114 h). Cessation of varenicline administration did not lead to elevations in ICSS thresholds in the rats that were chronically treated with saline (saline-pump group). One possible explanation for the elevations in ICSS thresholds after the cessation of varenicline administration is that nicotine addiction is at least partly mediated by a nicotine-induced upregulation and desensitization of nAChRs (Dani and Balfour, 2011). Varenicline might maintain this state and therefore cessation of varenicline administration might lead to delayed nicotine withdrawal signs (Hussmann *et al*, 2012). The present finding would suggest that cessation of varenicline use in former smokers could lead to a dysphoric state. To our knowledge, the effect of varenicline withdrawal on mood states has not been thoroughly investigated in large clinical trials. There are, however, some case reports that indicate that cessation of varenicline intake in former smokers can lead to agitation, anxiety, and hallucinations and these symptoms gradually resolve over time (May and Rose, 2010; Laine *et al*, 2009).

The present study showed that cytisine is as effective as varenicline in diminishing nicotine withdrawal and there are no delayed withdrawal signs. Cytisine might also be given additional consideration as smoking cessation aid because cytisine has a lower sensitivity than varenicline for human $\alpha 3\beta 4^*$ nAChRs (ganglionic receptor) (Stokes and Papke, 2012). The $\alpha 3\beta 4^*$ nAChR is highly expressed in brain stem areas involved in cardiovascular control and stimulation of these receptors may increase the risk for cardiovascular disorders (Sobieraj *et al*, 2013; Perry *et al*, 2002).

At the end of the studies, the AUC was calculated for the various nicotine and saline groups. This was carried out to investigate the cumulative effects of the various treatments on ICSS thresholds during the drug treatment period (12–72 h). These data confirm that varenicline and cytisine diminish the elevations in ICSS thresholds associated with nicotine withdrawal to a similar degree. It was interesting to note that the ICSS thresholds in the saline-pump/cytisine-inj group were elevated. This indicates that repeated administration of a high dose of cytisine lowers ICSS thresholds in the nicotine-withdrawing rats, but elevates ICSS thresholds in the drug-naive control rats. This is in line with the observation that high doses of nicotinic receptor agonists have aversive effects (Spiller *et al*, 2009; Le Foll and Goldberg, 2005; Fudala *et al*, 1985). However, as can be seen in Figure 4a, the rats rapidly develop tolerance to the aversive effects of cytisine, whereas cytisine continues to prevent the elevations in ICSS thresholds associated with nicotine withdrawal.

It has been suggested that drugs that lower ICSS thresholds are more likely to be abused than drugs that do not affect ICSS thresholds or elevate ICSS thresholds (Kornetsky *et al*, 1979). Indeed, widely abused drugs such as cocaine and amphetamine lower ICSS thresholds (Kornetsky and Esposito, 1979; Cryan *et al*, 2003). However, despite the fact that varenicline lowers ICSS thresholds, it has been suggested that its abuse liability is extremely low (McCull *et al*, 2008). This might be because the dose-response window for nicotinic receptor agonists to potentiate brain reward function in humans is very narrow and higher doses induce negative side effects such as nausea (McCull *et al*, 2008). Although the abuse liability of varenicline is low, there is evidence that varenicline can

have unintended side effects in certain psychiatric patients. For example, clinical evidence indicates that varenicline can precipitate manic episodes in people with bipolar disorder (Hussain *et al*, 2011; Alhatem and Black, 2009; Francois *et al*, 2011; Knibbs and Tsoi, 2011). Therefore, some caution with the use of varenicline in psychiatric patients is warranted.

In conclusion, the present studies indicate that varenicline and cytisine diminish the dysphoric-like state associated with spontaneous nicotine withdrawal in rats. It is suggested that these smoking cessation aids may prevent relapse to smoking in humans by diminishing the negative mood state associated with smoking cessation.

FUNDING AND DISCLOSURE

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was funded by a James and Esther King Biomedical Research Program Grant (IKG12) to RP and AB, and a National Institute on Drug Abuse (DA023575) and Flight Attendant Medical Research Institute (Grant 52312) to AB.

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