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Dopamine $D_{1/5}$ and $D_{2/3}$ agonists differentially attenuate somatic signs of nicotine withdrawal in rats

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ABSTRACT

Abrupt tobacco/nicotine cessation after chronic use causes various withdrawal symptoms/signs. There is evidence that dysfunction of brain dopaminergic system might be responsible for some nicotine withdrawal symptoms. The hypothesis for the present study was that different dopaminergic agonists would relieve different nicotine withdrawal signs. Adult male Sprague–Dawley rats were used. (−)-Nicotine bitartrate (9 mg/kg/day, salt content) or equimolar sodium tartrate was infused into each rat via a subcutaneous (s.c.) osmotic minipump for 7 days. To assess nicotine withdrawal signs, several somatic abstinence signs including teeth-chattering/chews, stretches/gasps, ptosis, shakes, and yawns were counted one day after removal of pumps. These signs were attenuated by the s.c. injection of 0.4 mg/kg nicotine bitartrate. Both a dopamine $D_{1/5}$ agonist (SKF81297) and a $D_{2/3}$ agonist (pramipexole) relieved abstinence signs dose-dependently but differentially. SKF81297 (0.32 mg/kg, s.c.) reduced teeth-chattering/chews but not shakes. Pramipexole (1 mg/kg, s.c.) decreased both teeth-chattering/chews and shakes. A low dose of pramipexole (0.1 mg/kg, s.c.) significantly increased yawns, consistent with previous studies that the stimulation of D_3 receptors induces yawning. These results indicate that a D_2-selective agonist should be considered a candidate to relieve nicotine withdrawal symptoms.

1. Background

A role of dopamine (DA) in the reinforcing effects of nicotine/tobacco smoking is well established. On the other hand, the precise role of DA during nicotine/tobacco abstinence is less well known. Fung et al. (1996) found a significant reduction of DA content in nucleus accumbens in rats after 24 h withdrawal from chronic nicotine administration. Duchemin et al. (2009) showed that basal DA release in mice striatal slices was decreased 12 and 24 h after chronic nicotine discontinuation. Some chronic nicotine rat studies using *in vivo* microdialysis have demonstrated that abrupt nicotine cessation or mecamylamine decreases basal levels of DA release in the nucleus accumbens (Hildebrand et al., 1998; Takahashi et al., 1998; Rada et al., 2001; Rahman et al., 2004). A monkey study showed that basal levels of DA release in the dorsal striatum also decreased after overnight abstinence from daily nicotine (Domino and Tsukada, 2009). A human study demonstrated that smokers abstinent from tobacco for 11 to 17 h have only 54% of the cerebrospinal fluid concentration of the DA metabolic homovanillic acid (HVA) of nonsmokers (Geracioti et al., 1999). Dagher et al. (2001) reported reduced D_1 receptor binding in the ventral striatum of cigarette smokers.

Recently, we found that L-DOPA reduces signs of nicotine withdrawal in rats (Ohmura et al., 2011). The next question to answer was whether D_1 and D_2 receptor families affect different signs of nicotine abstinence. Therefore, we examined whether a dopamine $D_{1/5}$ agonist (SKF81297), and a DA $D_{2/3}$ agonist (pramipexole) are effective in relieving nicotine withdrawal signs. If decreased DA levels after nicotine abstinence were a cause of nicotine withdrawal signs, selective dopaminergic agonists would be differentially effective. This manuscript describes the results obtained.

2. Materials and methods

2.1. Animals

Male Sprague Dawley rats (Harlan, Indianapolis, IN), weighing 260–320 g at the beginning of the experiment, were housed 2–3 per cage at a constant temperature of 20–21 °C. Animals were maintained on a 12 h light:dark cycle (lights on at 7:00, lights off at 19:00). Each animal had free access to rodent chow and water. Animal treatment complied with the NIH Animal Care Guidelines, and all procedures were performed according to a protocol approved by the University of Michigan Committee for Use and Care of Animals.

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2.2. Drugs

(−)-Nicotine bitartrate salt, sodium tartrate and SKF81297 were purchased from Sigma-Aldrich, St. Louis, MO, USA. Pramipexole was obtained from Boehringer Ingelheim Co. SKF81297 (R-(+)-6-Chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrobromide) was dissolved in distilled water and other drugs were dissolved in saline. The pH of the nicotine solution was adjusted using NaOH to approximately 7.0. Equimolar sodium tartrate dissolved in saline was used as the control solution for nicotine solution. All doses are expressed as salt. They were administered s.c. in a volume of 1 ml/kg.

2.3. Surgical procedure

2.3.1. Osmotic minipump implantations and removals

For chronic nicotine administration, osmotic minipumps (Model 2ML2, Durect Corporation, Cupertino, CA, USA) for drug infusion were surgically implanted s.c. between the scapulae under ketamine (90 mg/kg i.p.) and xylazine (10 mg/kg i.p.) anesthesia. The pumps were filled with either control solution or nicotine. The nicotine concentration was adjusted to deliver a dose of 9 mg/kg/day of nicotine salt (3.16 mg/kg/day nicotine base). Nicotine or the control solution was infused via implanted pumps at 5 μl/h for 1 week. This dose and duration has been used in previous studies of nicotine withdrawal (Malin et al., 1992; Hamilton et al., 2009). Moreover, the blood concentrations resulting from this dose in rats are almost the same concentrations as those measured in heavy smokers (Benowitz et al., 1982; LeSage et al., 2002). One week after implantation of osmotic minipumps, minipumps were surgically removed under ketamine (90 mg/kg i.p.) and xylazine (10 mg/kg i.p.) anesthesia.

2.3.2. Spontaneous somatic signs of nicotine withdrawal

Behavioral observations were performed from 9 to 12 a.m. in a clear plastic observation chamber (48×23×20 cm). Rat behaviors were observed 20–24 h after pump removal, based upon previous studies (Malin et al., 1992; Rylkova et al., 2008; Paterson et al., 2008). Rat behaviors were counted for 30 min by observers blind to the experimental conditions. Teeth-chattering/chews with empty mouth, stretches/gasps, shakes, ptosis, and miscellaneous other less frequent signs (*e.g.* diarrhea and yawns) were counted. Although the counting method is based on previous studies (Malin et al., 1992, 2006), "stretches" instead of "writhes" were noted because writhes were not observed. Tremors were not recorded because none was observed. To accurately assess teeth-chattering/chews with an empty mouth, bedding was not used in the observation chamber. Ptosis was not counted when a rat took a resting position to avoid confounding ptosis with sleeping. Ptosis was counted only once per min.

2.4. Experiment 1: effects of acute nicotine administration on nicotine withdrawal signs

Nicotine or equimolar sodium tartrate was infused into each rat via an s.c. osmotic minipump for 7 days. One day after removal of the pump, behavioral signs were counted. Rats received an s.c. injection of 0.4 mg/kg nicotine or saline 3 min prior to behavioral observation. Malin et al. (1992) showed that this dose of nicotine alleviated nicotine withdrawal signs.

2.5. Experiment 2: effects of acute $D_{1/5}$ agonist administration on nicotine withdrawal signs

Nicotine was infused into each rat via an s.c. osmotic minipump for 7 days. One day after removal of the pump, behavioral signs were counted. Rats received an s.c. injection of SKF81297 (0.032 mg/kg or 0.32 mg/kg) or sterilized water 10 min prior to behavioral observation.

2.6. Experiment 3: effects of acute $D_{2/3}$ agonist administration on nicotine withdrawal signs

Nicotine was infused into each rat via an s.c. osmotic minipump for 7 days. Rats received s.c. injection of pramipexole (0.1 mg/kg or 1 mg/kg) or saline 10 min prior to behavioral observation.

2.7. Data analysis

The number of nicotine withdrawal signs was analyzed by using one-way analysis of variance (ANOVA). Multiple comparisons with Bonferroni's correction were also conducted following each ANOVA if necessary. There was no significant difference among groups that received 7 day control tartrate infusion and acute saline or sterile water injection in each experiment despite the different time points of the injection (see above). Hence, that data were combined and used as the control groups for statistical analysis to decrease the number of sacrificed animals. The *alpha* level was set at 0.05 for all comparisons. All statistical procedures were conducted using SPSS (version 15.0 J).

3. Results

3.1. Experiment 1: effects of acute nicotine administration on nicotine withdrawal signs

One-way ANOVA showed a significant main effect of treatment conditions on overall withdrawal signs ($F(2, 30) = 24.95$, $P<0.01$, see

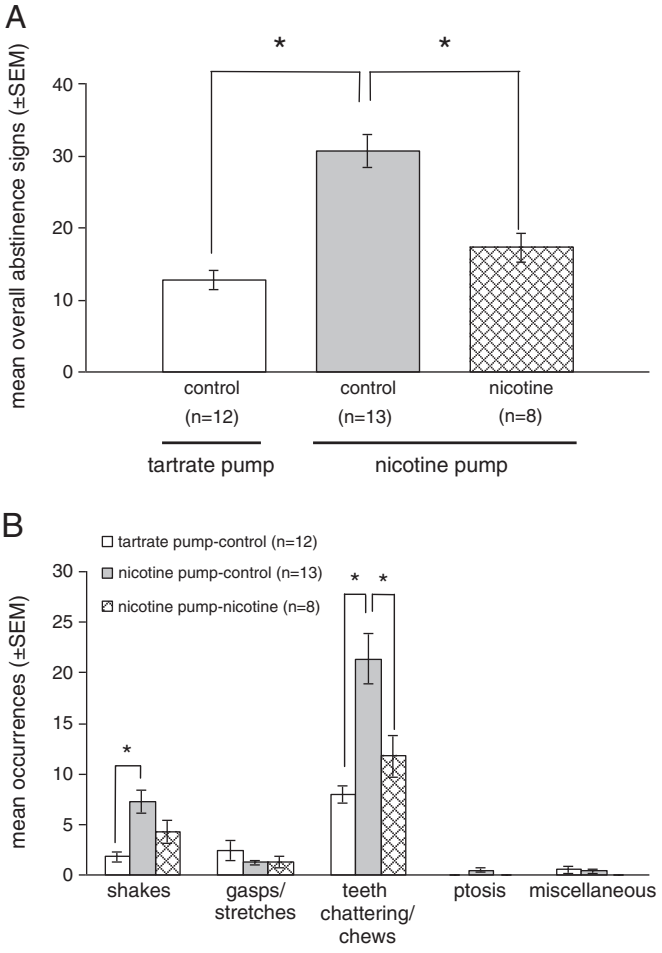

Fig. 1. Effects of nicotine on (A) overall somatic abstinence signs and (B) each category of somatic signs. Behavioral observation was conducted 20–24 h after removal of pump. Nicotine (0.4 mg/kg, s.c.) was injected 3 min before the observation. * $P<0.05$.

Fig. 1A). Post hoc comparisons showed that 0.4 mg/kg nicotine significantly attenuated overall withdrawal signs ($P<0.05$, Fig. 1A).

When withdrawal signs were divided into each category, one-way ANOVA indicated a significant main effect of treatment conditions on teeth-chattering/chews ($F(2, 30)=13.66$, $P<0.01$, Fig. 1B) and shakes ($F(2, 30)=8.91$, $P<0.01$, Fig. 1B), but not other signs. Post hoc comparisons showed that 0.4 mg/kg nicotine significantly reduced teeth-chattering/chews ($P<0.05$, Fig. 1B). Acute nicotine administration decreased shakes somewhat but this did not reach statistical significance in post hoc tests.

3.2. Experiment 2: effects of acute $D_{1/5}$ agonist administration on nicotine withdrawal signs

One-way ANOVA revealed a significant main effect of treatment conditions on overall withdrawal signs ($F(3, 37)=18.76$, $P<0.01$, see Fig. 2A). Post hoc comparisons showed that 0.32 mg/kg of SKF81297 significantly decreased overall withdrawal signs ($P<0.05$, Fig. 2A) while 0.032 mg/kg of SKF81297 did not affect them.

When withdrawal signs were divided into each category, one-way ANOVA indicated a significant main effect of treatment conditions on teeth-chattering/chews ($F(3, 37)=11.79$, $P<0.01$, see Fig. 1B) and shakes ($F(3, 37)=6.33$, $P<0.01$, see Fig. 2B), but not other signs. Post hoc comparisons showed that 0.32 mg/kg of SKF81297 significantly alleviated teeth-chattering/chews ($P<0.05$, Fig. 2B). Although SKF81297 administration somewhat reduced shakes, this did not reach statistical significance in post hoc tests.

3.3. Experiment 3: effects of acute $D_{2/3}$ agonist administration on nicotine withdrawal signs

One-way ANOVA indicated a significant main effect of treatment conditions on overall withdrawal signs ($F(3, 37)=23.36$, $P<0.01$, see Fig. 3A). Post hoc comparisons showed that 1 mg/kg pramipexole significantly mitigated overall withdrawal signs while 0.1 mg/kg pramipexole worsened them ($P<0.05$, Fig. 3A).

When withdrawal signs were divided into each category, one-way ANOVA revealed a significant main effect of treatment conditions on shakes ($F(3, 37)=19.30$, $P<0.01$, Fig. 3B), teeth-chattering/chews ($F(3, 37)=13.49$, $P<0.01$, Fig. 3B), ptosis ($F(3, 37)=8.87$, $P<0.01$, Fig. 3B), miscellaneous other signs ($F(3, 37)=39.74$, $P<0.01$, see Fig. 3B). In Experiment 3, "miscellaneous other signs" were equal to the number of yawns. Post hoc comparisons showed that 0.1 and 1 mg/kg pramipexole significantly decreased shakes, and that 1 mg/kg pramipexole significantly decreased teeth-chattering/chews ($P<0.05$, see Fig. 3B) while 0.1 mg/kg pramipexole did not affect them. Moreover, 0.1 mg/kg pramipexole significantly increased ptosis and yawning ($P<0.05$, see Fig. 3B).

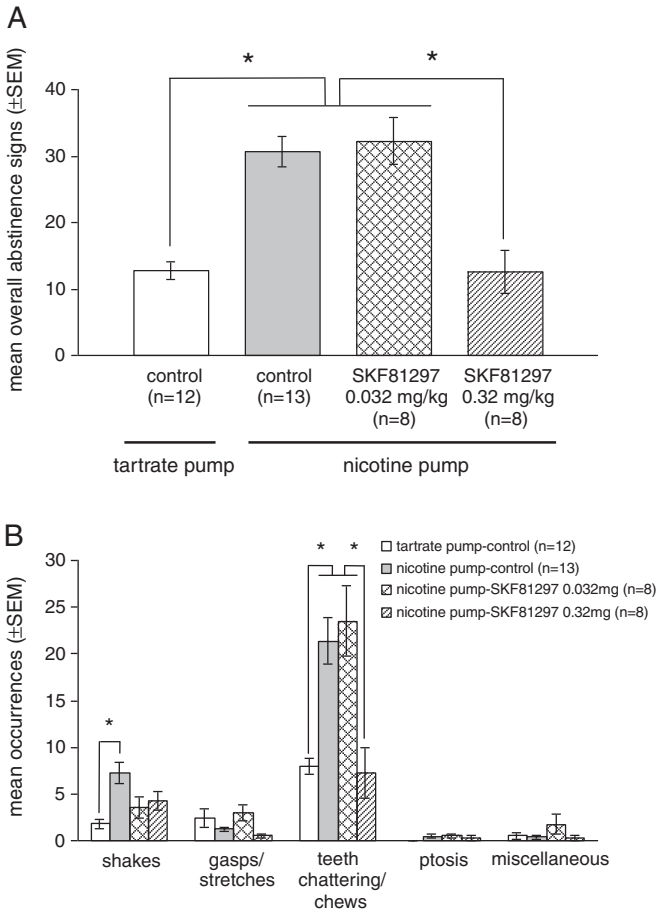

Fig. 2. Effects of $D_{1/5}$ agonist on (A) overall somatic abstinence signs and (B) each category of somatic signs. Behavioral observation was conducted 20–24 h after removal of pump. SKF81297 (0.032 or 0.32 mg/kg, s.c.) was injected 10 min before the observation. * $P<0.05$.

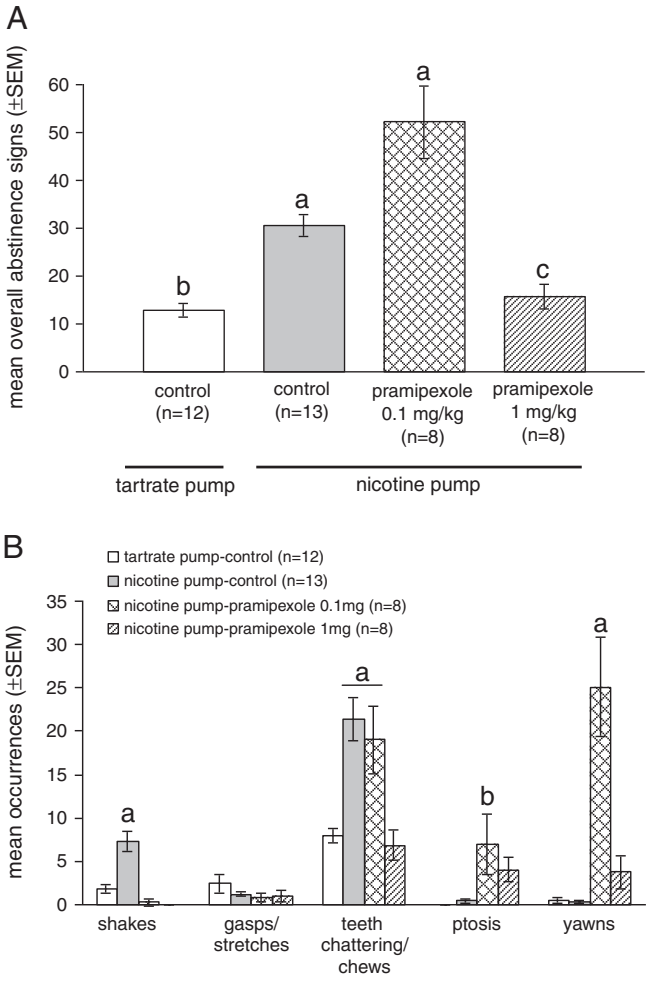

Fig. 3. Effects of $D_{2/3}$ agonist on (A) overall somatic abstinence signs and (B) each category of somatic signs. Behavioral observation was conducted 20–24 h after removal of pump. Pramipexole (0.1 or 1 mg/kg, s.c.) was injected 10 min before the observation. a: $P<0.05$, to all other groups, b: $P<0.05$, to all other groups except to pramipexole 1 mg group, c: $P<0.05$, to all other groups except to tartrate pump group.

4. Discussion

Both a D_1 agonist (SKF81297) and a $D_{2/3}$ agonist (pramipexole) mitigated nicotine withdrawal-induced somatic signs dose dependently, but differentially (Figs. 2 and 3). These results further support our hypothesis that decreased DA levels after nicotine abstinence are a cause of nicotine withdrawal signs. Moreover, small doses of nicotine as control alleviated nicotine withdrawal signs (Fig. 1), indicating that these signs appropriately reflected nicotine withdrawal.

SKF81297 0.32 mg/kg reduced overall somatic signs while 0.032 mg/kg did not. Moreover, SKF81297 did not reduce nicotine withdrawal-induced wet dog shakes while it attenuated teeth-chattering/chews (Fig. 2B). Reavill et al. (1993) demonstrated that 0.2–0.4 mg/kg of SKF81297 stimulate responses to the lever associated with SKF81297 beforehand while 0.025 mg/kg did not. Our results are consistent with this study. However, SKF81297 has discriminative stimulus effects (Reavill et al., 1993; Rosenzweig-Lipson and Bergman, 1993) and might cause drug abuse when treated chronically. SKF81297 also attenuates cue-evoked reinstatement of cocaine-seeking behavior in rats (Alleweireldt et al., 2002).

Although 1 mg/kg pramipexole attenuated nicotine withdrawal signs, 0.1 mg/kg pramipexole worsened them by increasing ptosis and yawning. Collins et al. (2005, 2007, 2008, 2009) have demonstrated that s.c. injection of 0.1 mg/kg pramipexole induces yawning while 1 mg/kg pramipexole does not. Our results are consistent with their results. They also showed that the effects of 0.1 mg/kg pramipexole are largely due to the stimulation of D_3 receptor while the effects of 1 mg/kg pramipexole are due to both D_2 and D_3 receptors. Thus, D_2 selective agonists, but not D_3 agonists, could be therapeutic agents for nicotine withdrawal syndromes.

It is notable that only one significant withdrawal effect (teeth chattering/chews) was obtained by nicotine. This raises an issue regarding how well "withdrawal" was modeled in this study. Furthermore, the large doses of praxipexole tended to induce yawning and ptosis, indicating possible drug induced side effects.

Withdrawal signs assessed in the present study are probably reflecting irritability (Malin et al., 1992; Malin and Goyarzu, 2009). Irritability is the most frequently reported symptom during smoking cessation (Hughes, 2007). To alleviate it would help smokers who want to quit smoking. However, it should be noted that depressed mood and craving for tobacco are better predictors of cessation than other symptoms including irritability (Paperwalla et al., 2004; Hughes, 2007, 2008).

Pramipexole produces antidepressant-like effects in rodents via activation of D_2 receptors (Maj et al., 1997; Siuciak and Fujiwara, 2004). These findings also support the idea that D_2 selective agonists, but not D_3 agonists, could be a therapeutic agent for nicotine withdrawal syndrome. It should be noted that pramipexole also has discriminative stimulus effects (Koffarnus et al., 2009). Although our results show that a $D_{1/5}$ agonist also attenuates some withdrawal signs, some $D_{1/5}$ agonists easily cause tolerance (Asin and Wirtshafter, 1992; Asin et al., 1995).

In summary, a D_2 receptor agonist is relatively effective for reducing nicotine withdrawal-induced somatic signs compared to a $DA_{1/5}$ receptor agonist. A US patent #6410579, filed February 14, 2001, was assigned to the former Pharmacia and Upjohn Company for the use of pramipexole and derivatives for the treatment of addictive disorders including nicotine/tobacco dependence. To our knowledge, the current holders of that patent have not pursued the use of pramipexole as an adjunct therapy for nicotine/tobacco withdrawal.

Disclosure/conflict of interest

The authors have no conflict of interest.

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References

Alleweireldt AT, Weber SM, Kirschner KF, Bullock BL, Neisewander JL. Blockade or stimulation of D_1 dopamine receptors attenuates cue reinstatement of extinguished cocaine-seeking behavior in rats. Psychopharmacol 2002;159:284–93.
Asin KE, Bednarz L, Nikkei A, Perner R. Rotation and striatal c-fos expression after repeated daily treatment with selective dopamine receptor agonists and levodopa. J Pharmacol Exper Ther 1995;273:1483–90.
Asin KE, Wirtshafter D. Effects of repeated dopamine D_1 receptor stimulation on rotation and c-fos expression. Eur J Pharmacol 1992;235:167–8.
Benowitz NL, Kuyt F, Jacob III P. Circadian blood nicotine concentrations during cigarette smoking. Clin Pharmacol Ther 1982;32:758–64.
Collins GT, Witkin JM, Newman AH, Svensson KA, Grundt P, Cao J, Woods JH. Dopamine agonist-induced yawning in rats: a dopamine D_3 receptor-mediated behavior. J Pharmacol Exp Ther 2005;314:310–9.
Collins GT, Newman AH, Grundt P, Rice KC, Husbands SM, Chauvignac C, Chen J, Wang S, Woods JH. Yawning and hypothermia in rats: effects of dopamine D_3 and D_2 agonists and antagonists. Psychopharmacol 2007;193:159–70.
Collins GT, Calinski DM, Newman AH, Grundt P, Woods JH. Food restriction alters N′-propyl-4,5,6,7-tetrahydrobenzothiazole-2,6-diamine dihydrochloride (pramipexole)-induced yawning, hypothermia, and locomotor activity in rats: evidence for sensitization of dopamine D2 receptor-mediated effects. J Pharmacol Exp Ther 2008;325:691–7.
Collins GT, Truccone A, Haji-Abdi F, Newman AH, Grundt P, Rice KC, Husbands SM, Greedy BM, Enguehard-Gueiffier C, Gueiffier A, Chen J, Wang S, Katz JL, Grandy DK, Sunahara RK, Woods JH. Proerectile effects of dopamine D_2-like agonists are mediated by the D_3 receptor in rats and mice. J Pharmacol Exp Ther 2009;329:210–7.
Dagher A, Bleicher C, Aston JA, Gunn RN, Clarke PB, Cumming P. Reduced dopamine D_1 receptor binding in the ventral striatum of cigarette smokers. Synapse 2001;42:48–53.
Domino EF, Tsukada H. Nicotine sensitization of monkey striatal dopamine release. Eur J Pharmacol 2009;607:91–5.
Duchemin AM, Zhang H, Neff NH, Hadjiconstantinou M. Increased expression of VMAT2 in dopaminergic neurons during nicotine withdrawal. Neurosci Lett 2009;467:182–6.
Fung YK, Schmid MJ, Anderson TM, Lau YS. Effects of nicotine withdrawal on central dopaminergic systems. Pharmacol Biochem Behav 1996;53:635–40.
Geracioti TD, West AS, Baker DG, Hill KK, Ekhator NN, Wortman MD, Keck PE, Norman AB. Low CSF concentration of dopamine metabolite in tobacco smokers. Am J Psychiat 1999;156:130–2.
Hamilton KR, Berger SS, Perry ME, Grunberg NE. Behavioral effects of nicotine withdrawal in adult male and female rats. Pharmacol Biochem Behav 2009;92:51–9.
Hildebrand BE, Nomikos GG, Hertel P, Schilström B, Svensson TH. Reduced dopamine output in the nucleus accumbens but not in the medial prefrontal cortex in rats displaying a mecamylamine-precipitated nicotine withdrawal syndrome. Brain Res 1998;779:214–25.
Hughes JR. Effects of abstinence from tobacco: etiology, animal models, epidemiology, and significance: a subjective review. Nicotine Tob Res 2007;9:329–39.
Hughes JR. Smoking and suicide: a brief overview. Drug Alcohol Depend 2008;98:169–78.
Koffarnus MN, Greedy B, Husbands SM, Grundt P, Newman AH, Woods JH. The discriminative stimulus effects of dopamine D_2- and D_3-preferring agonists in rats. Psychopharmacol 2009;203:317–27.
LeSage MG, Keyler DE, Shoeman D, Raphael D, Collins G, Pentel PR. Continuous nicotine infusion reduces nicotine self-administration in rats with 23-h/day access to nicotine. Pharmacol Biochem Behav 2002;72:279–89.
Maj J, Rogóz Z, Skuza G, Kołodziejczyk K. Antidepressant effects of pramipexole, a novel dopamine receptor agonist. J Neural Transm 1997;104:525–33.
Malin DH, Goyarzu P. Rodent models of nicotine withdrawal syndrome. Handb Exp Pharmacol 2009;192:401–34.
Malin DH, Lake JR, Newlin-Maultsby P, Roberts LK, Lanier JG, Carter VA, Cunningham JS, Wilson OB. Rodent model of nicotine abstinence syndrome. Pharmacol Biochem Behav 1992;43:779–84.
Malin DH, Lake JR, Smith TD, Khambati HN, Meyers-Paal RL, Montellano AL, Jennings RE, Erwin DS, Presley SE, Perales BA. Bupropion attenuates nicotine abstinence syndrome in the rat. Psychopharmacol 2006;184:494–503.
Ohmura Y, Jutkiewicz E Domino EF: L-DOPA attenuates rat nicotine withdrawal-induced behaviors. Pharmacol. Biochem. Behav. In press, 2011.
Paperwalla KN, Levin TT, Weiner J, Saravay SM. Smoking and depression. Med Clin North Am 2004;88:1483–94.
Paterson NE, Semenova S, Markou A. The effects of chronic versus acute desipramine on nicotine withdrawal and nicotine self-administration in the rat. Psychopharmacol 2008;198:351–62.
Rada P, Jensen K, Hoebel BG. Effects of nicotine and mecamylamine-induced withdrawal on extracellular dopamine and acetylcholine in the rat nucleus accumbens. Psychopharmacology 2001;157:105–10.

Rahman S, Zhang J, Engleman EA, Corrigall WA. Neuroadaptive changes in the mesoaccumbens dopamine system after chronic nicotine self-administration: a microdialysis study. Neurosci 2004;129:415–24.

Reavill C, Bond B, Overend P, Hunter AJ. Pharmacological characterization of the discriminative stimulus properties of the dopamine D1 agonist, SKF 81297. Behav Pharmacol 1993;4:135–46.

Rosenzweig-Lipson S, Bergman J. Dopamine D1 receptor involvement in the discriminative-stimulus effects of SKF 81297 in squirrel monkeys. J Pharmacol Exp Ther 1993;267:765–75.

Rylkova D, Boissoneault J, Isaac S, Prado M, Shah HP, Bruijnzeel AW. Effects of NPY and the specific Y1 receptor agonist [D-His^{26}]-NPY on the deficit in brain reward function and somatic signs associated with nicotine withdrawal in rats. Neuropeptides 2008;42:215–27.

Siuciak JA, Fujiwara RA. The activity of pramipexole in the mouse forced swim test is mediated by D2 rather than D3 receptors. Psychopharmacol 2004;175:163–9.

Takahashi H, Takada Y, Nagai N, Urano T, Takada A. Effects of nicotine and footshock stress on dopamine release in the striatum and nucleus accumbens. Brain Res Bull 1998;45:157–62.