Brief report

Reversal of Nicotine Withdrawal Signs Through Positive Allosteric Modulation of $\alpha 4\beta 2$ Nicotinic Acetylcholine Receptors in Male Mice

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Abstract

Introduction: Nicotine withdrawal symptoms are important factors in determining the relapse rate to tobacco smoking and drugs that diminish these symptoms would potentially have a higher success rate as smoking cessation aids. Unlike US Food and Drug administration approved smoke cessation aids (nicotine and varenicline) which act as nicotinic acetylcholine receptors (nAChRs) agonists, desformylflustrabromine (dFBr) acts as a nAChR positive allosteric modulator with higher selectivity to the $\alpha 4\beta 2$ nAChR. In animal studies, dFBr was well tolerated and reduced intravenous nicotine self-administration. In this study, we use behavioral test in mouse model of spontaneous nicotine withdrawal to assess the effect of dFBr on nicotine withdrawal symptoms.

Methods: Spontaneous nicotine withdrawal in nicotine-dependent ICR male mice was established 18–24 h after termination (minipump removal) of 14 days infusion of nicotine. After that (day 15), spontaneous signs of nicotine withdrawal were examined in the following order: anxiety-like behaviors, somatic signs, and then hyperalgesia using previously published behavioral protocols. Fifteen minutes before withdrawal signs testing, mice received a subcutaneous acute injection of vehicle or dFBr at the doses of 0.02, 0.1, and 1 mg/kg to determine the effect of dFBr on nicotine withdrawal symptoms.

Results: dFBr produced dose-dependent reversal of nicotine withdrawal signs in mouse model of spontaneous nicotine withdrawal.

Implications: Positive allosteric modulators of nAChR such as dFBr reduce nicotine withdrawal symptoms supporting the potential clinical use of this novel class of nAChR-based therapeutics as smoking cessation aid.

Introduction

Tobacco smoking raises the risk of cancer, cardiovascular, and pulmonary diseases and contributes to several other preventable diseases and disabilities. While tobacco smoke contains hundreds of chemicals, nicotine is the main active ingredient in tobacco smoke that leads to and maintains tobacco addiction.¹ Nicotine acts on nicotinic acetylcholine

receptors (nAChRs) expressed within the brain reward pathways including dopaminergic, GABAergic, and glutamatergic neurons that project from or to the ventral tegmental area (VTA).² Activation of nAChRs, especially the $\alpha 4\beta 2^*$ subtype, increases the firing rate of VTA dopaminergic neurons and increases dopamine level in the nucleus accumbens which is believed to mediate the rewarding effects associated with tobacco smoking.^{3–5} Upon continuous use of tobacco

products and sustained brain exposure to nicotine, dependence develops and smokers find it too difficult to quit even with the help of smoking cessation aids.⁶ However, currently available smoking cessation aids such as nAChR agonists (nicotine replacement therapy and varenicline) are modestly efficacious and associated with side effects. Therefore, more effective and safer pharmacological interventions for smoking cessation are still needed. To this end, there are increasing efforts to develop positive allosteric modulators (PAMs) that are selective for the $\alpha 4\beta 2^*$ nAChR subtype for treatment of nicotine dependence and neurological disorders.^{7,8} Unlike agonists, nAChR PAMs do not directly activate nAChRs; instead they enhance nAChR responses to the endogenously released ACh. As such, nAChR PAMs represent a more physiological alternative to classical nAChR agonists and potentially have higher nAChR subtype selectivity.⁹

To date, a number of nAChR PAMs with higher selectivity to the $\alpha 4\beta 2$ nAChRs, the primary nAChRs subtype mediating the addictive properties of nicotine, have been identified including desformylflustrabromine (dFBr). dFBr is a naturally occurring alkaloid that first isolated from *Flustra foliacea* then synthesized and its effect on nAChRs was demonstrated.^{10,11} dFBr enhances ACh-induced responses of $\alpha 4\beta 2$ nAChRs increasing ACh efficacy at the two known isoforms of $\alpha 4\beta 2$ nAChRs [($\alpha 4$)3($\beta 2$)2 and ($\alpha 4$)2($\beta 2$)3 nAChRs].^{11–13} Furthermore, dFBr has been shown to reduce intravenous nicotine self-administration in rats and to modify the discriminative stimulus effects of nicotine.^{14,15}

Withdrawal symptoms are one of the primary causes of high tobacco smoking relapse rates,¹⁶ and it is important to test the effects of potential nicotine dependence pharmacotherapies on nicotine withdrawal symptoms. Because the $\alpha 4\beta 2^*$ nAChRs play an important role in nicotine withdrawal, we hypothesized that $\alpha 4\beta 2$ PAMs like dFBr, would reverse withdrawal signs. To this end, in this study we examined the effect of acute dFBr administration in a well-established mouse model of nicotine withdrawal. Our results demonstrate that dFBr reverses important aspects of nicotine withdrawal extending in vivo characterization of dFBr as a potential drug for the treatment of nicotine addiction.

Methods

Subjects

All animal experiments described in this study were conducted on adult (20–25 g; 8–10 weeks old) male ICR mice using protocols approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. ICR mice were purchased from Harlan Laboratories (Indianapolis, IN) and housed in groups of five with free access to food and water in an Association for Assessment and Accreditation of Laboratory Animal Care approved facility. Mice received corn cob bedding and were fed Envigo Teklad mouse diet 7102 (LM-485) and were kept at 12 light/12 dark cycle and behavioral experiments were performed during the light cycle.

Drugs

(–)-Nicotine hydrogen tartrate salt [(–)-1-Methyl-2-(3-pyridyl) pyrrolidine (+)-bitartrate salt], was purchased from Sigma-Aldrich (St. Louis, MO). dFBr was obtained from Tocris Biosciences (Minneapolis, MN). All drugs were dissolved in physiological saline (0.9% sodium chloride) to the required doses expressed as the free base of the drug. dFBr at doses (0.02, 0.1, and 1 mg/kg body weight) were injected subcutaneously (s.c.) in a total volume of 1 mL/100 g body weight.

Chronic Administration of Nicotine

Subcutaneous Alzet osmotic minipumps [(model 2002); Durect Corporation, Cupertino, CA] were used to achieve continuous nicotine administration to ICR mice. Nicotine and saline minipumps were implanted in mice under anesthesia (45 mg/kg sodium pentobarbital, i.p.) and kept at a constant flow rate to deliver 24 mg nicotine/kg animal body weight/day for 14 days.

Behavioral Assessment of Nicotine Withdrawal

Following 14 days minipump infusion of 24 mg nicotine/kg animal body weight/day, minipumps were removed from mice (in the evening of day 14) and behavioral observation were performed on day 15, 18–24 h after minipump removal. Mice were pretreated 15 min before behavioral observations with either vehicle or dFBr (0.02, 0.1, and 1 mg/kg; s.c.) to assess the effects of acute dFBr administration on spontaneous withdrawal from nicotine. Behavioral tests were performed by an observer blinded to experimental treatment and in specific testing sequence known to produce the most consistent results with minimal within-group variability.¹⁷ The mice were first evaluated for 5 min in the plus maze test for anxiety-related behavior, followed by a 20-min observation of somatic signs measured as paw and body tremors, head shakes, backing, jumps, curls, and ptosis. Hyperalgesia was evaluated in the hot-plate test (52°C) immediately following the somatic sign observation period as previously described.¹⁷

Data Analysis

All behavioral data analyses were performed using the GraphPad software, version 6.0 (GraphPad Software, Inc., La Jolla, CA). Data were expressed as the mean $\pm$ SEM and assessed for the normality of the residuals and equal variance using either the *F*-test or the Brown–Forsythe test and the Bartlett's test before statistical analyses. All data passed these tests as indicated by similar Variances among groups. Statistical analyses included a one-way analysis of variance (ANOVA) followed by the Tukey post hoc correction with *p*-values $< .05$ considered significant.

Results

Anxiety-Related Signs of Nicotine Withdrawal

Anxiety-related behavior signs were measured in mice following 18–24 h withdrawal from 14 days infusion of nicotine (24 mg/kg/day) or saline and following acute treatment with dFBr or vehicle. Consistent with previous reports,¹⁸ nicotine withdrawal (nicotine minipump) mice demonstrated a significant ($p < .05$) increase in anxiety-related behavior in the plus maze (assessed as a decrease in time spent in the open arms) compared to control (saline minipump) mice (Figure 1A). Acute s.c. administration of 1 mg/kg dFBr or vehicle to saline minipump (nicotine naïve) mice did not significantly ($p > .05$) alter anxiety-related behavior indicating that dFBr does not induce anxiety-related behavior in the absence of nicotine. In contrast, acute s.c. administration of 0.02, 0.1, and 1 mg/kg dFBr to nicotine minipump mice (nicotine withdrawal group) produced a dose-dependent reversal of anxiety-related behavior (Figure 1A). One way ANOVA followed by Tukey post hoc revealed that dFBr at 1 mg/kg significantly ($F_{5,42} = 9.837$, $p < .0001$) reverse nicotine withdrawal anxiety-related behavior. Analyses of the average total number of arm crosses for each treatment group revealed no significant difference among groups (Table 1) indicating that the observed

Figure 1. Effects of dFBr on anxiety-like withdrawal signs in the mouse. Male ICR mice were chronically infused with saline or nicotine (24 mg/kg/day) for 14 days. After minipumps (MPs) removal, mice received dFBr (0.02, 0.1, and 1 mg/kg, s.c.) or vehicle. Fifteen min later, (A) anxiety-like behaviors (time spent in the open arm), (B) somatic signs, and (C) hyperalgesia (latency time) were measured. SV = (saline MP + vehicle); d1 = (saline MP + 1 mg/kg dFBr); NV = (nicotine MP + vehicle). Each point represents the mean $\pm$ SEM of 8 mice per group. *Significant difference ($p < .05$) compared to control SV group and #significant difference ($p < .0001$) compared to NV group.

Table 1. dFBr Does Not Have an Effect on the Average Number of Arm Crosses in the Elevated Plus Maze Test

Treatment	Average number of arm crosses $\pm$ SEM
Saline MP-vehicle	9.1 $\pm$ 0.5
Saline MP-dFBr (1)	8.6 $\pm$ 0.6
Nicotine MP-vehicle	8.4 $\pm$ 0.7
Nicotine MP-dFBr (0.02)	8.1 $\pm$ 0.6
Nicotine MP-dFBr (0.1)	8.8 $\pm$ 0.6
Nicotine MP-dFBr (1)	9.0 $\pm$ 0.5

Mice undergoing nicotine withdrawal received dFBr (0.02, 0.1, and 1 mg/kg, s.c.) or vehicle. The average numbers of arm crosses were recorded in the plus maze test. The numbers are presented as the total number of arm crosses $\pm$ SEM ($n = 8$). dFBr = desformylflustrabromine; MP = minipump; SEM = standard error of the mean.

reductions in time mice spent in the open arms of the plus maze were not related to changes in mice locomotor activity.

Somatic Signs of Nicotine Withdrawal

The effects of dFBr administration on somatic signs of spontaneous nicotine withdrawal were assessed using same nicotine withdrawal (nicotine minipumps) and control (saline minipumps) mice described above. Following pretreatment with dFBr or vehicle and plus maze test, somatic signs (paw and body tremors, head shakes, backing, jumps, curls, and ptosis) were observed for 20 min. Nicotine minipumps mice developed somatic signs (Figure 1B), indicated by a significant ($p < .05$) increase in number of observations compared to the

control (saline minipumps) mice. Similar to anxiety-related behavior, acute s.c. administration of the highest dose of dFBr did not alter somatic signs in the control (saline minipump) mice ($p > .05$) whereas dFBr pretreatment produce dose-dependent reversal of somatic signs in nicotine minipump mice (Figure 1B). One way ANOVA followed by Tukey post hoc revealed significant ($F_{5,42} = 86.34$, $p < .0001$) dFBr effect at both 0.1 and 1 mg/kg.

Hyperalgesia Signs of Nicotine Withdrawal

Following testing anxiety-related and somatic signs of nicotine withdrawal, same mice groups (nicotine or saline minipumps then acute dFBr or vehicle pretreatment) were evaluated for hyperalgesia using the hot-plate test (Figure 1C). Withdrawal from chronic nicotine exposure (nicotine minipump mice) resulted in significant ($p < .05$) decrease in hot plate latency (hyperalgesia) compared to saline minipump mice. While s.c. of 1 mg/kg dFBr with produced no change in hot plate latency in saline minipump mice, s.c. injection of 0.02 or 0.1 mg/kg dFBr in nicotine minipump mice produced, respectively, ~50 and ~100% reversal in nicotine withdrawal induced reduction in hot plate latency (Figure 1C). One-way ANOVA followed by Tukey post hoc revealed that the effect of 0.1 mg/kg dFBr on nicotine withdrawal induced hyperalgesia was ($F_{5,42} = 4.325$, $p = .0029$).

Discussion

Our results showed that dFBr, an $\alpha 4\beta 2$ PAM, reversed withdrawal signs in mice undergoing spontaneous withdrawal after chronic

nicotine infusion. The majority of high-affinity nicotine binding sites in the brain are associated with the $\alpha 4\beta 2^*$ nAChR subtype.¹⁹ Mice lacking the $\alpha 4\beta 2^*$ nAChR (eg, $\beta 2$ knock-out mice) lack the majority of nicotine binding sites; do not self-administer nicotine; and exhibit minimal VTA dopaminergic activity in response to exogenously administered nicotine.^{3,4} They also exhibit a loss of anxiety-related behavior and a loss of aversion in spontaneous and conditioned place aversion nicotine withdrawal models indicating that $\beta 2$ containing nAChRs are involved in the affective signs of nicotine withdrawal.¹⁷ Therefore, PAMs of $\alpha 4\beta 2$ nAChR such as dFBr represent an emerging class of nAChR-based therapeutics for nicotine dependence. They potentially provide selective modulation of $\alpha 4\beta 2$ nAChR subtype while avoiding side effects seen with nAChR agonists due to binding at multiple nAChR subtypes. In addition, dFBr has been shown to replace nicotine in rat model without exerting reinforcing actions of its own suggesting that dFBr reduces the need for tobacco intake with minimal abuse liability.¹⁴

Nicotine withdrawal symptoms include intense cravings for tobacco smoking and a profound state of dissatisfaction, anxiety, irritability, insomnia, and depression. These symptoms begin within days after nicotine abstinence and can continue for months and are considered one of the primary causes of high tobacco smoking relapse rates.¹⁶ Diminishing the dysphoric-like state associated with nicotine withdrawal is believed to prevent relapse to tobacco use and has been shown to contribute to the effect of varenicline and cytisine as smoking cessation aids.²⁰ In this study, we evaluated the effects of dFBr, a nAChR PAM with apparent selectivity to the $\alpha 4\beta 2$ nAChR subtype, on nicotine withdrawal symptoms in mice.

As previously published,¹⁸ nicotine withdrawal significantly increased anxiety-related behaviors, increased expression of somatic withdrawal signs, and decreased response latencies in the hot-plate test. Acute administration of dFBr 18–24 h following reversed, in a dose dependent manner, anxiety-related behavior and somatic signs of nicotine withdrawal with >90% of these signs reversed at 1 mg/kg dFBr. Furthermore, dFBr was even more potent in alleviating nicotine withdrawal induced hyperalgesia where almost full reversal obtained at 0.1 mg/kg dFBr. These dFBr effects were specific to in chronically nicotine-exposed mice as dFBr at equal doses produced little effect, if any, in a parallel control (saline minipump) mice group. Also, dFBr effects were specific to nicotine withdrawal anxiety-related behaviors and did not alter mice locomotor activities as measured by the number of crosses in the plus maze. Altogether, these results establish that dFBr alleviated important aspects of spontaneous withdrawal signs in chronically nicotine-exposed but not naïve mice. These findings, to the best of our knowledge, the first in vivo experimental results to support a role of an $\alpha 4\beta 2$ nAChR PAM in diminishing dystrophic-like state and hyperalgesia associated with nicotine withdrawal.

Conclusion

The low success rate, the high prevalence of side effects, and the high relapse rates associated with current smoking cessation aids demand the development of more selective and safer medication for the management of nicotine dependence. One emerging strategy is the use of nAChR PAMs which potentially have more favorable pharmacological profiles than currently used nAChR agonists. Because drugs that diminish nicotine withdrawal symptoms will more likely to have a lower tobacco smoking relapse rates, in this study we sought to provide in vivo evidence that nAChR PAMs such as dFBr have

this desirable effect. Our results show that dFBr diminish affective behavior associated with nicotine withdrawal supporting a promising role of $\alpha 4\beta 2$ nAChR PAM as a pharmacological strategy for tobacco smoking cessation aid.

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Declaration of Interests

None declared.

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References

- Benowitz NL. Neurobiology of nicotine addiction: implications for smoking cessation treatment. *Am J Med*. 2008;121(4 suppl 1):S3–S10.
- Changeux JP. Nicotine addiction and nicotinic receptors: lessons from genetically modified mice. *Nat Rev Neurosci*. 2010;11(6):389–401.
- Picciotto MR, Zoli M, Léna C, et al. Abnormal avoidance learning in mice lacking functional high-affinity nicotine receptor in the brain. *Nature*. 1995;374(6517):65–67.
- Picciotto MR, Zoli M, Rimondini R, et al. Acetylcholine receptors containing the beta2 subunit are involved in the reinforcing properties of nicotine. *Nature*. 1998;391(6663):173–177.
- Laviolette SR, van der Kooy D. The neurobiology of nicotine addiction: bridging the gap from molecules to behaviour. *Nat Rev Neurosci*. 2004;5(1):55–65.
- Gonzales D, Rennard SI, Nides M, et al.; Varenicline Phase 3 Study Group. Varenicline, an alpha4beta2 nicotinic acetylcholine receptor partial agonist, vs sustained-release bupropion and placebo for smoking cessation: a randomized controlled trial. *JAMA*. 2006;296(1):47–55.
- Mohamed TS, Jayakar SS, Hamouda AK. Orthosteric and allosteric ligands of nicotinic acetylcholine receptors for smoking cessation. *Front Mol Neurosci*. 2015;8:71.
- Uteshev VV. The therapeutic promise of positive allosteric modulation of nicotinic receptors. *Eur J Pharmacol*. 2014;727:181–185.
- Williams DK, Wang J, Papke RL. Positive allosteric modulators as an approach to nicotinic acetylcholine receptor-targeted therapeutics: advantages and limitations. *Biochem Pharmacol*. 2011;82:915–930.
- Peters L, König GM, Terlau H, Wright AD. Four new bromotryptamine derivatives from the marine bryozoan *Flustra foliacea*. *J Nat Prod*. 2002;65:1633–1637.
- Kim JS, Padnya A, Weltzin M, Edmonds BW, Schulte MK, Glennon RA. Synthesis of desformylflustrabromine and its evaluation as an alpha4beta2 and alpha7 nACh receptor modulator. *Bioorg Med Chem Lett*. 2007;17:4855–4860.
- Weltzin MM, Schulte MK. Desformylflustrabromine modulates $\alpha 4\beta 2$ neuronal nicotinic acetylcholine receptor high- and low-sensitivity isoforms at allosteric clefts containing the $\beta 2$ subunit. *J Pharmacol Exp Ther*. 2015;354(2):184–194.
- Hamouda AK, Deba F, Wang ZJ, Cohen JB. Photolabeling a nicotinic acetylcholine receptor (nAChR) with an ($\alpha 4$)/($\beta 2$)/2 nAChR-selective positive allosteric modulator. *Mol Pharmacol*. 2016;89(5):575–584.
- Liu X. Positive allosteric modulation of $\alpha 4\beta 2$ nicotinic acetylcholine receptors as a new approach to smoking reduction: evidence from a rat model of nicotine self-administration. *Psychopharmacology (Berl)*. 2013;230(2):203–213.

15. Moerke MJ, de Moura FB, Koek W, McMahon LR. Effects of nicotine in combination with drugs described as positive allosteric nicotinic acetylcholine receptor modulators *in vitro*: discriminative stimulus and hypothermic effects in mice. *Eur J Pharmacol*. 2016;786:169–178.
16. Le Foll B, Goldberg SR. Effects of nicotine in experimental animals and humans: an update on addictive properties. *Handb Exp Pharmacol*. 2009;192:335–367.
17. Jackson KJ, Martin BR, Changeux JP, Damaj MI. Differential role of nicotinic acetylcholine receptor subunits in physical and affective nicotine withdrawal signs. *J Pharmacol Exp Ther*. 2008;325(1):302–312.
18. Elhassan S, Bagdas D, Damaj MI. Effects of nicotine metabolites on nicotine withdrawal behaviors in mice. *Nicotine Tob Res*. 2017;19(6):763–766.
19. Flores CM, Rogers SW, Pabreza LA, Wolfe BB, Kellar KJ. A subtype of nicotinic cholinergic receptor in rat brain is composed of alpha 4 and beta 2 subunits and is up-regulated by chronic nicotine treatment. *Mol Pharmacol*. 1992;41:31–37.
20. Igari M, Alexander JC, Ji Y, Qi X, Papke RL, Bruijnzeel AW. Varenicline and cytosine diminish the dysphoric-like state associated with spontaneous nicotine withdrawal in rats. *Neuropsychopharmacology*. 2014;39(2):455–465.