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In vivo Interactions between $\alpha 7$ Nicotinic Acetylcholine Receptor and Nuclear Peroxisome Proliferator-Activated Receptor- α : Implication for Nicotine Dependence

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Abstract

Chronic tobacco use dramatically increases health burdens and financial costs. Limitations of current smoking cessation therapies indicate the need for improved molecular targets. The main addictive component of tobacco, nicotine, exerts its dependency effects *via* nicotinic acetylcholine receptors (nAChRs). Activation of the homomeric $\alpha 7$ nAChR reduces nicotine's rewarding properties in conditioned place preference (CPP) test and i.v. self-administration models, but the mechanism underlying these effects is unknown. Recently, the nuclear receptor peroxisome proliferator-activated receptor type- α (PPAR α) has been implicated as a downstream signaling target of the $\alpha 7$ nAChR in ventral tegmental area dopamine cells. The present study investigated PPAR α as a possible mediator of the effect of $\alpha 7$ nAChR activation in nicotine dependence. Our results demonstrate the PPAR α antagonist GW6471 blocks actions of the $\alpha 7$ nAChR agonist PNU282987 on nicotine reward in an unbiased CPP test in male ICR adult mice. These findings suggest that $\alpha 7$ nAChR activation attenuates nicotine CPP in a PPAR α -dependent manner. To evaluate PPAR α activation in nicotine dependence we used the selective and potent PPAR α agonist, WY-14643 and the clinically used PPAR α activator, fenofibrate, in nicotine CPP and we observed attenuation of nicotine preference, but fenofibrate was less potent. We also studied PPAR α in nicotine dependence by evaluating its activation in nicotine withdrawal. WY-14643 reversed nicotine withdrawal signs whereas fenofibrate had modest efficacy. This suggests that PPAR α plays a role in nicotine reward and withdrawal and that further studies are warranted to elucidate its function in mediating the effects of $\alpha 7$ nAChRs in nicotine dependence.

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Introduction

The homomeric $\alpha 7$ nicotinic acetylcholine receptor (nAChR) has unique features of high calcium permeability, rapid desensitization and low probability of channel opening (Séguéla et al., 1993; Williams et al., 2011), and has been shown to play a role in cognition, inflammation, immunity and neuroprotection (Corradi and Bouzat, 2016). Recent findings suggest this low-affinity $\alpha 7$ nAChR modulates nicotine reward and reinforcement in rodents (Brunzell and McIntosh, 2012; Harenza et al., 2014). The $\alpha 7$ nAChR selective agonist PNU282987 infused locally into the nucleus accumbens (NAc) shell reduced intravenous (i.v.) self-administered nicotine in rats. In contrast, ArIB, an $\alpha 7$ selective nAChR antagonist, infused in the NAc increased nicotine intake (Brunzell and McIntosh, 2012). Similarly, the genetic deletion of $\alpha 7$ nAChRs in mice enhances nicotine reward as measured in the conditioned place preference (CPP) test, whereas $\alpha 7$ knock-in (producing mice heterozygous for a Leu250-to-Thr substitution in the channel domain of $\alpha 7$ subunit which creates a gain-of-function mutation) abolishes nicotine preference. In addition, the selective $\alpha 7$ agonist PHA-543613 blocked the development of nicotine CPP in mice (Harenza et al., 2014). Attenuation of nicotine reward and reinforcement by $\alpha 7$ nAChR agonists seems to be associated with a decreased nicotine-induced dopaminergic transmission in the brain, as PNU282987 blocks nicotine-induced increased firing activity of the ventral tegmental area (VTA) dopamine neurons in rats (Melis et al., 2013).

This important effect of $\alpha 7$ nAChR modulation of nicotine reward has prompted studies of the underlying mechanism. It has been suggested that $\alpha 7$ nAChR activation regulates VTA dopaminergic cells *via* the peroxisome proliferator activated receptor α (PPAR α) in the rat. The $\alpha 7$ nAChR agonist PNU282987 induced synthesis of two fatty acid PPAR α endogenous ligands, oleoylethanolamide (OEA) and palmitoylethanolamide (PEA), that in turn activate PPAR α and phosphorylate $\beta 2$ -containing nAChRs on dopamine neurons *via* a tyrosine kinase pathway (Melis et al., 2013). These findings suggest a pathway by which $\alpha 7$ nAChR pharmacological stimulation indirectly inactivates $\beta 2$ -containing nAChRs via PPAR α receptors. However, the above-noted study did not directly investigate this mechanism using a nicotine reward paradigm which is imperative because $\beta 2$ -containing nAChRs are required for nicotine reward (Picciotto et al., 1998; Walters et al., 2006).

PPAR α is a nuclear ligand-activated transcription factor that when activated, enhances transcription of various genes involved in modulating many peripheral physiological responses such as inflammation and lipolysis (Zhu et al., 2000). Importantly, PPAR α s, which are located in brain regions associated with reward (Moreno et al., 2004; Plaza-Zabala et al., 2010; Smaga et al., 2014), have been shown to modulate the rewarding properties of abused substances such as alcohol and nicotine (Bilbao et al., 2015; Melis et al., 2008). Acute administration of PPAR α agonists attenuates nicotine (Mascia et al., 2011; Muldoon et al., 2013; Panlilio et al., 2012) and alcohol reinforcement (Bilbao et al., 2015), alcohol intake (Blednov et al., 2016a, 2016b) and nicotine-induced dopamine firing in rodents (Melis et al., 2008). For example clofibrate, a lipid-lowering agent and PPAR α agonist (Staels et al., 1998), was shown in rats to block acquisition of nicotine seeking, decrease

nicotine i.v. self-administration and block nicotine-induced dopamine release into the NAc shell (Panlilio et al., 2012).

Therefore, we hypothesize that PPAR α may serve as a downstream mediator of $\alpha 7$ nAChR activation in nicotine reward. To test this hypothesis the present study investigated the interaction of the $\alpha 7$ nAChR and PPAR α in a preclinical mouse model of reward (nicotine CPP). Furthermore, we examined PPAR α activation in nicotine CPP and nicotine withdrawal, a behavioral outcome not measured before in preclinical studies with PPAR α activators. We compared effects of the selective and potent PPAR α agonist WY-14643 (Lo Verme et al., 2005; Willson et al., 2000) with a commonly used lipid lowering fibrate medication that activates PPAR α fenofibrate (Keating, 2011). Results from these experiments may provide insight into the roles of $\alpha 7$ nAChR and PPAR α in nicotine dependence.

Materials and Methods

Animals

ICR male mice (8 weeks upon arrival; Harlan Laboratories, Indianapolis, IN) served as subjects. Mice were housed four per cage with *ad libitum* access to food and water on a 12-h light cycle in a humidity and temperature controlled vivarium that was approved by the Association for Assessment and Accreditation of Laboratory Animal Care. Experiments were performed during the light cycle and were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Drugs

(-)-Nicotine hydrogen tartrate [(-)-1-methyl-2-(3-pyridyl) pyrrolidine (+)-bitartrate] and mecamylamine HCl (non-selective nAChR antagonist) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). PNU282987 ($\alpha 7$ nAChR agonist) and cocaine HCl were provided by the Drug Supply Program of the National Institute on Drug Abuse (Rockville, MD). Drugs were dissolved in physiological saline and administered systemically (s.c. for nicotine, mecamylamine, PNU282987 and i.p. for cocaine). Fenofibrate (PPAR α agonist), WY-14643 (PPAR α agonist), and GW6471 (PPAR α antagonist) were purchased from Tocris (Minneapolis, MN) and dissolved in a mixture of 1:1:18 [1 volume ethanol/1 volume Emulphor-620 (Sanofi-Aventis, Bridgewater, NJ) and 18 volumes saline] and administered i.p. Drug solutions were prepared in 10 ml solutions (i.e. 3mg of drug in 10ml of vehicle indicates 3mg/kg dose). Freshly prepared solutions were injected at a total volume of 1 ml/100g of body weight. Doses are expressed as the free base of the drug.

Nicotine and cocaine conditioned place preference studies

An unbiased CPP paradigm was performed as we previously described (Kota et al., 2007; Sanjakdar et al., 2015). Briefly, the CPP apparatus consisted of three chambers in a linear arrangement (ENV3013; Med Associates, St Albans, VT). The external white and black chambers (20×20×20 cm each) differed in overall color and floor texture (white mesh or black rod), and were separated by a smaller gray chamber with a smooth PVC floor.

Partitions could be removed to allow access from the gray chamber to the black and white chambers. On day 1 animals were confined to the middle chamber for a 5 min habituation and then allowed to freely move between all three chambers for 15 min. Time spent in each chamber was recorded and these data were used to populate groups of approximately equal bias in baseline chamber preference. Twenty-minute conditioning sessions occurred twice a day (days 2–4). During conditioning sessions mice were confined to one of the larger chambers. The saline groups received saline in one large chamber in the morning and saline in the other large chamber in the afternoon. The nicotine group received nicotine in one large chamber and saline in the other large chamber. Treatments were counterbalanced to ensure some mice received the unconditioned stimulus in the morning and others received it in the afternoon. The nicotine-paired chamber was randomized across groups. Sessions were 4 hr apart and were conducted by the same investigator. On test day (day 5) mice could access all chambers for 15 min in a drug free state. The preference score was calculated by determining the difference between time spent in the drug paired side on the test day versus the time in drug paired side on the baseline day. Any mouse showing preference for one side higher than 65% was not used in the study.

Nicotine Precipitated Withdrawal Studies

A well-established and validated nicotine withdrawal model was performed (Bagdas et al., 2014; Damaj et al., 2003; Muldoon et al., 2015; Salas et al., 2007) Mice were infused with 24 mg/kg/day nicotine or saline for 14 days using s.c. osmotic minipumps (model 2000; Alzet Corporation, Cupertino, CA) implanted under isoflurane anesthesia (Jackson et al., 2008). Nicotine concentration was adjusted according to animal weight and mini pump flow rate. On the morning of day 15 mice were pretreated with vehicle, WY-14643 (0.3, 1 and 5 mg/kg, i.p.; 15 min prior) or fenofibrate (50 and 100 mg/kg, i.p.; 1 hr prior) before challenge with the non-selective nAChR antagonist mecamylamine (2 mg/kg; s.c.) to precipitate withdrawal. Affective (anxiety-like behavior) and physical (somatic signs and hyperalgesia) nicotine withdrawal signs were evaluated 10 min later as described in (Jackson et al., 2008). Mice were first evaluated for 5 min in the elevated plus maze test for anxiety-related behavior. Time spent on the open arms of the plus maze was used as a measure of anxiety-related response. The number of crosses between open and closed arms was counted as a measure of locomotor activity. The plus maze assessment was immediately followed by a 20 min observation of somatic signs measured as paw and body tremors, head shakes, backing, jumps, curls and ptosis. Mice were placed in clear activity cages without bedding for the observation period. The total number of somatic signs was tallied for each mouse and the average number of somatic signs during the observation period was plotted for each test group. Hyperalgesia was evaluated using the hot plate test immediately following the somatic sign observation period. Mice were placed into a 10-cm wide glass cylinder on a hot plate (Thermojust Apparatus, Richmond, VA) maintained at 52°C. The latency to reaction time (jumping or paw licking) was recorded. The specific testing sequence was chosen based on our prior studies showing that this order of testing reduced within-group variability and produced the most consistent results (Jackson et al., 2008). All studies were performed by an observer blinded to experimental treatment.

Statistical analysis

Data were analyzed using the GraphPad software version 6.0 (GraphPad Software, Inc., La Jolla, CA) and expressed as the mean $\pm$ S.E.M. To determine if there was a nicotine effect (CPP and withdrawal studies), results with the nicotine control treatment groups were compared to the saline treatment groups with an unpaired two-tailed t-test. To determine if drugs given to alter/block the effects of nicotine (PNU282987, GW6471, WY14643, and fenofibrate) had an effect on their own, treatment groups were compared to their appropriate vehicle treatment groups with an unpaired two-tailed t-test or ordinary one-way analysis of variance (ANOVA) where applicable. To determine whether treatment with the above-mentioned compounds affected nicotine CPP or precipitated nicotine withdrawal, results with the dosage groups (i.e. fenofibrate+nicotine) were compared to the vehicle+nicotine treatment group using Holm-Šídák comparison tests in conjunction with an ANOVA (in which the vehicle and i.e. fenofibrate+vehicle groups were excluded). Two-way ANOVA followed by the Tukey multiple comparisons test was used in order to evaluate attenuation of dose response of nicotine CPP by PPAR α agonist WY-14643. Comparisons were considered statistically significant when $p < 0.05$.

Results

Development of Nicotine CPP Attenuated by α 7 nAChR Full Agonist PNU282987

Mice were conditioned with either saline or nicotine (0.5 mg/kg; s.c.) for 3 days in the CPP paradigm. The 0.5mg/kg dose of nicotine has been previously shown to produce a significant preference in the CPP test (Grabus et al., 2006; Walters et al., 2006). In Fig. 1 a robust CPP was observed in nicotine-conditioned mice pre-treated with vehicle ($t=14.86$, $df = 13$, $p<0.05$). PNU282987 given 15 min prior to nicotine, reduced nicotine reward in a dose-related manner [$F(2, 21) = 18.27$, $p < 0.0001$]. As revealed by the Holm-Šídák comparison tests, PNU282987 (3mg/kg) significantly altered nicotine CPP ($p < 0.05$), but was ineffective at the lower dose of 0.6mg/kg ($p > 0.05$). PNU282987 at the dose of 3 mg/kg did not produce a preference in saline treated-mice ($t=0.03760$, $df=12$, $p>0.05$).

PPAR α Antagonist Blocks α 7 nAChR Agonist PNU282987 in Nicotine CPP

The PPAR α antagonist GW6471 was utilized to evaluate the PPAR α dependency of α 7 nAChR activation in nicotine CPP. In Fig. 2 male ICR mice conditioned with 0.5mg/kg s.c. of nicotine for three days exhibited a significant preference ($t=5.796$, $df=14$, $p<0.05$). One-way ANOVA revealed that pretreatment with the α 7 nAChR agonist PNU282987 (3mg/kg; s.c.) given 15 min prior to nicotine attenuated nicotine CPP. This attenuation was significantly blocked by the PPAR α antagonist GW6471 (2mg/kg; i.p) administered 30 min prior to PNU282987 [$F(3, 28) = 6.301$, $p = 0.0021$], whereas GW6471 did not have an effect on nicotine CPP ($p > 0.05$). PNU282987 and GW6471 did not cause aversion or preference on their own or in combination [$F(3, 24) = 0.08290$, $p = 0.9687$].

The PPAR α Agonist WY-14643 Attenuated Nicotine CPP

We then tested the impact of direct activation of PPAR α using the selective and potent PPAR α agonist WY-14643 on nicotine CPP. Mice were conditioned with either saline or

nicotine (0.5 mg/kg) for 3 days in the CPP paradigm. In Fig. 3A a robust CPP was observed in nicotine-conditioned mice pre-treated with vehicle ($t=11.10$, $df=13$, $p<0.05$). WY-14643 reduced nicotine reward in a dose-dependent manner at all doses tested (0.3, 0.6 and 1 mg/kg) [$F(3, 28) = 15.19$, $p<0.0001$]. On its own WY-14643 did not produce a preference or aversion in saline treated-mice ($t=0.9787$, $df=11$; $p>0.05$).

WY-14643 Did Not Shift the Potency of Nicotine in Nicotine CPP

To test the effect of the PPAR α agonist WY-14643 on the potency of nicotine in the CPP test WY-14643 (1 mg/kg; i.p.) was administered 15 minutes prior to nicotine (0.1, 0.5 and 1 mg/kg; s.c.) in the CPP test. Two-way ANOVA revealed that a significant nicotine preference [$F(3, 53) = 9.225$, $p<0.0001$], a significant blockage of nicotine preference by WY-14643 [$F(1, 53) = 44.54$, $p<0.0001$] and interaction [$F(3, 53) = 4.315$, $p=0.0085$]. In Fig. 3B nicotine preference was significant at 0.5 and 1mg/kg doses after 3 days of conditioning ($p<0.001$). WY-14643 pretreatment significantly attenuated nicotine preference at 0.5 and 1mg/kg ($p<0.05$) and had no effect on the 0.1 mg/kg dose of nicotine ($p>0.05$) WY-14643 did not produce preference or aversion on its own ($p>0.05$).

PPAR α Agonist WY-14643 Did Not Attenuate Cocaine CPP

To test for the behavioral selectivity of WY-14643 on nicotine CPP, WY-14643 was evaluated in cocaine CPP as previously described (Sanjakdar et al, 2015; Zachariou et al, 2001). In Fig. 3C robust preferences for cocaine (10mg/kg; i.p.) and nicotine (0.5mg/kg; s.c.) were produced after 3 days of conditioning in mice [$F(2, 22) = 57.40$, $p<0.0001$]. The 10 mg/kg dose of cocaine has been previously shown to produce a significant preference in the CPP test (Alajaji et al, 2016; Ignatowska-Jankowska et al., 2013). Although WY-14643, with a 15 min pretreatment, totally reduced nicotine reward at 1mg/kg as previously observed in this study [$F(3, 25) = 21.18$, $p<0.0001$], it had no significant effect on cocaine preference ($p>0.05$).

Clinically Used PPAR α Agonist Fenofibrate Reduced Nicotine CPP

We utilized the clinically available PPAR α agonist fenofibrate in the nicotine CPP paradigm. As previously observed in this study one way ANOVA showed that nicotine induced a significant preference in comparison to saline-treated mice after the 3-day conditioning period ($t=9.883$, $df=13$; $p<0.05$). In Fig. 4 pretreatment with lower doses of fenofibrate (1 and 9 mg/kg) 1 hr prior to nicotine did not significantly alter nicotine CPP ($p>0.05$). However, the highest dose of 50 mg/kg of fenofibrate reduced nicotine preference significantly [$F(3, 24) = 3.652$, $p=0.0267$]. Fenofibrate had no effect on its own in saline treated-mice ($t=0.5570$, $df = 11$, $p>0.05$). Fenofibrate was administered at doses previously described (Blednov et al., 2016a, 2016b).

Nicotine Withdrawal Signs Attenuated by PPAR α Agonist WY-14643

The physical (somatic signs and hyperalgesia) and affective (anxiety-related behavior) signs of nicotine withdrawal were measured in mice following pretreatment with either WY-14643 or vehicle 15 min prior to mecamylamine administration on day 15. In Fig 5 nicotine withdrawn mice had a significantly increased anxiety-related behavior in the plus maze

($t=5.469, df=11, p < 0.05$; Fig. 5A), increased expression of somatic withdrawal signs ($t=6.801, df=12, p < 0.05$; Fig. 5B) and decreased response latencies in the hot-plate test ($t=3.047, df=12, p < 0.05$; Fig. 5C) compared to control mice implanted with saline minipumps. In Fig. 5A one-way ANOVA revealed that pretreatment with WY-14643 attenuated anxiety-like behavior (time in open arms in the plus-maze test) at the dose of 5 mg/kg [$F(3, 22) = 5.037, p=0.0083$]. As shown in Table 1 WY-14643 had no effect on the number of arm crosses in the plus maze [$F(5, 32) = 0.4386, p=0.8182$]. In addition, as shown in Fig. 5B pretreatment with 1 and 5 mg/kg of WY-14643 decreased nicotinic somatic withdrawal signs [$F(3, 23) = 12.52, p<0.0001$]. In our study somatic signs were expressed as followed: paw tremors (~70%), body tremors (~5%), head shakes (~10%), backing (~15%). WY-14643 reduced these individual somatic signs in a uniformed manner. Finally, in Fig. 5C pretreatment with WY-14643 also attenuated the expression of hyperalgesia (hot-plate latency) at 5 mg/kg [$F(3, 24) = 3.566, p=0.0290$]. The highest dose of WY-14643 tested (5 mg/kg) did not significantly affect behavioral responses in saline-infused mice in any withdrawal test.

Fenofibrate Modestly Attenuated Nicotine Withdrawal

Fenofibrate was administered 1 hr prior to mecamylamine on day 15 after 14 days of continuous nicotine exposure via osmotic minipumps. Following mecamylamine administration nicotine withdrawals signs (anxiety-like behavior, somatic signs and hyperalgesia) were measured in mice. In Fig. 6 nicotine withdrawn mice displayed an increase in anxiety-related behavior in the plus maze ($t=7.813, df=14, p<0.05$; Fig. 6A), enhanced expression of somatic withdrawal signs ($t=12.94, df=14, p<0.05$; Fig. 6B) and attenuated response latencies in the hot-plate test ($t=5.921, df=14, p<0.05$; Fig. 6C) in comparison to their saline minipump-implanted counterparts. In Fig. 6A one way ANOVA revealed that pretreatment with fenofibrate had no effect on anxiety-like behavior (time in open arms in the plus-maze test) at both doses tested (50 and 100 mg/kg) [$F(2, 21) = 1.089, p = 0.3547$]. As shown in Table 2 fenofibrate did not significantly alter the number of arm crosses in the plus maze test [$F(5, 42) = 0.5318, p = 0.7509$]. However, as shown in Fig. 6B pretreatment with fenofibrate partially attenuated nicotinic somatic withdrawal signs only at the highest dose used of 100 mg/kg [$F(2, 21) = 6.928, p = 0.0049$]. Somatic signs were expressed in the following distribution: paw tremors (~70%), body tremors (~5%), head shakes (~10%), backing (~15%). Fenofibrate partially attenuated these individual somatic signs in a uniformed manner. Lastly, as shown in Fig. 6C pretreatment with fenofibrate was ineffective at attenuating the expression of hyperalgesia (hot-plate latency) at both doses tested [$F(2, 21) = 2.026, p = 0.1569$]. The highest dose of fenofibrate tested (100 mg/kg) did not significantly affect behavioral responses in saline-infused mice in any withdrawal test. In the nicotine withdrawal studies fenofibrate was administered at doses previously described (Blednov et al., 2016a, 2016b)

Discussion

This is the first report demonstrating the ability of a PPAR α antagonist to block the inhibitory effects of an $\alpha 7$ nAChR agonist on nicotine reward in a mouse CPP paradigm (Fig. 2). This suggests that $\alpha 7$ nAChR activation attenuates nicotine CPP in a PPAR α -

dependent mechanism. We therefore compared the effects of a selective and potent PPAR α agonist, WY-14643, to fenofibrate, a clinically available PPAR α agonist in nicotine mouse models of reward and withdrawal. Our results provide some important and novel insights about the effects of PPAR α agonists in these nicotine dependence tests. The PPAR α agonists WY-14643 and fenofibrate attenuated nicotine preference as expected but fenofibrate was less potent (Fig. 3A and Fig.4). Also, in contrast to WY-14643, fenofibrate had a modest efficacy in reducing nicotine withdrawal signs (Fig. 5 and Fig. 6).

Our results indicated that attenuation by $\alpha 7$ nAChR activation in nicotine CPP is PPAR α mediated (Fig. 2). This finding is consistent with suggestion that an $\alpha 7$ nAChR agonist prevents nicotine-induced excitation of dopamine neurons via PPAR α mechanism (Melis et al., 2013). Indeed, the PPAR α agonist WY-14643 completely and dose-dependently blocked nicotine conditioned reward in the CPP test (Fig. 3A). In addition, WY-14643 at the highest effective dose (1 mg/kg) blocked all doses of nicotine in the CPP test (Fig. 3B). Furthermore, WY-14643 (1mg/kg) had no significant effect on cocaine CPP suggesting behavioral selectivity of WY-14643 for attenuating nicotine reward (Fig.3C). In support of our findings WY-14643 has been previously shown to be ineffective in reducing cocaine self-administration (Mascia et al., 2011). Our findings with WY-14643 are consistent with other PPAR α agonists such as clofibrate that was reported to attenuate nicotine reinforcement and reinstatement in rats through a PPAR α mechanism of action (Mascia et al., 2011; Muldoon et al., 2013; Panlilio et al., 2012). Our study with fenofibrate in nicotine CPP showed that fenofibrate blocked the development of nicotine CPP at a lower potency (a 9-fold difference estimate) than WY-14643, the selective and potent PPAR α agonist (Fig. 4). In fact, the dose of fenofibrate to completely block nicotine CPP was 50 mg/kg. At the higher dose of 100 mg/kg, fenofibrate-treated mice were no longer statistically different from the nicotine-treated mice.

Our nicotine withdrawal results suggest PPAR α activation by WY-14643 is effective at attenuating nicotine withdrawal signs in a mouse model. To our knowledge this is the first study to evaluate PPAR α agonists in a preclinical test for nicotine withdrawal. WY-14643 attenuated both the affective (anxiety-like behavior) and physical (somatic and hyperalgesia) signs of withdrawal (Fig. 5) whereas fenofibrate only partially and modestly reduced the somatic signs intensity at the highest dose used, 100 mg/kg (Fig. 6). Higher doses of fenofibrate were not investigated due to adverse locomotor effects (data not shown). Clinically available smoking cessation therapies act to a large extent by reducing the nicotine withdrawal signs/symptoms (Mooney and Sofuoglu, 2006), one of the primary causes of high tobacco relapse rates (Le Foll and Goldberg, 2009); consequently, our animal studies included a focus on nicotine withdrawal. Somatic signs have shown to contribute less to nicotine-seeking behavior than affective signs (De Biasi and Dani, 2011; Epping-Jordan et al., 1998); thus, the modest reduction of somatic signs by fenofibrate may not predict its efficacy as a smoking cessation aid.

The $\alpha 7$ nAChR full agonist PNU282987 used in the CPP studies is selective for the $\alpha 7$ nAChR (Bodnar et al., 2005; Hajós et al., 2005; Taslim and Saeed Dar, 2011). However, it has been suggested that $\alpha 7$ nAChR activation might indirectly lead to downregulation of $\beta 2$ -nicotinic subunits via PPAR α -induced phosphorylation of these subunits (Melis et al., 2013,

2010). Indeed, $\alpha 7$ nAChR pharmacological activation by PNU282987 enhanced the neuronal levels of endogenous PPAR α ligands OEA and PEA in the VTA (Melis et al., 2013). Therefore, PPAR α activation by WY-14643 may attenuate nicotine conditioned reward in the CPP test via a similar mechanism leading to a functional downregulation of $\beta 2$ subunits. $\beta 2$ -containing nAChRs are well known to play an important role in nicotine reward in the CPP test (Walters et al., 2006). The lack of reduction of cocaine CPP by PPAR α agonist WY-14643 is somewhat surprising if we assume an important role for $\beta 2$ -containing nAChRs in the effect of PPAR α activation. Nevertheless, it is possible that this mechanism (i.e. $\beta 2$ -containing nAChR downregulation) may not be involved in cocaine CPP. Unlike nicotine CPP, genetic and pharmacological activation of $\alpha 7$ nAChRs does not alter cocaine preference (Harenza et al., 2014). It has been reported that cocaine CPP is partially reduced in $\beta 2$ knockout mice (Zachariou et al., 2001) at 5mg/kg of cocaine, suggesting that $\beta 2$ -containing nAChRs play a role in cocaine CPP. However, at the higher dose of 10mg/kg, the dose used in our study, no reduction of cocaine CPP was observed (Zachariou et al., 2001). Another possibility is the degree of phosphorylation of the $\beta 2$ subunit may not be sufficient enough to alter cocaine CPP in comparison to a complete genetic ablation of the $\beta 2$ subunit ($\beta 2$ knockout mice). Therefore, the proposed mechanism of $\alpha 7$ nAChR activation indirectly downregulating $\beta 2$ -containing nAChRs may not play a role in cocaine CPP. In nicotine withdrawal, it is possible that regulation of $\beta 2$ nAChR subunits influences the reversal of nicotine withdrawal-related signs by the PPAR α agonist WY-14643. Indeed, $\beta 2$ -containing nAChRs are important for the affective signs of nicotine withdrawal (Jackson et al., 2008). In addition, animal studies reported a correlation between the time-course of brain $\beta 2$ -containing nAChRs upregulation and nicotine withdrawal signs (Gould et al., 2014). Furthermore, nicotine withdrawn smokers have upregulated $\beta 2$ -containing nAChRs (Cosgrove et al., 2010).

Collectively our preclinical findings on fenofibrate are consistent with its lack of effectiveness seen in a recent clinical study (Perkins et al., 2015) as a smoking cessation aid. That pilot study was a 4-week evaluation of fenofibrate using a within-subjects crossover design with nicotine-dependent volunteers ($n=38$). Although that experiment had limitations in sample size, duration and used only one dose of fenofibrate, our data suggest that fenofibrate might not be the appropriate PPAR α drug to use because it has modest effects on nicotine withdrawal and has been shown to be a weak and non-selective PPAR α agonist ($EC_{50} > 10 \mu M$) (Lo Verme et al., 2005; Willson et al., 2000). Importantly, our data with WY-14643 and those reported with clofibrate (Panlilio et al., 2012) suggests that PPAR α is a potential molecular target to evaluate for smoking cessation. Notably, PPAR α s undergo different structural conformations upon interaction with different ligands and each ligand-receptor conformation may lead to different patterns of gene expression modulation. For example activation of PPAR α by WY-14643 and fenofibrate activate different set of genes as well as a small set of overlapping genes (Guo et al., 2006). Therefore, evaluation of more selective and potent PPAR α agonists such as LY518674 (>2000 -fold more potent and >300 -fold more selective than fenofibrate) and PPAR α biased agonists such as the selective PPAR modulators (SPPARMS) K-877 (Pemafibrate®) (Liu et al., 2015) should be considered. SPPARMS are thought to interact with the large binding pocket of PPAR α to induce a different co-factor recruitment, resulting in higher potency and fewer adverse side effects

than the original fibrates compounds (Fruchart, 2013). LY518674 and K-877 are currently in phase II trials with promising results in treating dyslipidemia (Ishibashi et al., 2016; Raza-Iqbal et al., 2015). These compounds may prove to be more efficacious candidates for smoking cessation therapy; however, preclinical studies are imperative to investigate this hypothesis. In summary, our findings build on the understanding of the underlying mechanism of $\alpha 7$ nAChR activation in nicotine reward. Further investigation needs to be conducted to elucidate the role of PPAR α mediation of $\alpha 7$ nAChR in nicotine dependence.

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- $\alpha 7$ nAChR activation in nicotine CPP is PPAR α mediated.
- Fenofibrate is less potent than WY-14643 in nicotine dependence tests.
- PPAR α agonists attenuate nicotine withdrawal

Figure 1. Attenuation of the Development of Nicotine CPP by $\alpha 7$ nAChR Orthosteric Full Agonist PNU282987

Mice were conditioned with either subcutaneous (s.c.) saline or nicotine (0.5mg/kg) for 3 days. A robust CPP was observed in nicotine-conditioned mice pre-treated with vehicle. The $\alpha 7$ agonist, PNU282987 (0.6 and 3 mg/kg; s.c.) reduced nicotine reward as measured by the CPP test in a dose-related fashion. * Denotes $p < 0.05$ from vehicle-vehicle; # Denotes $p < 0.05$ from vehicle-nicotine. Each point represents the mean $\pm$ SEM of $n = 6-8$ mice per group.

Figure 2. Interaction between PPAR α and α 7 nAChR in the Nicotine Reward

Mice were conditioned with either s.c. saline or nicotine (0.5mg/kg) for 3 days. A robust CPP was observed in nicotine-conditioned mice pre-treated with vehicle. The α 7 agonist PNU282987 (mg/kg; s.c.) reduced nicotine reward. The PPAR α antagonist GW6471 (2 mg/kg; i.p.) blocked the effect of the α 7 nAChR agonist in nicotine CPP. * Denotes $p < 0.05$ from vehicle-vehicle; # Denotes $p < 0.05$ from vehicle-nicotine; Φ Denotes $p < 0.05$ from vehicle-PNU282987-nicotine. Each point represents the mean $\pm$ SEM of $n=6-9$ mice per group.

Figure 3. Effects of PPAR α Agonist WY-14643 on Nicotine and Cocaine CPP

Mice were conditioned with either s.c. saline or nicotine (0.5 mg/kg) for 3 days. A robust CPP was observed in nicotine-conditioned mice pre-treated with vehicle. A) Intraperitoneal (i.p.) injection of PPAR α agonist WY-14643 (0.3, 0.6, and 1 mg/kg) reduced nicotine reward as measured by the CPP test in a dose-related fashion. B) To evaluate blockade of dose response of nicotine CPP by PPAR α agonist mice were conditioned with either saline or nicotine (0.1, 0.5 and 1 mg/kg; s.c.) for 3 days. A robust CPP was observed in nicotine-conditioned mice pre-treated with vehicle by the dose of 0.5 mg/kg or above. Pretreatment with WY-14643 (1 mg/kg; i.p.) reduced nicotine-CPP at the dose of 0.5 and 1 mg/kg nicotine. C) To test the selectivity of the attenuating effect of the PPAR α agonist in nicotine CPP a separate group of mice was conditioned by saline, cocaine (10 mg/kg; i.p.) or nicotine (0.5 mg/kg; s.c.) for 3 days. A robust CPP was observed in both nicotine-conditioned and cocaine-conditioned mice pre-treated with vehicle. The PPAR α agonist WY-14643 (1mg/kg; i.p.) reduced nicotine reward, but not cocaine reward as measured by the CPP test. * Denotes $p < 0.05$ from vehicle control; # Denotes $p < 0.05$ from nicotine control. Each point represents the mean $\pm$ SEM of $n=6-8$ mice per group.

Figure 4. Effect of PPAR α Agonist Fenofibrate on Nicotine CPP

Mice were conditioned with either s.c. saline or nicotine (0.5mg/kg) for 3 days. A robust CPP was observed in nicotine-conditioned mice pre-treated with vehicle. Fenofibrate (1, 9 and 50 mg/kg; i.p.), clinically used PPAR α agonist, reduced nicotine reward as measured by the CPP test in a dose-related fashion. * Denotes $p < 0.05$ from vehicle control; # Denotes $p < 0.05$ from nicotine control. Each point represents the mean $\pm$ SEM of $n=6-8$ mice per group.

Figure 5. Effects of PPAR α Agonist WY-14643 on Physical and Affective Signs of Precipitated Nicotine Withdrawal

Mice were chronically infused with saline or nicotine (24 mg/kg/day) for 14 days. On day 15 mice received i.p. injection of WY-14643 (0.3, 1 and 5 mg/kg) or vehicle. Mice then were administered mecamylamine (2mg/kg; s.c.) 10 min prior to behavioral assessment of A) anxiety-like behaviors (Time spent in the open arm), B) somatic signs, and C) hyperalgesia (hot plate latency). Nicotine induced withdrawal symptoms: increased anxiety-related behavior and somatic signs, but decreased hot plate latency. Compared to vehicle, pretreatment with WY-14643: A) attenuated the anxiety-like behavior at 5mg/kg; B) reduced somatic signs at 1 and 5mg/kg; and C) significantly increased hot plate latency at 5mg/kg in nicotine withdrawn mice. Each point represents the mean $\pm$ S.E.M. of $n=6-8$ mice per group. * Denotes $p < 0.05$ vs. Saline minipump group, # Denotes $p < 0.05$ vs. Nicotine minipump group. MP: minipump

Figure 6. Effects of PPAR α Agonist Fenofibrate on Physical and Affective Signs of Precipitated Nicotine Withdrawal

Mice were chronically infused with saline or nicotine (24 mg/kg/day) for 14 days. On day 15 mice received fenofibrate 1 hr pretreatment (50 and 100 mg/kg; i.p.) or vehicle. Withdrawal was precipitated by administration of mecamylamine (2mg/kg; s.c.) 10 min prior to behavioral testing of: A) anxiety-like behaviors (Time spent in the open arm); B) somatic signs; and C) hyperalgesia (hot plate latency). Nicotine induced withdrawal symptoms increase anxiety-related behavior and somatic signs, but decrease hot plate latency. Compared to vehicle, pretreatment with fenofibrate: A) had no effect on the anxiety-like behavior; B) reduced somatic signs at 100 mg/kg; and C) did not alter hot plate latency in nicotine withdrawn mice. Each point represents the mean $\pm$ S.E.M. of 8 mice per group. * Denotes $p < 0.05$ vs. Saline minipump group, # Denotes $p < 0.05$ vs. Nicotine minipump group. MP: minipump

Table 1
WY-14643 does not significantly alter the average number of arm crosses in the elevated plus maze test

Mice undergoing nicotine withdrawal received WY-14643 (0.3, 1, 5; i.p.) or vehicle. The average number of arm crosses were recorded in the plus maze test. The numbers are presented as the total number of arm crosses $\pm$ SEM ($n=6-7$). MP, minipump.

<u>Treatment</u>	<u>Average number of arm crosses $\pm$SEM</u>
Saline MP-vehicle	7.8 $\pm$ 0.9
Saline MP- WY-14643 (5)	8 $\pm$ 0.8
Nicotine MP-vehicle	7.1 $\pm$ 0.4
Nicotine MP-WY-14643(0.3)	7.2 $\pm$ 0.3
Nicotine MP-WY-14643 (1)	7.2 $\pm$ 0.3
Nicotine MP-WY-14643 (5)	7.7 $\pm$ 0.5

Table 2
Fenofibrate does not have an effect on the average number of arm crosses in the elevated plus maze test

Mice undergoing nicotine withdrawal received fenofibrate (50 and 100 mg/kg; i.p.) or vehicle. The average number of arm crosses were recorded in the plus maze test. The numbers are presented as the total number of arm crosses $\pm$ SEM ($n=8$). MP, minipump.

Treatment	Average number of arm crosses $\pm$ SEM
Saline MP-vehicle	8.3 $\pm$ 0.6
Saline MP- Fenofibrate (50)	7.6 $\pm$ 0.5
Saline MP- Fenofibrate (100)	7.4 $\pm$ 0.3
Nicotine MP-vehicle	7.1 $\pm$ 0.5
Nicotine MP-Fenofibrate (50)	7.8 $\pm$ 0.5
Nicotine MP-Fenofibrate (100)	8 $\pm$ 0.8