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Potentiation of (α4)2(β2)3, but not (α4)3(β2)2, Nicotinic Acetylcholine Receptors Reduces Nicotine Self-Administration and Withdrawal Symptoms

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Abstract

The low sensitivity (α4)3(β2)2 (LS) and high sensitivity (α4)2(β2)3 (HS) nAChR isoforms may contribute to a variety of brain functions, pathophysiological processes, and pharmacological effects associated with nicotine use. In this study, we examined the contributions of the LS and HS α4β2 nAChR isoforms in nicotine self-administration, withdrawal symptoms, antinociceptive and hypothermic effects. We utilized two nAChR positive allosteric modulators (PAMs): desformylflustrabromine (dFBr), a PAM of both the LS and HS α4β2 nAChRs, and CMPI, a PAM selective for the LS nAChR. We found that dFBr, but not CMPI, decreased intravenous nicotine self-administration in male mice in a dose-dependent manner. Unlike dFBr, which fully reverses somatic and affective symptoms of nicotine withdrawal, CMPI at doses up to 15 mg/kg in male mice only partially reduced nicotine withdrawal-induced somatic signs, anxiety-like behavior and sucrose preference, but had no effects on nicotine withdrawal-induced hyperalgesia. These results indicate that potentiation of HS α4β2 nAChRs is necessary to modulate nicotine's reinforcing properties that underlie nicotine intake and to reverse nicotine withdrawal symptoms that influence nicotine abstinence. In contrast, both dFBr and CMPI enhanced nicotine's hypothermic effect and

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Contributed reagents or analytic tools: Thakur, Garai
Performed data analysis: Akinola, Bautista, Alkhlaif, Jackson, Damaj, Hamouda, Fowler
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reduced nicotine's antinociceptive effects in male mice. Therefore, these results indicate a more prevalent role of HS α4β2 nAChR isoforms in mediating various behavioral effects associated with nicotine, whereas the LS α4β2 nAChR isoform has a limited role in mediating body temperature and nociceptive responses. These findings will facilitate the development of more selective, efficacious, and safe nAChR-based therapeutics for nicotine addiction treatment.

Keywords

Nicotinic Acetylcholine Receptors; Positive Allosteric Modulators; Nicotine Self-Administration; Nicotine Withdrawal Symptoms; Nicotine-induced Hypothermia; Acute thermal nociception

1. INTRODUCTION

Neuronal nicotinic acetylcholine receptors (nAChRs) are a heterogeneous group of ligand-gated ion channels expressed throughout the nervous system and in non-neuronal tissues (Dani and Bertrand, 2007; Gotti and Clementi, 2004). Brain nAChRs are present at the postsynaptic and presynaptic membranes of various nerve endings and consequently play a role in modulating higher brain functions, such as learning and memory, reward and reinforcement, and pain perception (Jensen et al., 2005; Kalamida et al., 2007). The nAChRs are also involved in the pathophysiological processes underlying various neuropsychiatric conditions, such as nicotine addiction and neuropathic pain (Hurst et al., 2013; Jain, 2004; Taly et al., 2009; Ueda et al., 2010; Umana et al., 2013).

The α4β2 nAChR subtypes are the most abundant nAChRs in the brain and are present in two distinct isoforms: (α4)2(β2)3 nAChR, the high sensitivity (HS) isoform, has a high affinity for acetylcholine (ACh) and nicotine, and (α4)3(β2)2 nAChR, the low sensitivity (LS) form, has a lower affinity for ACh and nicotine (Gotti et al., 2009; Moroni et al., 2006; Nelson et al., 2003). The (α4)3(β2)2 and (α4)2(β2)3 nAChRs exhibit unique structural features that result in differences in the number of agonist binding sites, agonist affinity, allosteric binding sites, channel kinetics, and other biochemical/pharmacological properties (Bertrand and Terry, 2018). Interestingly, both isoforms differ in their brain region expression patterns, with the LS (α4)3(β2)2 being the major isoform expressed in the cortex (DeDominicis et al., 2017; Marks et al., 2007). However, the physiological and pharmacological significance of the different α4β2 nAChR isoforms are not well understood.

A substantial body of research has established the indispensable role of α4β2* (* denotes that additional subunits other than α4 and β2 may be present) nAChRs in mediating the effects of nicotine. The α4β2* nAChRs have a higher affinity to nicotine than other nAChR subtypes, and mice lacking either the α4 or β2 subunit lack high-affinity nicotine binding sites (Flores et al., 1997; Flores et al., 1992; Marubio et al., 1999; Picciotto et al., 1995). The facilitating effects of nicotine on the firing rate of striatal dopaminergic neurons and dopamine release at nucleus accumbens were reduced in β2 knockout mice (Changeux, 2010; Picciotto et al., 1995; Picciotto et al., 1998; Zoli et al., 2002), and the β2 knockout mice also exhibit a significant reduction in nicotine intravenous and oral self-administration compared to wild-type mice (Picciotto et al., 1995; Picciotto et al., 1998). Further, α4β2*

nAChRs are expressed at multiple anatomical locations within the pain transmission pathways and mediate the antinociceptive effects of nicotine and other nAChR agonists in animal models (Kyte and Gewirtz, 2018; Nirogi et al., 2013; Umana et al., 2013). Nicotine-induced antinociception in the tail-flick and hot-plate tests were substantially reduced in α4 and β2 knockout mice, respectively (Marubio et al., 1999). In addition, α4β2* nAChRs were shown to play an essential role in nicotine-induced hypothermia in rodents (Tapper et al., 2007; Tritto et al., 2004). However, given that the α4 and β2 nAChR subunits may combine to form either the HS or LS isoforms, the exact contributions of either of these subtypes in the above-noted behaviors are unclear.

Positive allosteric modulators (PAMs) of nAChRs have recently emerged as a promising drug class to selectively target α4β2* nAChRs for potential therapeutic uses (Mohamed et al., 2015; Uteshev, 2016; Wilkerson et al., 2020). PAMs do not directly activate nAChRs on their own; rather, they enhance the potency and/or efficacy of the ligand (Fowler and Kenny, 2014; Lee et al., 2011; Moerke et al., 2016; Mohler et al., 2014; Nirogi et al., 2013; Pandya and Yakel, 2011; Rode et al., 2012). Desformylflustrabromine (dFBr) and 3-(2-chlorophenyl)-5-(5-methyl-1-(piperidin-4-yl)-1H-pyrazol-4-yl)isoxazole (CMPI) are nAChR PAMs that preferentially potentiate the α4β2 nAChRs (Albrecht et al., 2008; Sala et al., 2005; Wilkerson et al., 2020). *In vitro* expression studies have established dFBr as a PAM for both (α4)3(β2)2 and (α4)2(β2)3 nAChRs isoforms, whereas CMPI is a selective PAM for the (α4)3(β2)2 nAChR isoform (Hamouda et al., 2016; Wang et al., 2017; Weltzin and Schulte, 2015). To date, CMPI is one of the only three nAChR PAMs that are reported to distinguish between the HS and LS (α4)3(β2)2 nAChR isoforms; the other two are NS9283 (Timmermann et al., 2012) and galantamine (Jin et al., 2014). Previously, dFBr was shown to modulate nicotine intake and nicotine-induced antinociception in animal models (Bagdas et al., 2018a; Liu, 2013; Weggel and Pandya, 2019). However, little is known about the *in vivo* effects of CMPI and/or dFBr across the variety of nicotine-associated behavioral profiles related to reinforcement, withdrawal, pain and thermoregulation.

In this study, we sought to utilize the preferential selectivity of dFBr and CMPI to delineate the contributions of the LS and HS α4β2 nAChR isoforms in the various behavioral effects associated with nicotine use and withdrawal. The results reported herein significantly contribute to our understanding of the role of nAChR subpopulations involved in the underlying mechanism of nicotine behaviors, which will facilitate the development of more selective, efficacious, and safe nAChR-based therapeutics for the treatment of nicotine addiction.

2. Materials and methods

2.1. Animals

Adult male C57BL/6J mice were used for the intravenous nicotine self-administration and withdrawal studies (Jackson Laboratory, Bar Harbor, ME). All other studies were conducted in adult male Institute of Cancer Research (ICR) mice (Harlan Laboratories, Indianapolis, IN). Animals were housed in groups in standard environmental conditions (12 h light/ 12h dark cycle) at Association for Assessment and Accreditation of Laboratory Animal Care (AALAC)-accredited facilities. Food and water were provided ad libitum, except during self-

administration studies in which mice were mildly food restricted. Animal study procedures were carried out using protocols approved by the Institutional Animal Care and Use Committees at the Virginia Commonwealth University and the University of California, Irvine.

2.2. Drugs

(−)-Nicotine hydrogen tartrate salt [(−)-1-Methyl-2-(3-pyridyl) pyrrolidine (+)-bitartrate salt] and dihydro-β-erythroidine hydrobromide (DHβE) were purchased from Sigma-Aldrich (St. Louis, MO). Desformylflustrabromine (dFBr) and 3-(2-chlorophenyl)-5-(5-methyl-1-(piperidin-4-yl)-1H-pyrazol-4-yl) isoxazole (CMPI) were obtained from Tocris Biosciences (Minneapolis, MN). The vehicle was a mixture of 1:1:18 [1 volume propylene glycol, 1 volume Tween-80 and 18 volumes saline]. Drug doses were chosen based on in vitro potency and available *in vivo* literature (Bagdas et al., 2018a; Moerke et al., 2016; Weggel and Pandya, 2019). Nicotine doses are represented in the free base form. Drugs were injected intraperitoneally (i.p.) for a total volume of 1 ml/100 g body weight unless noted otherwise.

2.3. Intravenous nicotine self-administration

Subjects were food trained and then surgically catheterized as previously described (Chen et al., 2018; Chen et al., 2020; Fowler and Kenny, 2011). Briefly, mice were anesthetized with an isoflurane (1-3%)/oxygen vapor mixture and prepared with intravenous catheters. Catheters consisted of a 6 cm length of silastic tubing fitted to a guide cannula (Plastics One, Roanoke, VA) bent at a curved right angle and encased in dental acrylic. The catheter tubing was passed subcutaneously from the animal's back to the right jugular vein, and a 1 cm length of the catheter tip was inserted into the vein and tied with surgical silk suture. Following the surgical procedure, animals were allowed ≥48 h to recover from surgery, then provided access to respond for food reward until criteria were achieved. Mice were then permitted to acquire intravenous nicotine self-administration during 1 h daily sessions, 6-7 days per week, at the standard training dose of nicotine (0.03 mg/kg/infusion) for seven days and were then transitioned onto the moderate 0.1 mg/kg/infusion dose of nicotine. This dose was chosen since mice exhibit the highest level of responding for this dose across the dose response-curve, and it also results in a similar level of intake as that found at higher doses with behavioral titration via self-administration (Fowler and Kenny, 2011). Nicotine was delivered through tubing into the intravenous catheter by a Razel syringe pump (Med Associates). Each session was performed using 2 retractable levers (1 active, 1 inactive). Five lever presses on the active lever resulted in the delivery of an intravenous nicotine infusion (0.03 mL infusion volume), followed by a 20 second time out period with cue light activation (FR5TO20 sec schedule). Responses on the inactive lever were recorded but had no scheduled consequences. After acquisition and achieving stable responding on the 0.1 mg/kg/infusion dose, subjects were examined for the effects of dFBr or CMPI on nicotine intake. For Cohort 1 (n=8 males), mice were administered dFBr, or vehicle control, 15 minutes prior to each session utilizing a within subject Latin-square design. For Cohort 2 (n=10 males), mice were treated as above, but were injected with CMPI, or vehicle control, 15 minutes prior to each session utilizing a within subject Latin-square design. This study was then replicated with an additional cohort of 5 mice, resulting in a total of n=10 males for

the CMPI study. In between each dFBr, CMPI or vehicle administration session, subjects were provided baseline days to ensure stable, reinstated responding prior to the next dose. Catheters were flushed daily with physiological sterile saline solution (0.9% w/v) containing heparin (100 units/mL). Catheter integrity was verified with the ultra short-acting barbiturate anesthetic Brevital (2%, methohexital sodium, Eli Lilly, Indianapolis, IN) at the end of the study. Behavioral responses were automatically recorded by MedAssociates software.

2.4. Food self-administration

To ensure the selectivity of the effects of dFBr on nicotine intake, a separate set of mice (n=11 males) were examined for the effects of dFBr on lever pressing behavior to earn food pellets (5TUM grain-based pellets, TestDiet, St. Louis, MO). Mice were first trained to acquire operant food self-administration across ascending fixed ratio schedules from 1 to 5 lever presses to earn a reward, as previously described (Fowler and Kenny, 2011), up to a fixed ratio 5 time out 20 sec schedule of reinforcement (FR5TO20 sec). Responses on the inactive lever were recorded but had no scheduled consequences. After demonstrating stable baseline responding for food pellets across three consecutive sessions, mice were administered dFBr, or vehicle control, 15 minutes prior to each session utilizing a within subject Latin-square design. Behavioral responses were automatically recorded by MedAssociates software.

2.5. Nicotine withdrawal testing

2.5.1. Chronic administration of nicotine.—

Subcutaneous Alzet osmotic minipumps [(model 2002); Durect Corporation, Cupertino, CA] were used to achieve continuous nicotine administration to mice. Nicotine and saline minipumps were implanted in mice under anesthesia [with 4% isoflurane and maintained with 1.5-2% isoflurane in oxygen using a face mask and a vaporizer (VetEquip Inc, Pleasanton, CA)]. The minipumps were kept at a constant flow rate to deliver 24 mg nicotine bitartrate/kg animal body weight/day for 14 days which known to maintain stable nicotine level and elicit nicotine withdrawal symptoms upon withdrawal (Damaj et al., 2003).

2.5.2. 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 observations were performed on day 15, 18-24 hr after minipump removal. Mice were pretreated 15 min prior to behavioral observations with either vehicle or CMPI (10 and 15 mg/kg; s.c.) to assess the effects of acute CMPI administration on spontaneous withdrawal from nicotine. Behavioral testing was performed by an observer blinded to the experimental treatment and in a specific testing sequence known to produce the most consistent results with minimal within-group variability (Jackson et al., 2008). The mice were first evaluated for 5 min in the light-dark box (LDB) test for anxiety-related behavior [the total time spent in the light compartment were recorded for 5 min by a video monitoring technique and ANY-MAZE software (Stoelting Co., Wood Dale, IL)]. The LDB apparatus is composed of a small, enclosed dark or black compartment (36 x 10 x 34 cm) with a passageway (6 x 6 cm) extending to a larger, light or white compartment (36 X 21 X 34 cm). The mice were habituated to the experiment room for 30 minutes before testing. We then followed the LDB test by a 20-min observation

of somatic signs measured as paw and body tremors, head shakes, backing, jumps, curls, and ptosis. The total number of signs were measured. Hyperalgesia was evaluated in the hot-plate test (52°C) immediately following the somatic sign observation period as previously described (Jackson et al., 2008). Finally, sucrose preference (2% sucrose concentration) using the two-bottle choice was determined. Animals were exposed to two 30 ml sipper tubes, one with tap water and the other with 2% sucrose solution and the measurements were taken at 24 hr after removal of nicotine or saline minipumps. Sucrose preference (percentage) was calculated as follows: preference = [sucrose solution intake (ml)/total fluid intake (ml)] × 100.

2.6 Acute thermal nociceptive test

2.6.1. Hot plate test—Mice were placed into a Plexiglas cylinder on a Hot Plate Analgesia Meter (Thermojust Apparatus or IITC Inc) maintained at 55.0°C. Two control baseline latencies at least 10 min apart were obtained for each animal prior to drug administration. Animals were injected with the specified dose of drug(s) and tested at various times thereafter. The reaction time was scored when the animal jumped or licked its paw. If there is no reaction observed, the trial was ended at 20 sec, and the animal was removed from the hot plate. Antinociceptive response was calculated as percentage of maximum possible effect (%MPE, where %MPE = [(test- control)/(20-control) × 100]. For experiments testing the effect of dFBr and CPMI on nicotine-induced effects, mice were pretreated with nAChR PAMs and received nicotine at different times. Mice were then tested 5 min later after nicotine administration.

2.6.2. Tail flick test—Tail Flick Analgesia Meter (IITC Inc.) was used to test tail flick response to thermal nociception. If there is no tail flick response, the trial ended after 10 sec. Antinociceptive response was calculated as percentage of maximum possible effect (%MPE, where %MPE = [(test- control)/(10-control) × 100]. Animals were injected with the specified dose of drug(s) and tested at various times after that. Mice were pretreated with nAChR PAMs and received nicotine at different times. Mice were then tested 5 min later after nicotine administration.

2.7. Body temperature

Rectal body temperatures were measured for mice using a thermistor probe (inserted 24 mm) and a digital thermometer (Yellow Springs Instrument Co., Yellow Springs, OH, USA). Baseline measurements were obtained prior to injections of nAChR PAMs and then after 15, 30, and 60 min of the administration of treatments. Data were calculated as the difference in rectal temperature before and after treatment. The variation of ambient temperature in the laboratory ranged from 21 °C to 24 °C. In a second set of experiments, mice were tested in combination of DHβE (2 mg/kg, s.c.), dFBr (6mg/kg, s.c.) or CMPI (15 mg/kg, i.p.) and nicotine (0.5 mg/kg). Pretreatment times were chosen based on previous studies (Bagdas et al., 2015; Walters et al., 2006). Drugs are injected 15 min intervals, and body temperatures were examined before injections and 30 min after nicotine or saline injection.

2.8. Data analysis

All data analyses were performed using the GraphPad Prism software, version 8 (GraphPad Software, Inc., La Jolla, CA), and are expressed as the mean ± SEM. Behavioral data were first assessed for normality of residuals and equal variance using either the F-test or the Brown-Forsythe test and the Barlett's test. All data passed this test. Statistical analyses include ordinary one-way, two-way, and repeated measure (RM) two-way analyses of variance (ANOVA) where appropriate, followed by the Holm-Sidak test post hoc correction with p-values < 0.05 considered significant.

3. RESULTS

3.1. dFBr, but not CMPI, Decreases Intravenous Nicotine Self-Administration

The first set of studies sought to examine whether voluntary nicotine intake would become altered with differential modulation of the high affinity and low affinity α4β2 nAChRs (Figure 1A). Treatment with higher doses of dFBr (6 and 9 mg/kg) induced a significant decrease in the number of intravenous nicotine infusions, as compared to the vehicle control [One-way ANOVA, F_{Dose} (1.602,11.22) = 8.551, $p = 0.008$] (Figure 1B). A decrease in lever pressing behavior was selective for the active lever and greatest at the 9 mg/kg dFBr dose, as the mice did not differ in their responses between the active and inactive lever at this dose [Two-way RM ANOVA, F_{Dose} (3,18) = 6.96, $p = 0.003$; F_{Lever} (1,6) = 231.2, $p < 0.0001$; $F_{DosexLever}$ (3,18) = 6.15, $p = 0.005$] (Figure 1C). To further ensure that the attenuated responses on the active lever was selectively due to the animal's directed response to decrease consumption of nicotine, a separate cohort of mice was examined for dFBr's effects on food self-administration in the operant lever-pressing task. Importantly, the mice did not exhibit a difference in the number of food pellets earned [One-way ANOVA, F_{Dose} (1.695,16.95) = 0.898, $p = 0.410$] (Figure 1D), or in their lever pressing behavior [Two-way RM ANOVA, F_{Dose} (2,20) = 0.542, $p = 0.590$; F_{Lever} (1,10) = 92.96, $p < 0.0001$; $F_{DosexLever}$ (2,20) = 1.08, $p = 0.359$] (Figure 1E), following pretreatment of dFBr. These data provide direct evidence that dFBr exerts specific effects by decreasing nicotine intake during intravenous nicotine self-administration.

Next, we examined whether selective PAM modulation of the LS (α4)3(β2)2 nAChR isoform with CMPI would elicit a similar effect on nicotine self-administration behavior. Surprisingly, the 10 and 15 mg/kg doses of CMPI did not alter the number of nicotine infusions earned, as compared to vehicle treatment [One-way ANOVA, F_{Dose} (2,18) = 0.479, $p = 0.627$] (Figure 1F). In addition, differences were also not found across doses for the number of lever presses on either the active or inactive lever during nicotine self-administration [Two-way RM ANOVA, F_{Dose} (2,18) = 1.481, $p = 0.254$; F_{Lever} (1,9) = 44.44, $p < 0.0001$; $F_{DosexLever}$ (2,18) = 0.414, $p = 0.667$] (Figure 1G). Together, these findings indicate that the modulation of the HS (α4)2(β2)3 nAChR isoform mediates the reinforcing properties of nicotine that regulate drug intake.

3.2. Effects of CMPI on nicotine withdrawal

Since we recently showed that dFBr fully reverses somatic and affective nicotine withdrawal signs in mice (Hamouda et al., 2018), we investigated in this study the effects of the LS

(α4)3(β2)2 PAM CMPI on nicotine withdrawal in mice. Anxiety-related behavior signs were measured in mice following 24 hr withdrawal from 14 days infusion of nicotine (24 mg/kg/day) or saline and following acute treatment with CMPI or vehicle. Consistent with previous reports (Alkhlaif et al., 2017), mice undergoing nicotine withdrawal demonstrated a significant ($p<0.05$) increase in anxiety-related behavior in the LDB test (assessed as a decrease in time spent in the light side) compared to saline minipump control mice (Figure 2A). Acute administration of 15 mg/kg CMPI or vehicle to saline minipump (nicotine naïve) mice did not significantly ($p>0.05$) alter anxiety-related behavior, importantly indicating that CMPI does not induce anxiety-related behavior in the absence of nicotine. In contrast, administration of 10 and 15 mg/kg dFBr to mice undergoing nicotine withdrawal produced a partial reversal of anxiety-related behavior (Figure 2A). One way ANOVA [$F_{Dose}(4,35)=21.49$, $p<0.0001$] followed by Tukey post hoc revealed that CMPI at 15 mg/kg partially reverse nicotine's withdrawal anxiety-related behavior.

The effects of CMPI administration on somatic signs of spontaneous nicotine withdrawal were assessed using same nicotine-dependent mice described above. Following pretreatment with CMPI or vehicle, somatic signs (paw and body tremors, head shakes, backing, jumps, curls, and ptosis) were observed for 20 min. Nicotine withdrawal mice developed somatic signs (Figure 2B), indicated by a significant ($p<0.05$) increase in the number of observations compared to the saline minipumps control mice. Similar to anxiety-related behavior, acute administration of the highest dose of CMPI did not alter somatic signs in the control mice ($p>0.05$), whereas CMPI pretreatment produced a partial reversal of somatic signs in nicotine withdrawal mice at the highest dose tested (Figure 2B). One way ANOVA followed by Tukey post hoc revealed significant [$F_{Dose}(4,35)=38.51$, $p<0.0001$] CMPI effect at 15 mg/kg.

Following testing anxiety-related and somatic signs of nicotine withdrawal, the groups were evaluated for hyperalgesia using the hot plate test (Figure 2C). Withdrawal from chronic nicotine exposure (nicotine minipump mice) resulted in significant [$F(4, 35) = 6.870$; $p<0.05$) decrease in hot plate latency (hyperalgesia) compared to saline minipump control mice. Administration of 15 mg/kg CMPI produced no change in hot plate latency in the saline minipump mice. In addition, 10 or 15 mg/kg CMPI in nicotine minipump mice did not produce a significant reversal of in nicotine withdrawal-induced reduction in hot plate latency (Figure 2C).

Finally, 24 hr after removal of minipumps, mice implanted with nicotine minipumps showed a significant decrease in sucrose preference when compared to the group that received saline [$F(4, 35) = 10.77$; $p<0.05$) (Figure 2D). Administration of 15 mg/kg CMPI produced no change in sucrose preference in saline minipump mice. In addition, 10 or 15 mg/kg CMPI in nicotine minipump mice did not produce a significant reversal of nicotine withdrawal-induced reduction in sucrose preference (Figure 2D).

3.3. dFBr or CPMI Enhance Nicotine-induced Hypothermia

The next set of experiments was carried out to evaluate the ability of dFBr and CMPI to directly induce changes in body temperature and their ability to modulate nicotine-induced changes in body temperature in mice (Figure 3). To examine the direct effects of dFBr and

CMPI, mice were treated with vehicle, dFBr (1, 3, 6, and 9 mg/kg, s.c.), or CMPI (5 and 15 mg/kg, i.p.) and subsequently assessed for body temperature changes at 15, 30, and 60 min post-treatment. In a separate experiment, mice were pretreated with varying doses of dFBr (1, 3, and 6 mg/kg s.c.) or CMPI (5, 10, and 15 mg/kg i.p.) 15 min before administering 0.5 mg/kg nicotine s.c., and subsequently assessed for body temperature changes at 15, 30, and 60 min. When administered alone, dFBr up to 9 mg/kg or CMPI up to 15 mg/kg did not produce significant body temperature changes compared to their vehicle-treated counterparts at any time points tested (Figure 3A and 3B). In contrast, treatment with nicotine produced statistically significant decreases in body temperature with maximum effects observed 30 min post-treatment. The significance of nicotine's effect was [$F_{Nicotine}$ (1,14) = 33.28, p < 0.0001; F_{Time} (1.343,18.80) = 7.919, p = 0.0069; $F_{TimexNicotine}$ (2,28) = 12.15, p = 0.0002] when analyzed with a two-way repeated-measures analyses of variance (RM ANOVA, GraphPad Prism) (Figure 3C and 3D). Furthermore, pretreatment with dFBr or CMPI 15 min prior to nicotine administration resulted in dose-dependent enhancement of nicotine-induced hypothermia. In mice treated with dFBr then nicotine, the maximum effect on body temperature was observed at 15 min post-treatment and remained higher than the effect observed in mice treated with nicotine alone up to 60 min post-treatment (Figure 3C). dFBr potentiation of nicotine-induced hypothermia was significant based on two-way RM ANOVA [F_{dFBr} (4,35) = 30.25, p < 0.0001; F_{Time} (1.979,69.26) = 26.25, p < 0.0001; $F_{dFBrxTime}$ (8,70) = 4.564, p = 0.0002]. Likewise, mice treated with CMPI then nicotine exhibited a higher degree of hypothermia that was statistically significant when compared to mice treated with vehicle or with nicotine alone using two-way RM ANOVA [F_{CMPI} (4,27) = 20.24, p < 0.0001; F_{Time} (1.963, 53.01) = 16.79, p <0.0001; $F_{CMPIxTime}$ (8,54) = 2.689, p = 0.0145] (Figure 3D).

To evaluate the possible role of α4β2* nAChRs in dFBr and CMPI enhancement of nicotine-induced hypothermia, we tested the effect of CMPI and dFBr in the presence of dihydro-β-erythroidine hydrobromide (DHβE). DHβE is a nAChR antagonist that preferentially inhibits α4β2, α4β4, and α2β2 nAChRs (Harvey et al., 1996; Khiroug et al., 2004; Papke et al., 2008). Mice were pretreated with 2 mg/kg DHβE prior to treatment with 0.5 mg/kg nicotine in combination with 6 mg/kg dFBr or 15 mg/kg CMPI (Figure 4). When compared to control (saline + vehicle), change in body temperature following treatment with nicotine, nicotine+dFBr, or nicotine+CMPI was statistically significant [one-way ANOVA, $F_{treatment}$ (5,42) = 47.14, p < 0.0001] with p values of 0.0013, <0.0001, and <0.0001, respectively, Pretreatment with dFBr or CMPI before nicotine significantly enhanced nicotine's hypothermic effect (p<0.0001 in Holm-Sidak's multiple comparison test) which was fully blocked in the presence of DhβE. Body temperature in the nicotine+dFBr+DHβE and nicotine+CMPI+DHβE treatment groups were non-significantly different (p>0.05) from the control group and statistically significant (p<0.0001) when compared to nicotine+dFBr and nicotine+CMPI, respectively.

3.4. Effect of dFBr and CMPI on Acute Thermal Nociception Behavior

Since PAMs of α4β2* nAChR subtypes are predicted to produce antinociceptive effects on their own, experiments were designed to test the direct effects of dFBr and CMPI on acute thermal nociceptive behavior using hot plate and tail flick tests in adult male ICR mice. The

effects of dFBr and CMPI on the latency to acute thermal sensitivity in hot plate and tail flick tests were measured at 15, 30, 45, and 60 min. Figure 5 shows the latency (MPE%) of hot plate and tail flick tests at 15 min after injection of vehicle or varying doses of dFBr or CMPI. Neither dFBr nor CMPI significantly altered sensitivity to thermal nociception in mice in both assays compared to their vehicle-treated counterparts. Similarly, no significant antinociceptive effect in both was observed at 30, 45, and 60 min post-injection of dFBr and CMPI (Data not shown).

3.4.1. dFBr or CPMI Pretreatment Leads to a Reduction of Nicotine-induced Acute Thermal Antinociception in Mice—Subsequently, we investigated the effects of dFBr or CMPI on nicotine-induced acute thermal antinociception. Our initial hypothesis was that administration of these nAChR PAMs would enhance the effects of nicotine on acute thermal antinociception. Therefore, we started our investigation with low doses of nicotine. Mice were treated with two different doses of nicotine, 0.5 mg/kg in the hot plate, and 1.0 mg/kg in the tail flick test due to the differential potencies of nicotine in each respective test. In addition, mice were pretreated with dFBr (1, 3 and 6 mg/kg s.c.) or CMPI (5,10,15 mg/kg i.p.) 15 min before nicotine injection (0.5 or 1 mg/kg s.c.) after which they were tested 5 min later. Contrary to our initial hypothesis that CMPI and dFBr would enhance nicotine's effect, coadministration of dFBr or CMPI did not increase nicotine's antinociceptive effect by in either the hot plate or tail flick tests (Figures 6 and 7). A two-way ANOVA revealed only significant effects of nicotine in the hot plate [$F_{Nicotine}$ (1,56) = 44.75, p<0.0001; Figure 6A] and tail flick tests in the dFBr study [$F_{Nicotine}$ (1,56) = 9.556, p<0.0031; Figure 7A], and significant effects in response to nicotine in the hot plate test [$F_{Nicotine}$ (1,46) = 13.91, p=0.0005; Figure 6B] and in the tail flick test [$F_{Nicotine}$ (2,38) = 8.242, p=0.0067; Figure 7B] in the CMPI study. However, no significant effects of dFBr on nicotine-induced acute thermal antinociception were observed. Similarly, a two-way ANOVA revealed no significant effects of CMPI on nicotine-induced acute thermal antinociception in either the hot plate or tail flick test.

The next series of experiments investigated a higher dose of nicotine producing approximately 80% MPE in each respective test, 2.0 mg/kg in the hot plate and 2.5 mg/kg in the tail flick test. Following the same dosing paradigm as above, mice were pretreated with dFBr (1, 3 and 6 mg/kg s.c.) or CMPI (5,10, and 15 mg/kg i.p.) 15 min before nicotine (2.0 or 2.5 mg/kg s.c.) and tested 5 min later. As shown in Figures 6 and 7, pretreatment with dFBr or CMPI attenuated rather than augmented nicotine-induced antinociception in both the hot plate and tail flick assays in a dose-related manner. In the hot plate test, two-way ANOVA analysis revealed significant effects of dFBr treatment [F_{dFBr} (3,56) = 6.308, p=0.0009] and nicotine [$F_{Nicotine}$ (1,56) = 146.2, p<0.0001] (Figure 6A). A Holm-Sidak post hoc analysis revealed marked attenuation of nicotine-induced antinociception by dFBr only at the highest doses (3 and 6 mg/kg, s.c). Correspondingly, in the tail flick test, a two-way ANOVA analysis also revealed significant effects of dFBr treatment [F_{dFBr} (3,56) = 28.27, p<0.0001] and nicotine [$F_{Nicotine}$ (1,56) = 134.8, p<0.0001] (Figure 7A). A Holm-Sidak post hoc analysis shows significant attenuation of nicotine-induced antinociception at all doses of dFBr tested (0.1, 1, and 3 mg/kg, s.c.).

In response to CMPI treatment at a high nicotine dose, data reveals significant attenuation of nicotine-induced antinociception in mice in a dose-related fashion (Figures 6 and 7). In the hot plate test, significant effects were observed for both CMPI treatment [F_{CMPI} (3,54) = 18.32, p<0.0001] and nicotine [$F_{Nicotine}$ (1,54) = 108.2, p<0.0001] (Figure 6B). A Holm-Sidak post hoc analysis shows significant attenuation of nicotine-induced antinociception at all doses of CMPI tested (10 and 15 mg/kg). Likewise, in the tail flick test, significant effects were observed for CMPI treatment [F_{CMPI} (2,42) = 23.98, p<0.0001] and nicotine [$F_{Nicotine}$ (1,42) = 137.3, p<0.0001] (Figure 7B). Post hoc analysis revealed significant attenuation of nicotine-induced antinociception at all doses of CMPI tested (5, 10, and 15 mg/kg).

3.4.2. Effects of Intrathecal administration of dFBr on Nicotine-induced Antinociceptive Behavior—To rule out the possible involvement of metabolite(s) in the effects of s.c. dFBr seen above, we examined the effects of intrathecal administration of dFBr on acute thermal nociceptive behavior in mice in the tail flick test (Figure 8). Mice were administered dFBr (0.5 or 5 ug/5ul) intrathecally 5 min before being administered nicotine or saline s.c. in the tail flick test. Nicotine (1.0 or 2.5 mg/kg) was given subcutaneously following the administration of dFBr and then tested 5 min later. Nicotine produced significant increases in tail flick latency across all nicotine treated groups (1.0 and 2.5 mg/kg) compared to saline-treated groups [$F_{Nicotine}$ (2,63) = 72.82, p < 0.0001]. Intrathecal dFBr, much like subcutaneously administered dFBr, produced a significant antinociceptive effect after intrathecal administration at 0.5 and 5 μg/mouse. However, dFBr completely blocked nicotine antinociceptive effects in the tail-flick test in a dose-related manner [F_{dFBr} (2,63) = p < 0.0001].

4. DISCUSSION

The α4 and β2 subunits assemble to form the most widely expressed neuronal nicotinic acetylcholine receptors (nAChRs) in the brain (Taly et al., 2009). Two functional nAChR isoforms are created when assembled alone: the low sensitivity (LS) (α4)3(β2)2 nAChR and the high sensitivity (HS) (α4)2(β2)3 nAChR (Nelson et al., 2003; Zwart et al., 2006). These subunits also form other nAChR subtypes when (α4)2(β2)2 assemble with an accessory subunit, such as α5, α6, and β3 (Gotti et al., 2009). These diverse nAChR subpopulations differ in their expression pattern in various cell types and regions within the central nervous system, their functional contributions to various physiological processes, and their response to exogenously administered nicotinic ligands (agonists, antagonists, and allosteric modulators).

The two compounds, desformylflustrabromine (dFBr) and 3-(2-chlorophenyl)-5-(5-methyl-1-(piperidin-4-yl)-1H-pyrazol-4-yl) isoxazole (CMPI), have been previously characterized *in vitro* using heterologously expressed nAChRs. They are both categorized as α4β2* nAChR PAMs, but their nAChR subtype selectivity was found to be dissimilar. dFBr potentiates nAChRs containing the α4 or α2 subunits that assemble with a β2, but not β4 subunit (Deba et al., 2018; Pandya and Yakel, 2011; Sala et al., 2005). In contrast, CMPI only potentiates nAChRs containing three copies of α4 subunits, resulting in the presence of two adjacent α4 subunits harboring CMPI at their extracellular interface (Hamouda et al.,

2016; Wang et al., 2017). This, along with that fact that a minimum of two ACh binding sites (i.e., two of α4:β2 pair) are required for a functional α4β2* nAChR, limits the binding spectrum of CMPI to the LS (α4)3(β2)2 nAChR. Thus, CMPI represents an excellent tool to study the physiological role and pharmacological contributions of the LS (α4)3(β2)2 nAChR. At the (α4)3(β2)2 nAChR, dFBr and CMPI were equipotent, potentiating responses to low ACh concentrations with a potentiation EC_{50}s of ~0.3 μM and were similar in their maximal potentiation (~400 fold potentiation at 1 μM PAM)(Deba et al., 2018; Hamouda et al., 2016; Hamouda et al., 2015). Despite their similar concentration-dependence effects at the (α4)3(β2)2 nAChR, CMPI and dFBr exerted different effects on the potency and efficacy in the presence of a ligand. At the (α4)3(β2)2 nAChR, dFBr enhanced the efficacy but not the potency of ACh, whereas CMPI enhanced ACh potency by ~100 fold without significant effect on its efficacy (Deba et al., 2018; Hamouda et al., 2016; Hamouda et al., 2015). Thus far, dFBr has been subject of several *in vivo* studies and was found to modulate antinociception, nicotine reinforcement, and withdrawal signs associated with nicotine in rodents (Bagdas et al., 2018a; Liu, 2013; Weggel and Pandya, 2019). In this study, we took advantage of the subtype selectivity of dFBr and CMPI to assess the contribution of the two α4β2* nAChRs isoforms, LS (α4)3(β2)2 and HS (α4)2(β2)3, on nicotine self-administration, nicotine somatic and affective withdrawal symptoms, nicotine-induced hypothermia and acute thermal hyperalgesia. Comparison of our results for the effects of dFBr and CMPI on nicotine-induced behaviors reveals a differential role of α4β2 nAChR isoforms in the underlying mechanisms of nicotine's actions with the HS (α4)2(β2)3 nAChR positively linked to all aspects of nicotine effects. In contrast, the LS (α4)2(β2)3 nAChR was only positively linked to nicotine-induced hypothermia, negatively linked to nicotine-induced antinociception, and did not influence nicotine intake.

4.1. HS α4β2 nAChR isoform plays a vital role in nicotine self-administration and nicotine withdrawal symptoms.

Our results show that dFBr, but not CMPI, reduces intravenous nicotine self-administration in C57BL/6J mice, thereby establishing a critical role of the HS α4β2 nAChRs isoform, but not the LS α4β2 nAChR isoform, in mediating mechanisms underlying nicotine reinforcement. Our results also demonstrate that CMPI cannot fully reverse nicotine withdrawal symptoms at doses up to 15 mg/kg, which was in clear contrast to dFBr's full reversal of nicotine withdrawal somatic and affective-like signs that we have previously reported (Hamouda et al., 2018). The high brain permeability of CMPI (brain:plasma concentrations ~9 folds 1 hr following intraperitoneal injection in rat) (Albrecht et al., 2008) and the effects of CMPI on other nicotine-induced effects (see below) provides direct evidence that the CMPI doses administered were in a pharmacologically appropriate range. Thus, these data establish a profound role of the HS α4β2 nAChRs isoform, rather than the LS α4β2 nAChR isoform, in both nicotine intake and the development of nicotine withdrawal signs. PAMs such as dFBr and CMPI require agonist occupancy at the ligand binding site to exert their pharmacological effects, and nicotine binds with a higher affinity at the HS α4β2 nAChR isoform compared to the LS α4β2 nAChR isoform (EC_{50}s of 0.3-0.8 and 18-38 μM, respectively) (Nelson et al., 2003; Tavares Xda et al., 2012). Therefore, the inability of CMPI to alter nicotine self-administration or fully reverse nicotine withdrawal symptoms provides direct evidence of the important role of the HS α4β2 nAChR

isoform in these actions. These results further support the hypothesis that the level of nicotine required to elicit a reinforcing effect is due to nicotine binding at the high affinity sites in the HS (α4)2(β2)3 nAChR, with little nicotine occupancy at low affinity sites in the LS (α4)3(β2)2 nAChR. It is also important to note that the α4 and β2 nAChR subunits expressed in dopaminergic and other neurotransmitter systems within the reward and reinforcement-related pathways can assemble alone (HS and LS isoforms) or with additional subunits (e.g. α2), and as such, potentiation of these subtypes by dFBr could have also contributed the observed effects on nicotine self-administration and withdrawal symptoms. However, while prior reports have found that dFBr can potentiate nAChRs containing the α2 and β2 subunits (Pandya and Yakel, 2011), our prior findings indicate that α2-containing nAChRs do not mediate the reinforcing properties of nicotine during sustained self-administration (Lotfipour et al., 2013), thereby supporting the involvement of the HS (α4)2(β2)3 nAChR isoform in regulating nicotine consumption. In addition, chronic nicotine has been shown to selectively upregulate α4β2* nAChRs in a variety of biological systems (Flores et al., 1992; Nashmi et al., 2007) and to stabilize the HS (α4)2(β2)3 nAChR isoform (Lester et al., 2009; Srinivasan et al., 2011; Vallejo et al., 2005). Since both of these studies examined the effects of the PAMs under conditions of chronic nicotine exposure, the cholinergic system was likely biased towards predominate activation of the HS (α4)2(β2)3 nAChR isoform. As such, it will be important in further studies to develop a highly selective PAM targeting the HS (α4)2(β2)3 nAChR to better delineate aspects of these contributions as a means of guiding therapeutic development.

Both HS and LS α4β2 nAChR isoforms contribute to nicotine-induced hypothermia.—When administered alone, neither dFBr nor CMPI had a significant effect on body temperature; however, both significantly potentiated the nicotine-induced reduction in body temperature in a dose-dependent manner. The dose-dependent influence of dFBr and CMPI on nicotine-induced hypothermia were mediated via β2-dependent mechanisms, as coadministration of the β2 selective antagonist DhβE prevented these effects. The ineffectiveness of dFBr to modulate body temperature when administered alone at doses of 1 to 6 mg/kg is consistent with prior findings, which demonstrated that off-target hypothermic effects occur at much higher doses (32 and 100 mg/kg) (Moerke et al., 2016). Moreover, the dFBr enhancement of nicotine-induced hypothermia is also consistent with prior findings (Moerke et al., 2016). Given that CMPI enhanced nicotine's hypothermic effect to the same extent as dFBr, the LS (α4)3(β2)2 nAChR appears to be sufficient to alter nicotine-induced hypothermia, and thus, a main finding of the current study establishes an important role for the LS (α4)3(β2)2 nAChR subtype in the cholinergic regulation of body temperature.

4.2. HS and LS α4β2 nAChR isoforms play differential roles in nicotine-induced acute thermal antinociception.

In contrast to the *enhancing* effects of the PAMs with nicotine on body temperature, nicotine-induced antinociception was *attenuated* by either dFBr or CMPI in both the tail flick and hot plate tests. Of relevance, CMPI elicited a notably pronounced blockade in the hot plate test, with the 15 mg/kg dose almost altogether abolishing the nicotine-induced effect. Further, both PAMs were able to completely abolish nicotine-induced antinociception in the tail flick test. The dFBr blockade of nicotine-induced antinociception in the tail flick

test was also observed after intrathecal administration of the drug, ruling out that this effect is a result of a pharmacokinetic factor (e.g. production of metabolite(s) of dFBr with α4β2* nAChRs blocking effect). Of note, the hot plate paw withdrawal response is believed to involve mainly supraspinal sites, whereas the tail flick reflex is thought to be spinally-mediated (Langerman et al., 1995). Thus, the pronounced effect of CMPI in the hot plate test may indicate a greater role of the LS (α4)3(β2)2 nAChR in the supraspinal regulation of acute pain nociception. This finding further supports the contention that LS (α4)3(β2)2 is the major isoform expressed in cortical regions (DeDominicis et al., 2017; Marks et al., 2007). Alternatively, the LS (α4)3(β2)2 and HS (α4)2(β2)3 nAChRs may play differential roles in the regulation of nicotine's acute antinociception due to differences in nicotine affinity and efficacy, effects on the stability of the receptor conformation for channel gating, and/or expression patterns with cell-specific localization at pre- and post-synaptic membranes in nociceptive circuits. For instance, the receptors may be localized differentially on excitatory and inhibitory circuits, with the inhibitory effects being more pronounced due to higher levels of ligand activation via PAM modulation. Thus, to further understand each isoform's respective contributions, future efforts will need to focus on drug development to derive a HS (α4)2(β2)3 nAChR selective PAM and/or novel methods to characterize membrane expression of the two different isoforms within specific cell types.

The direct effects of CMPI up to 15 mg/kg and dFBr up to 6 mg/kg that we observed in the hot plate and tail flick tests (<20 and 10% MPE, respectively, Figure 3) are comparable to results reported in rats, in which CMPI up to 20 mg/kg and dFBr up to 10 mg/kg produced <20% MPE (Deba et al., 2020). However, dFBr at a higher dose (20 mg/kg) exerted a direct effect (~60% MPE) on acute thermal pain in rats (Deba et al., 2020), indicating the lower doses of dFBr used in the current study were not sufficient to induce independent effects for these pain-related assessments in mice. In prior reports, dFBr was found to enhance nicotine-mediated antinociception in a mouse model of chronic nerve injury (Bagdas et al., 2018a; Weggel and Pandya, 2019), which suggests that α4β2* nAChRs may differentially contribute to acute versus persistent pain conditions. Indeed, since α4β2* nAChRs PAMs, like dFBr, have been shown to enhance acute nicotine's antinociceptive effects in chronic pain conditions, the discrepancy in dFBr's effects with acute vs. chronic nicotine could potentially be attributed to changes in the expression level and/or the regulation of the different LS and HS α4β2 nAChR isoforms. Chronic nicotine has been shown to selectively upregulate α4β2* nAChRs in a variety of biological systems (Flores et al., 1992; Nashmi et al., 2007) and to stabilize the HS (α4)2(β2)3 nAChR isoform (Lester et al., 2009; Srinivasan et al., 2011; Vallejo et al., 2005). Therefore, under chronic nicotine exposure conditions, the HS (α4)2(β2)3 nAChR isoform may predominate with cholinergic signaling, leading to a biased response in the presence of the dFBr PAM. While no statistically significant sex difference was observed in the direct effects of CMPI and dFBr up to 20 mg/kg in the tail flick and hot plate tests in rats (Deba et al., 2020), the use of only male mice remains a limitation of work presented in this study. Further work is needed to evaluate CMPI modulation of nicotine effects under chronic pain conditions in male and female mice.

4.3. Relevance to the development of α4β2 nAChR-based therapeutics.

Traditionally, drug development efforts for nAChR based therapeutics have been agonist-focused in their approach (Hurst et al., 2013; Umana et al., 2013). However, with few exceptions (e.g., varenicline for smoking cessation), efforts to develop nAChR agonists that meet clinical criteria were mostly unsuccessful (Hurst et al., 2013), and side effects associated with the use of nAChR agonists as analgesics were often reported (Jain, 2004). The diversity of neuronal nAChRs, each with unique biochemical and pharmacological characteristics, substantially contributes to the challenge of developing nAChR agonists suitable for clinical application. For example, ABT-594, the first selective α4β2 nAChR agonist to be tested as an analgesic in a clinical trial, was found to be clinically efficacious, but poorly tolerated, due to an α3β4* nAChR-mediated ganglionic side effects (Lee et al., 2011; Zhu et al., 2011). The fact that multiple nAChR subtypes have been proposed as suitable targets for antinociceptive and anti-allodynic effects, including the α4β2, α6β4, α7, and α9, makes it difficult to discern the subunit selectivity that is optimal to achieve a more efficacious nAChR-based analgesic (Bagdas et al., 2018b; Hone and McIntosh, 2018). In the recent years, PAMs have emerged as a drug class to selectively target nAChRs for potential therapeutic uses (Fowler and Kenny, 2014; Mohamed et al., 2015; Uteshev, 2016; Wilkerson et al., 2020). As noted above, PAMs do not directly activate the nAChR on their own; however, they enhance an agonists' potency and/or efficacy, thereby potentially enhancing cholinergic signaling upon the release of endogenous ACh or presence of nicotine. Therefore, PAMs do not produce non-physiological continuous activation and desensitization of nAChRs, which is evidenced with agonists (Uteshev, 2016; Williams et al., 2011). As such, PAMs are expected to have less undesirable side effects than agonists (Fowler and Kenny, 2014; Lee et al., 2011; Moerke et al., 2016; Mohler et al., 2014; Nirogi et al., 2013; Pandya and Yakel, 2011; Rode et al., 2012). Since nAChR PAMs, unlike agonists, do not bind at the conserved ACh binding site but bind to more diverse binding sites within the nAChR structure, they have more diverse structural motifs and higher potential of subtype selectivity (Bertrand and Gopalakrishnan, 2007; Taly et al., 2011). Indeed, nAChR PAMs like CMPI have exhibited higher nAChR subtype selectivity than agonists (Wang et al., 2017). Therefore, based on the current findings, future structure-based design of novel PAMs carry the potential to derive more efficacious nAChR-based therapeutics with the goal of attenuating nicotine self-administration, alleviating withdrawal symptomology, and mediating pain responsivity.

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Abbreviations:

nAChRs	nicotinic acetylcholine receptors
PAMs	positive allosteric modulators
dFBr	desformylflustrabromine

CMPI	3-(2-chlorophenyl)-5-(5-methyl-1-(piperidin-4-yl)-1H-pyrazol-4-yl)isoxazole
MPE%	maximum possible effect

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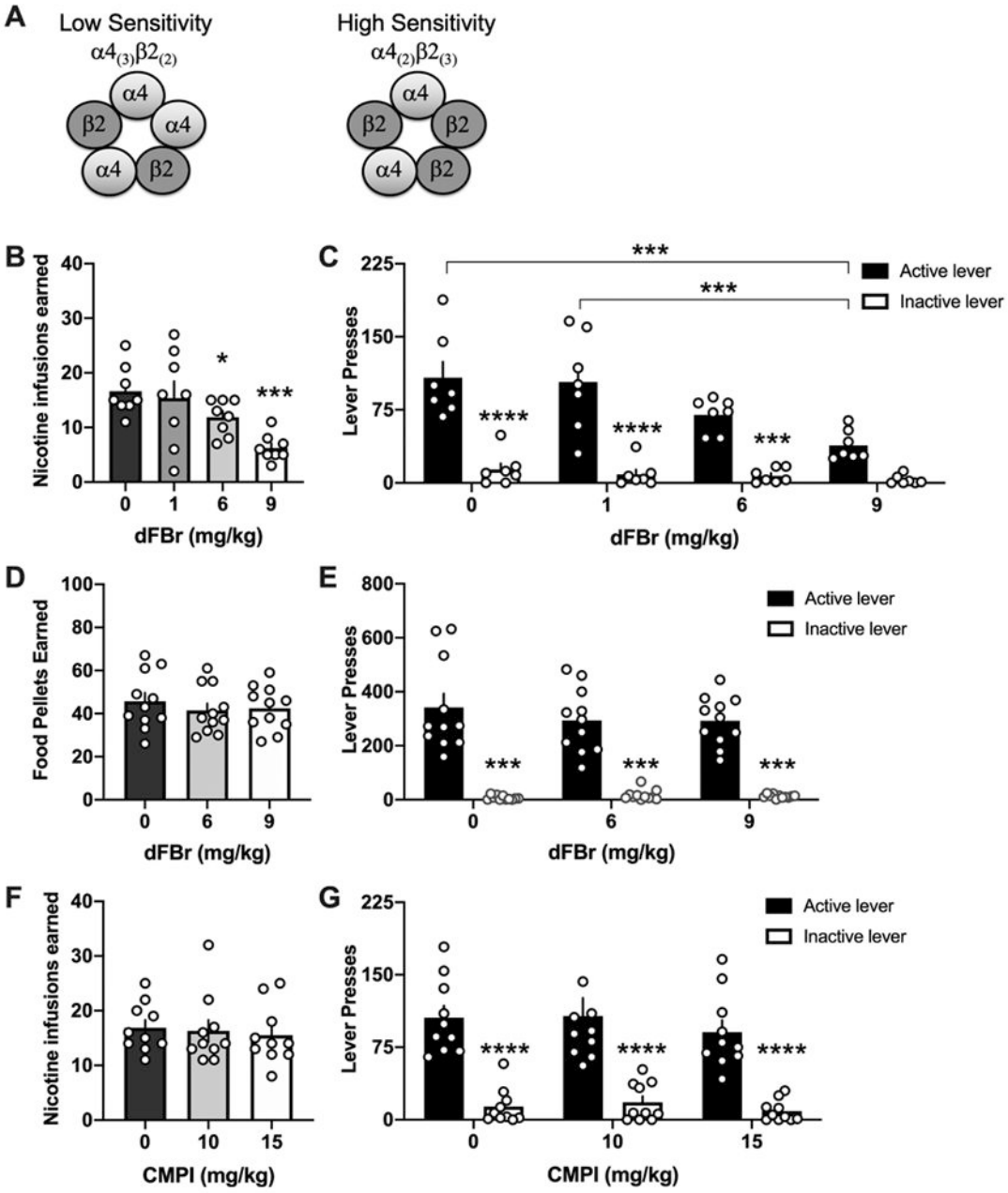

Figure 1: dFBr, but not CMPI, dose-dependently attenuates intravenous nicotine self-administration in mice.

(A) Schematic illustrates the subunit assembly of high- and low-sensitivity α4β2 nAChRs. **(B)** dFBr dose dependently decreased the number of intravenous nicotine infusions earned by self-administering mice, with the greatest effects found at 9 mg/kg. **(C)** When examining the active and inactive lever presses during the nicotine self-administration sessions, mice significantly attenuated their responding on the active lever for the 9 mg/kg dose, as compared to the vehicle and 1 mg/kg doses. Mice maintained a preference for the nicotine-associated active lever, compared to the inactive lever, for all doses except 9 mg/kg. **(D)** During food self-administration, dFBr did not alter the number of food rewards earned

across all doses. **(E)** Mice maintained a preference for the food-associated active lever, compared to the inactive lever, across all dFBr dose administrations. **(F)** CMPI did not alter the number of nicotine infusions earned in self-administering mice, compared to vehicle treatment. **(G)** Mice maintained preference for the nicotine-associated active lever across all CMPI doses, compared to the inactive lever. Data represent mean ± SEM of 8-11 mice/group. *$p < 0.05$, ***$p < 0.001$, **** $p < 0.0001$

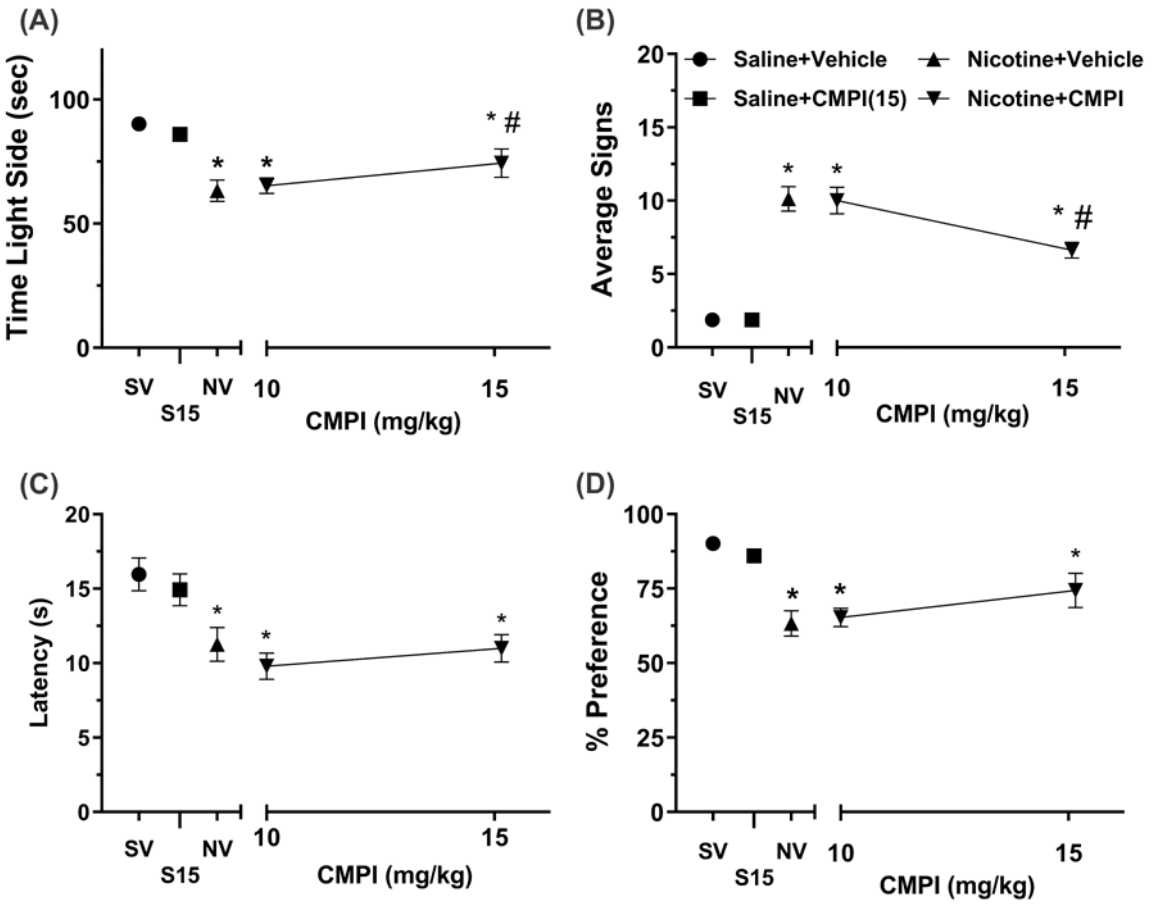

Figure 2: CMPI slightly modulates nicotine withdrawal in mice.

Nicotine-dependent mice were injected with CMPI (10 or 15 mg/kg i.p.) and tested for various withdrawal signs **(A)** Effect of CMPI on nicotine withdrawal-induced anxiety-like behavior as measured by time spent in the light side of the light-dark box; **(B)** Effect of CMP on somatic signs of nicotine withdrawal; **(C)** Effect of CMP on nicotine withdrawal-induced hyperalgesia as measured in the hot plate test and **(D)** Effect of CMP on nicotine withdrawal-induced decrease in sucrose preference as measured in the two-bottle choice test. *Denotes significantly different from Saline+Vehicle (S/V), $p<0.05$; #Denotes significantly different from Nicotine+Vehicle (N/V), $p<0.05$. “S15” denotes mice treated with saline+15 mg/kg CMPI. Each point represents the mean ± S.E.M. of 8 mice per group.

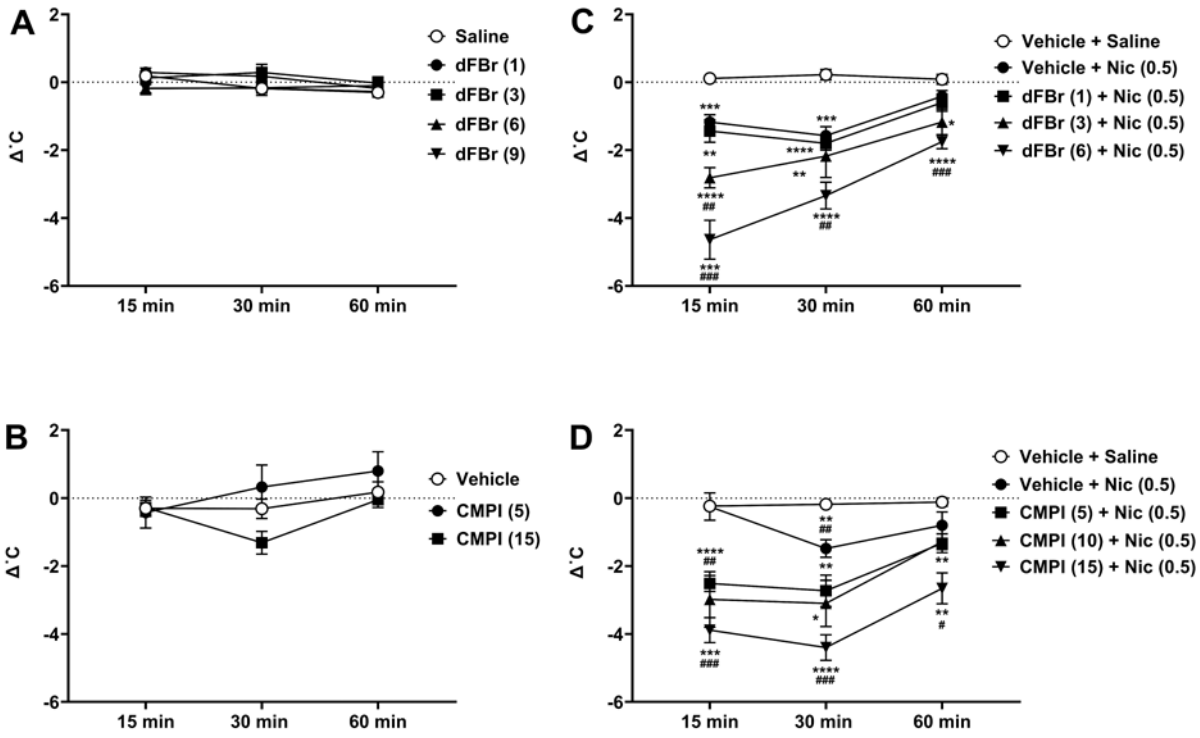

Figure 3: dFBr and CMPI enhance nicotine-induced hypothermia in mice.

Time course of effects of administered nAChR PAMs **(A)** dFBr and **(B)** CMPI on body temperature in mice at 15, 30, and 60 min. Mice were pretreated with dFBr or CMPI for 15 minutes, after which they were assessed for body temperature changes at 15, 30- and 60-min posttreatment. **(C)** and **(D)** show the effect of subcutaneously administered dFBr and CMPI on nicotine-induced hypothermia in mice. Mice were pretreated with dFBr (1, 3, and 6 mg/kg s.c.) or CMPI (5,10, and 15 mg/kg i.p.) 15 min before administering 0.5 mg/kg nicotine, after which they were assessed for body temperature changes at 15, 30, and 60 min. Data is represented by the mean change in body temperature (Δ˚C) ± SEM of 6-8 mice/ treatment/group. Significant differences are based on Holm-Sidak post-hoc test following two-way ANOVA. $^{**}p < 0.01$, $^{***}p < 0.001$ versus vehicle + saline. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$ versus vehicle + nic (0.5).

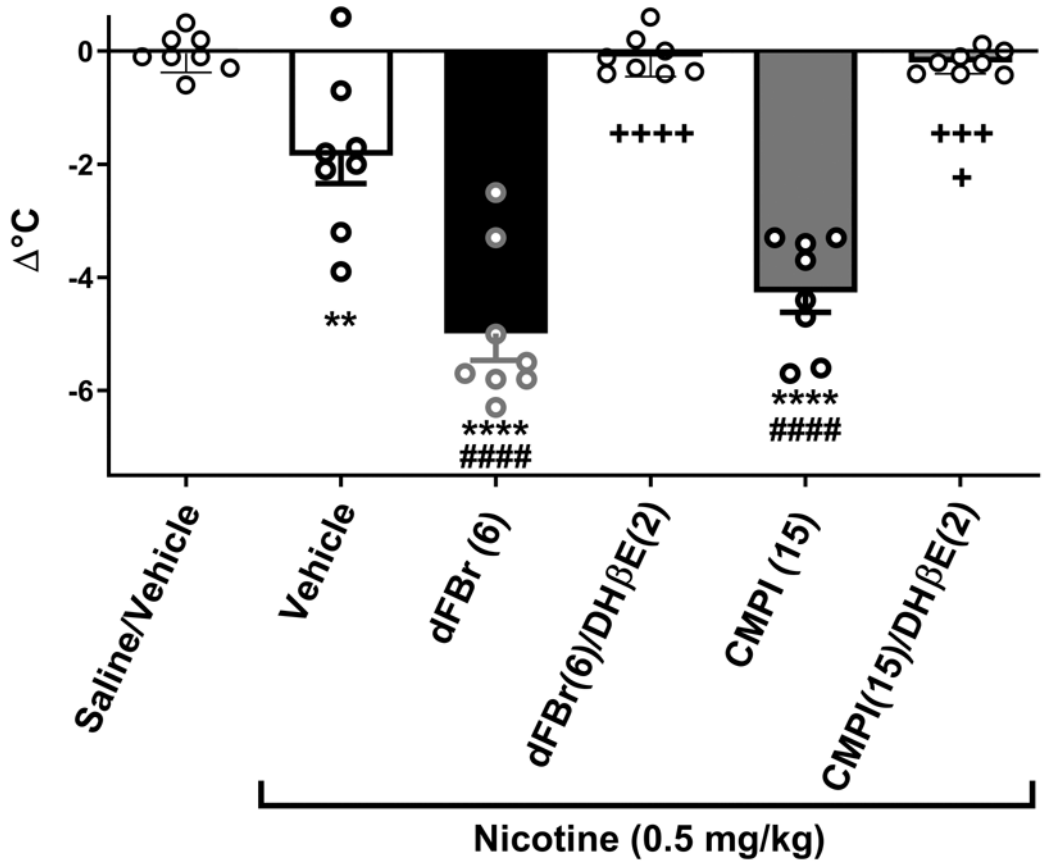

Figure 4: α4β2* nAChRs mediate the effects of nicotine, dFBr and CMPI on body temperature.

Mice body temperatures were recorded before and 30 min after treatment with vehicle, nicotine, or a combination of nicotine with CMPI, dFBr, and/or DHβE. Nicotine, dFBr, CMPI, and DHβE were used at 0.5, 6, 15 and 2 mg/kg, respectively. Data is represented by the mean change in body temperature (Δ˚C) ± SEM of 8 mice/treatment/group. Significant differences are based on Holm-Sidak post-hoc test following one-way ANOVA. $^{**}p < 0.01$, $^{****}p < 0.0001$ versus saline + vehicle. $^{\#\#\#\#}p < 0.0001$ versus vehicle + 0.5 mg/kg nicotine. $^{++++}p < 0.0001$ indicates statistical significance compared to same treatment without DHβE.

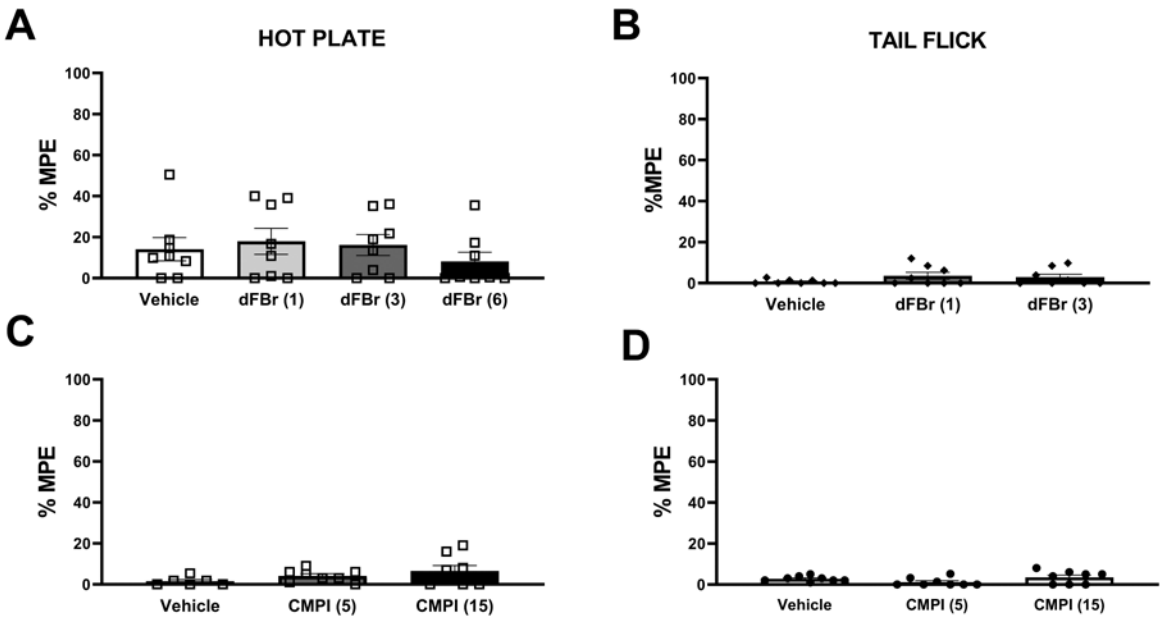

Figure 5: nAChR PAMs do not modulate acute thermal antinociception in mice.

Effect of systemically administered nAChR PAMs dFBr and CMPI on acute thermal nociception in mice in the hot plate **(A)** and **(C)** and tail flick test **(B)** and **(D)**. Mice were pretreated with varying concentrations of dFBr or CMPI 15 min before being subjected to the hot plate or tail flick tests. Doses are in represented in mg/kg. Data is represented by the mean %MPE + SEM of 6-8 mice/treatment/group. Results are non-significant based on two-way ANOVA.

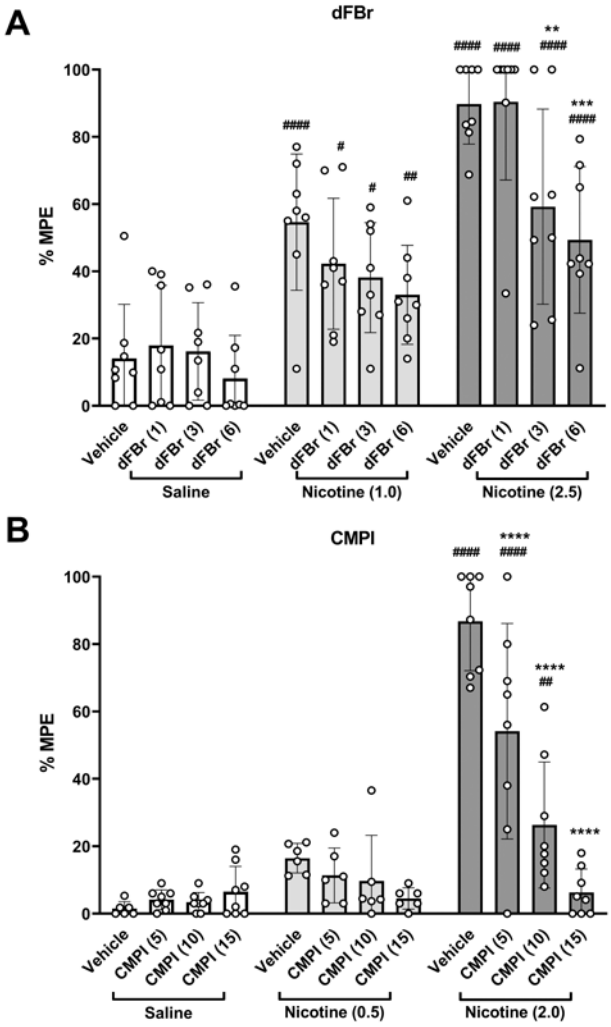

Figure 6: Effect of subcutaneously administered nAChR PAMs (A) dFBr and (B) CMPI on nicotine-induced acute thermal antinociception in mice in the hot plate test.

Mice were pretreated with dFBr (1, 3, and 6 mg/kg s.c.) or CMPI (5,10,15 mg/kg i.p.) 15 min before being subjected to the hot plate test. Nicotine (1 or 2.5 mg/kg s.c.) was introduced 5 min after pretreatment of nAChR PAM. Total pretreatment time is 15 min for nAChR PAM and 10 min for nicotine. Data is represented by the mean %MPE + SEM of 6-8 mice/treatment/group. Significant differences are based on Holm-Sidak post-hoc test following two-way ANOVA. $^{**}p < 0.01$, $^{***}p < 0.001$ versus dFBr vehicle within groups. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$ versus saline + vehicle.

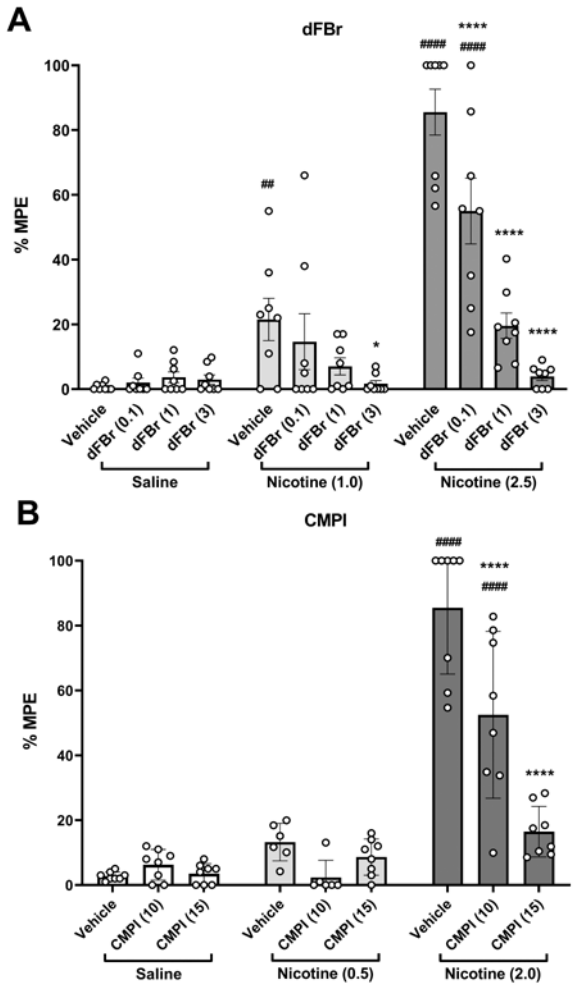

Figure 7: Effect of subcutaneously administered nAChR PAMs (A) dFBr and (B) CMPI on nicotine-induced acute thermal antinociception in mice in the tail flick test.

Mice were pretreated with dFBr (0.1, 1, and 3 mg/kg s.c.) or CMPI (5,10,15 mg/kg i.p.) 15 min before being subjected to the hot plate test. Nicotine (1 or 2.5 mg/kg s.c.) was introduced 5 min after pretreatment of nAChR PAM. Total pretreatment time is 15 min for nAChR PAM and 10 min for nicotine. Data is represented by the mean %MPE + SEM of 6-8 mice/treatment/group. Significant differences are based on Holm-Sidak post-hoc test following two-way ANOVA. $^{*}p < 0.05$, $^{****}p < 0.0001$ versus dFBr vehicle within groups. $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$ versus saline + vehicle.

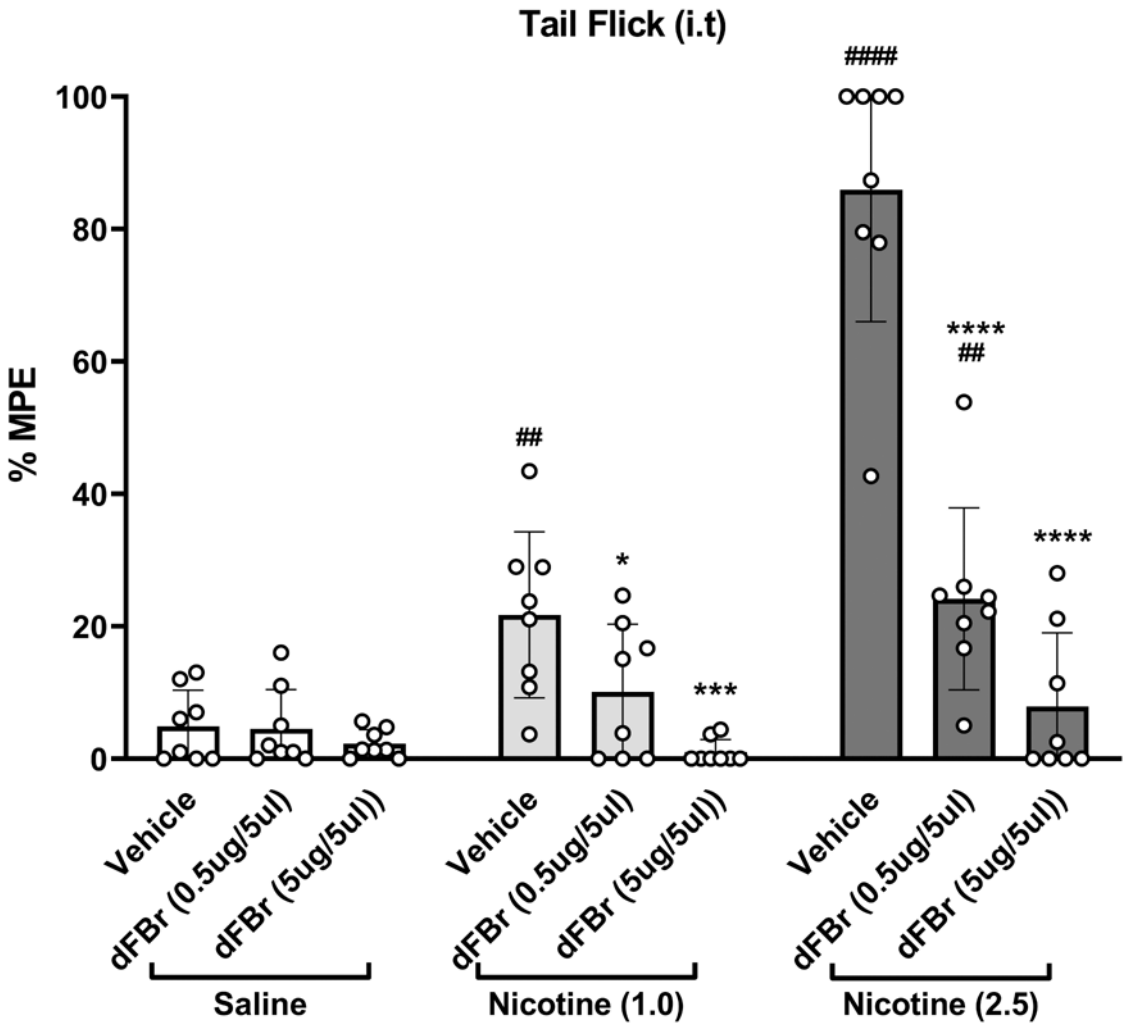

Figure 8: Effect of intrathecal administration of dFBr on nicotine-induced acute thermal antinociception in mice in the tail flick test.

Mice were given an intrathecal administration of dFBr (0.5, 5 ug/5ul) 5 min before being subjected to the tail flick test. Nicotine (1 or 2.5 mg/kg) was given subcutaneously immediately following administration of nAChR PAM. Total pretreatment time is 5 min for nAChR PAM and 5 min for nicotine. Data is represented by the mean %MPE + SEM of 6-8 mice/treatment/group. Significant differences are based on Holm-Sidak post-hoc test following two-way ANOVA. $^{*}p < 0.05$, $^{***}p < 0.001$ $^{****}p < 0.0001$ versus dFBr vehicle. $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$ versus saline + vehicle.