

The $\beta 3$ subunit of the nicotinic acetylcholine receptor is required for nicotine withdrawal-induced affective but not physical signs or nicotine reward in mice

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ABSTRACT

Nicotinic acetylcholine receptors (nAChRs) are the primary target for nicotine, the addictive component in tobacco products. These pentameric receptors are made up of various subunits which contribute to the diverse functions of nAChR subtypes. The $\beta 3$ subunit of the nAChR has been understudied in nicotine dependence, even though it is expressed in brain regions important for drug reward. Therefore, we assessed nicotine dependence behaviors in $\beta 3$ wildtype (WT) and knockout (KO) male and female mice. We evaluated nicotine reward in the conditioned place preference (CPP) test and then measured nicotine withdrawal signs after chronic exposure to the drug. For the withdrawal studies, mice were continuously infused with 24 mg/kg/day of nicotine using surgically implanted osmotic mini-pumps for 14 days. Mini-pumps were removed at day 15, and withdrawal signs (somatic signs, hyperalgesia, anhedonia-like measure using the sucrose preference test and anxiety-like behaviors using the light dark boxes) were collected at 24 h intervals for three days following spontaneous withdrawal of nicotine. Nicotine-induced CPP did not differ between $\beta 3$ KO and WT mice. $\beta 3$ KO mice displayed similar somatic symptoms and hyperalgesia compared to WT mice but showed significant absence in affective (anhedonia and anxiety-like behaviors) withdrawal signs in nicotine-dependent mice. These observations suggest that the $\beta 3$ nicotinic subunits do not seem to influence nicotine reward but plays an important role in affective nicotine withdrawal signs. Given the health burden of tobacco use disorder and the modest effect of smoking cessation aids, it is important to understand underlying factor contributing to nicotine dependence. The results of this study will further our knowledge of the role of the $\beta 3$ nAChR subunit in nicotine reward and withdrawal behaviors in hopes of finding new molecular targets for smoking cessation aids.

1. Introduction

Tobacco use disorder is associated with allelic variation in *CHRN3*, (Bierut, 2007; Chen et al., 2012; Saccone et al., 2007; Zeiger et al., 2008), a gene that encodes for the $\beta 3$ subunit of the nicotinic acetylcholine receptor (nAChR) (Thorgeirsson et al., 2010). The $\beta 3$ subunit is not widely distributed in the brain, but is expressed in brain regions important in nicotine reward and withdrawal such as the ventral tegmental area, substantia nigra, nucleus accumbens and medial habenula (Deneris et al., 1989; Grady et al., 2009, 2007). In addition, nicotinic receptors containing the $\beta 3$ subunit have been implicated in dopamine release from terminals in the mouse striatum (Cui et al., 2003; Sharon R. Grady et al., 2007). Further, research has shown that these brain regions are important for nicotine reward and withdrawal (Picciotto et al., 1998; Salas et al., 2004; Tapper et al., 2004; Zhao-shea et al., 2015; Zhao-shea et al., 2013).

Nevertheless, surprisingly little is known about the role of $\beta 3$ -containing nAChRs in nicotine reward and withdrawal. Genetically modified mice may serve as important tools to understand the impact of the $\beta 3$ subunit on nicotine reward and withdrawal behaviors. A recent study reported that oral nicotine consumption in the two-bottle choice

paradigm was decreased in $\beta 3$ knockout (KO) mice (Kamens et al., 2015) suggesting that the $\beta 3$ subunit may partially mediate oral nicotine intake.

To extend these findings, in this study, we assessed nicotine dependence behaviors in $\beta 3$ wildtype (WT) and KO male and female mice. We evaluated nicotine reward in the conditioned place preference (CPP) test and then measured nicotine withdrawal signs after chronic exposure to nicotine. The findings of this study will further elucidate the role of the nicotinic $\beta 3$ subunit in nicotine dependence.

2. Materials and methods

2.1. Animals

Healthy viable mice null for the $\beta 3$ nicotinic subunit were provided by Dr. Jerry Stitzel at Institute for Behavioral Genetics, University of Colorado (Boulder, CO). The $\beta 3$ KO and their WT littermates were bred in an animal care facility at Virginia Commonwealth University (Richmond, VA). The $\beta 3$ KO mice were originally reported by Cui et al. (2003). All mice used in each experiment were backcrossed to C57BL/6J (Jackson Laboratories, Bar Harbor, ME) at least for 9 generations.

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Mutants and WT controls were obtained by crossing with heterozygote mice to control for any irregularities that might arise with crossing only mutant mice. Mice were group-housed (1–4 per cage) in a temperature and humidity-controlled animal care facility approved by Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Mice had free access to food and water under a 12-h light/dark cycle (lights on at 6:00 am) schedule. Male and female mice were 8–10 weeks old at the start of the experiments. All experiments were performed during the light cycle and the study was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. All studies were carried out in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Drugs

(–)-Nicotine hydrogen tartrate [(–)-1-methyl-2-(3-pyridyl) pyrrolidine (+)-bitartrate] was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). All doses of nicotine refer to the free-base form. Freshly prepared solutions were given to mice at 10 ml/kg.

2.3. Nicotine conditioned place preference studies

An unbiased CPP paradigm was performed, as we previously described (Kota et al., 2007). Briefly, the CPP apparatus consisted of three chambers in a linear arrangement (Med Associates, St Albans, VT). The CPP apparatus (Med Associates, St. Albans, VT, ENV3013) consisted of white and black chambers (20 × 20 × 20 cm each), which differed in overall color and floor texture (white mesh or black rod). These chambers 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, male and female mice 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 (0.1, 0.5 or 1 mg/kg, s.c.) in one large chamber and saline in the other large chamber. Treatments were counterbalanced equally in order to ensure that some mice received the unconditioned stimulus in the morning while others received it in the afternoon. The nicotine-paired chamber was randomized among all groups. Sessions were 4 h apart and were conducted by the same investigator. On test day (day 5), mice were allowed access to 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 during test day versus the time in drug paired side during the baseline day. Below the specifics for the dose regimen and pretreatment time for each drug in the individual experiments are mentioned.

2.4. Spontaneous nicotine withdrawal studies

Male and female mice were anesthetized by inhaling isoflurane/oxygen vapor mixture (1–3%). Alzet osmotic minipumps (model 2000; Alzet Corporation, Cupertino, CA) were then implanted subcutaneously within the animals for 14 days. The Alzet minipumps were filled with either nicotine or saline solutions and inserted by making incision parallel to the spine at shoulder level of the mouse. The wound was closed using wound clips and the mice were placed in a surgery room for recovery before using them in experiments. Post-operative care was done for 14 days by observing the animals daily. For all of the procedures, the dose of nicotine was 24 mg/kg/day, calculated according to

the mouse weight. On day 15, spontaneous nicotine withdrawal was induced by removing the minipumps under isoflurane anesthesia in aseptic surgical conditions. The experiment was adapted as previously described (Damaj et al., 2003; Jackson et al., 2008). On day 15 mice were tested for anxiety-like behavior in the plus-maze test, somatic signs and hyperalgesia (day 15). On day 16 mice were tested for sucrose preference. Mice were first evaluated for 5 min in the plus maze test for anxiety-related behavior. Time spent on the open arms of the plus maze was assessed as a measure of anxiety-related response. The number of arm crosses between the open and closed arms was also 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. On day 16, sucrose preference test was used to investigate the anhedonia-like behavior in rodents after the induction of nicotine withdrawal. In this experiment, mice were individually housed and acclimated to cages with food and water. Mice had free access to two 30 ml sipper tubes containing tap water for 3 days as a baseline. Animals then were exposed to two 30 ml sipper tubes, one with tap water and the other with 2% sucrose solution. To prevent any bias, tube placement was switched daily. The measurements of sucrose preference were taken at day 2 after removal of osmotic minipumps. Sucrose preference was determined as the percentage of 2% sucrose volume consumed over the total fluid intake volume. Sucrose preference (percentage) was calculated as follows: preference = [sucrose solution intake (ml)/total fluid intake (ml)] × 100. The experiment was adapted as previously described (Alkhlaif et al., 2017; Pothion et al., 2004; Toma et al., 2017). 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.

2.5. Statistical analysis

The data were analyzed with GraphPad Prism software, version 6 (GraphPad Software, Inc., La Jolla, CA), and expressed as mean ± SEM. Mice were analyzed with an ordinary two-way analysis of variance (ANOVA) test with genotype (KO versus WT) and treatment (saline versus nicotine) as between-subject factors in conjunction with a Tukey post hoc test. Prior to the ANOVA test. All experiments animals with the same genotype were randomly allocated to experimental groups. Differences were considered significant at $p < 0.05$.

3. Results

3.1. The role of $\beta 3$ nicotinic subunits in nicotine CPP

$\beta 3$ WT and KO mice were conditioned with either saline or nicotine at doses of (0.1, 0.5, or 1 mg/kg; s.c.) for 3 days in the CPP paradigm. As displayed in Fig. 1, nicotine treated $\beta 3$ WT and KO mice showed a significant preference in comparison with saline treated counterparts [$F_{\text{treatment}}(3,73) = 20.29$; $p < 0.0001$]. There was no significant effect of genotype in nicotine CPP [$F_{\text{genotype}}(1, 73) = 0.04152$; $p = 0.8391$]. In addition, the interaction between nicotine treatment and genotype was not significant between the same subjects [$F_{\text{interaction}}(3, 73) = 0.2205$; $p = 0.8819$].

$\beta 3$ nicotinic subunits are involved in affective not physical signs of

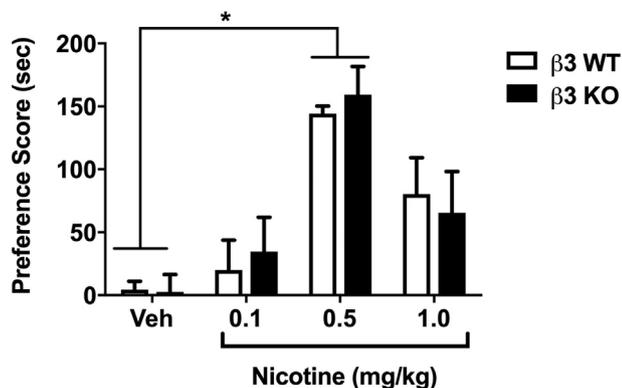


Fig. 1. Nicotine CPP in beta3 WT and KO mice.

beta3 WT and KO mice were conditioned with either s.c. saline or nicotine at doses of (0.1, 0.5, 1 mg/kg) for 3 days. A robust CPP was observed in nicotine-conditioned beta3 WT and KO mice in comparison with vehicle. *Denotes $p < 0.05$ vs saline control. #Denotes $p < 0.05$ nicotine dose of 0.5 mg/kg vs nicotine dose of 0.1 mg/kg in both WT and KO. Each point represents the mean \pm SEM of $n = 11$ (6 male and 5 female) mice per group.

nicotine withdrawal.

Spontaneous nicotine withdrawal was induced in beta3 WT and KO mice via removal of minipumps and nicotine withdrawals signs (anxiety-like behavior, somatic signs and hyperalgesia) were measured. In Fig. 2A, nicotine withdrawn beta3 WT mice displayed anxiety-related behavior in the plus maze compared to their saline treated counterparts [$F_{\text{treatment}}(1, 28) = 22.08$; $p < 0.0001$]. However, beta3 KO mice

undergoing nicotine withdrawal did not display signs of anxiety-like behavior. Indeed, nicotine withdrawal induced anxiety-like behavior in beta3 WT mice but not beta3 KO mice [$F_{\text{genotype}}(1, 28) = 23.12$; $p = 0.0001$]. Analyses of the average total number of arm crosses for each treatment group revealed no significant difference among groups (Supplementary Table 1) indicating that the observed reductions in time mice spent in the open arms of the plus maze were not related to changes in mice locomotor activity.

In addition, there was a significant interaction between genotype and treatment [$F_{\text{interaction}}(1, 28) = 15.45$; $p = 0.0005$]. However, as shown in Fig. 2B beta3 WT and KO mice undergoing spontaneous nicotine withdrawal both expressed somatic withdrawal signs compared to control littermates [$F_{\text{treatment}}(1, 28) = 117.1$; $p = 0.0001$]. There was no significant effect of genotype [$F_{\text{genotype}}(1, 28) = 0.2655$; $p = 0.6104$] or interaction [$F_{\text{interaction}}(1, 28) = 0.02950$; $p = 0.8649$]. In addition, nicotine withdrawn beta3 WT and KO mice displayed significant hyperalgesia compared to control littermates [$F_{\text{treatment}}(1, 28) = 94.67$; $p = 0.0001$; Fig. 2C]. There was no significant effect of genotype [$F_{\text{genotype}}(1, 28) = 0.01347$; $p = 0.9084$] or interaction [$F_{\text{interaction}}(1, 28) = 0.1811$; $p = 0.6737$]. Furthermore, we measured sucrose preference in beta3 WT and KO mice 48 h after induction of spontaneous withdrawal. Nicotine infused beta3 WT mice demonstrated an attenuation of sucrose preference compared to their saline controls [$F_{\text{treatment}}(1, 28) = 30.16$; $p < 0.0001$]. This effect was absent in nicotine withdrawn beta3 KO mice, indicating a significant difference in genotype [$F_{\text{genotype}}(1, 28) = 14.24$, $p = 0.0008$]. Two-way ANOVA revealed a significant effect of interaction between genotype and treatment [$F_{\text{interaction}}(1, 28) = 29.79$; $p < 0.0001$; Fig. 2D].

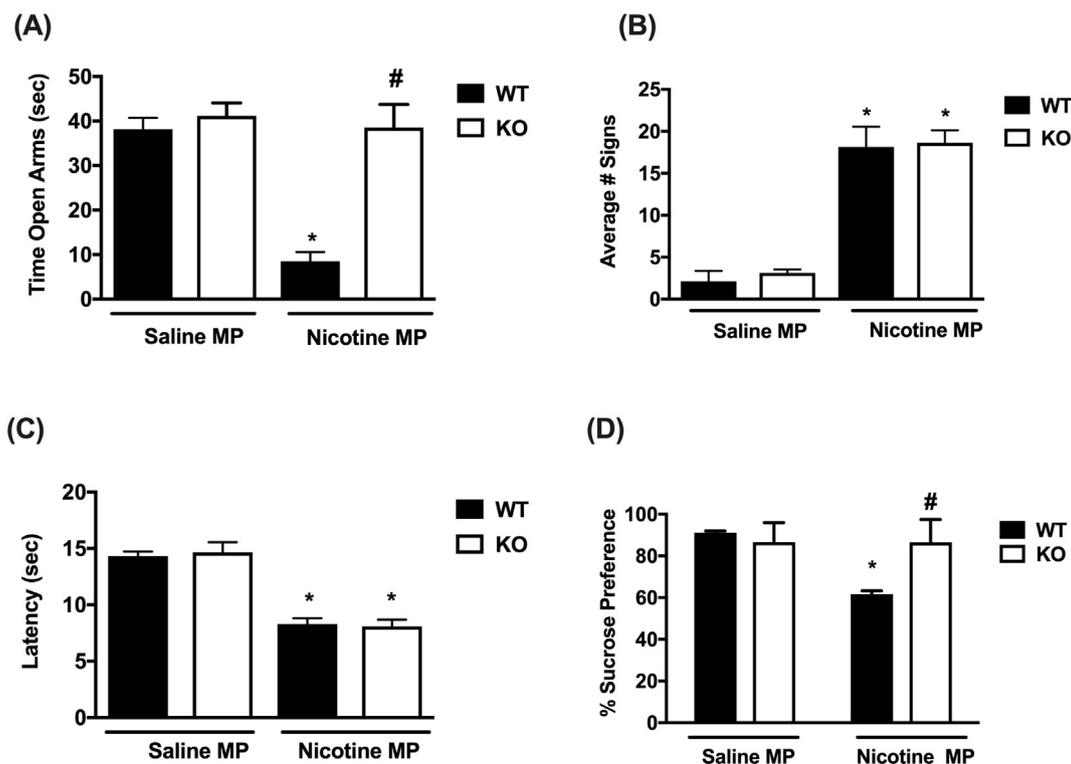


Fig. 2. Spontaneous nicotine withdrawal in beta3 WT and KO mice.

beta3 WT and KO mice were chronically infused with saline or nicotine (24 mg/kg/day) for 14 days. On day 15, minipumps were removed to induce spontaneous nicotine withdrawal. On day 15 mice were tested for: A) anxiety-like behaviors (time spent in the open arm), B) somatic signs, C) hyperalgesia (hot plate latency) and on day 16 mice were tested for: D) sucrose preference. A) Nicotine withdrawn beta3 WT mice exhibit increased anxiety-like behavior, but this effect is not shown in nicotine withdrawn beta3 KO mice. B) beta3 WT and KO mice render nicotine dependent both displayed increased somatic signs. C) Both beta3 WT and KO mice undergoing nicotine withdrawal exhibited decreased hot plate latencies. D) Nicotine withdrawn beta3 WT mice exhibit decreased sucrose preference, but this effect is not shown in nicotine withdrawn beta3 KO mice. Each point represents the mean \pm S.E.M. of $n = 8$ (4 male and 4 female) mice per group. *Denotes $p < 0.05$ vs. Saline MP group, #Denotes $p < 0.05$ vs. nicotine MP group.

4. Discussion

This study sought to examine the influence of $\beta 3$ nicotinic subunits on two main features of nicotine dependence, reward and withdrawal by using $\beta 3$ null mutant animals. To our knowledge, this is the first report demonstrating that $\beta 3$ nAChRs is not necessary for nicotine conditioned reward but seems to mediate affective but not somatic signs of withdrawal in nicotine-dependent mice. Together these data provide evidence of the importance of the $\beta 3$ subunit in nicotine dependence behaviors.

We determined the role of $\beta 3$ nAChRs in the CPP test of reward. In CPP experiments, the magnitude of nicotine preference achieved did not differ between the $\beta 3$ WT and KO mice following drug conditioning. Indeed, the dose of 0.5 mg/kg nicotine induced significant preference in both WT and KO mice. The lack of effect of the *Chrn3* gene on nicotine reward is in contrast with a recent report (Kamens et al., 2015) that showed a reduction in voluntary oral nicotine consumption in the 2-bottle choice test in mice lacking the $\beta 3$ subunit compared with wild-type animals. This difference could be related to differences in behavioral paradigms used (CPP vs oral consumption) in the two studies.

The lack of effects for $\beta 3$ nAChRs in CPP behaviors is in contrast with the results reported for the $\alpha 6$ nicotinic subunit. This is an important comparison since the gene that codes for the $\beta 3$ subunit is located adjacent to the gene coding for the $\alpha 6$ subunit on human chromosome 8. In addition, these subunits co-assemble to form functional receptors such as $\alpha 4\alpha 6\beta 2\beta 3^*$ nAChRs, in reward relevant brain regions (Cui et al., 2003; Gotti et al., 2005). Work from our labs and others using $\alpha 6$ KO and selective $\alpha 6$ -containing nAChRs showed an important role for this subunits in nicotine reward and reinforcement using the CPP test (Jackson et al., 2009; Sanjakdar et al., 2015) and IVSA in mice (Pons et al., 2008) and rats (Brunzell et al., 2010).

An important finding of our study is the observation that $\beta 3$ nAChRs are not involved in the physical signs of nicotine withdrawal, as indicated by the lack of a reduction in somatic signs and the presence of hyperalgesia in nicotine-withdrawn $\beta 3$ KO mice. In addition, we assessed two affective signs of nicotine withdrawal in $\beta 3$ KO mice and found that $\beta 3$ nAChRs are involved in both affective measures of nicotine withdrawal. Nicotine-dependent $\beta 3$ KO mice displayed a lack of anxiety-related behavior in the plus maze, as well as a lack of decrease in sucrose preference. Of interest, $\beta 3$ null mutant control mice in our withdrawal studies (Fig. 2) did not display any baseline significant behavioral differences from the WT counterpart animals in the plus-maze test. This is in contrast to an earlier study, where $\beta 3$ KO mice displayed decreased baseline anxiety-like behavior in the elevated plus maze, light-dark box in comparison with their wildtype counterparts (Booker et al., 2007). This difference can probably be explained by the differences in genetic background of $\beta 3$ KO mice between the studies. The $\beta 3$ KO mice were maintained on a mixed genetic background (129Svj \times C57BL/6) in the Booker et al. study, while our mice were backcrossed and maintained on C57BL/6J genetic background for > 9 generations. Indeed, The *Chrn3* gene was reported to influence nicotine oral consumption in $\beta 3$ KO mice on a C57BL/6 background, but not $\beta 3$ KO mice on 129S6/SvEvTac background (Kamens et al., 2015). Finally, our data on $\beta 3$ KO mice in withdrawal are consistent with previous studies assessing the role of $\beta 2$ and $\alpha 6$ nAChRs in nicotine withdrawal in mice that showed that these two subunits are important for affective but somatic signs of withdrawal (Besson et al., 2006; Jackson et al., 2008, 2009; Stoker et al., 2015). This study is not without limitations. While compensatory changes in other neuronal nicotinic receptors levels or function may have occurred and could have contributed to the behavioral outcomes in this study, deletion of the $\beta 3$ gene did not affect expression of mRNA for other neuronal nAChR subunits in various brain regions (Cui et al., 2003). However, given the lack of a selective $\beta 3$ nAChR antagonist available, these results are important for aiding in the understanding of potential $\beta 3$ subunit-dependent pathways. Of note, both male and female mice were used in

this study. Since no significant sex-differences were found, results were combined. Future studies with an a priori hypothesis focusing on sex differences are warranted.

Overall our results are consistent with several human genetic association studies where SNPs in *CHRN3* were found to be associated with “dizziness” experience from the first few cigarettes (Ehringer et al., 2010), the number of quit attempts (Hoft et al., 2009), nicotine dependence but not cigarette per day (Rice et al., 2012; Thorgeirsson et al., 2010).

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.pbb.2019.05.003>.

Funding

This research was supported by the National Institutes of Health – National Institute on Drug Abuse: R01 DA032246 (MID), and T32 DA007027 (AJ).

Declaration of Competing Interests

The authors declare there is no conflict of interest to declare regarding the publication of this article.

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