

Original Investigation

Mice Lacking the $\beta 4$ Subunit of the Nicotinic Acetylcholine Receptor Show Memory Deficits, Altered Anxiety- and Depression-Like Behavior, and Diminished Nicotine-Induced Analgesia

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Abstract

Rationale: The role of $\beta 4$ -containing nicotinic acetylcholine receptors (nAChRs) in cognition, anxiety, depression, and analgesia in the absence of nicotine is unclear.

Methods: Wild-type ($\beta 4^{+/+}$) and knockout ($\beta 4^{-/-}$) mice for the nAChR $\beta 4$ subunit were tested in behavioral tests assessing cognitive function, affective behaviors, and nociception.

Results: There were no learning and memory deficits in $\beta 4^{-/-}$ mice compared with $\beta 4^{+/+}$ mice during the acquisition of the Barnes maze, contextual fear conditioning, and Y maze tasks. In the Barnes maze memory retention test, male $\beta 4^{-/-}$ mice showed reduced use of the spatial search strategy, indicating small spatial memory deficits compared with $\beta 4^{+/+}$ mice. In the cue-induced fear conditioning memory retention test, $\beta 4^{-/-}$ mice exhibited reduced freezing time compared with $\beta 4^{+/+}$ mice. Compared with $\beta 4^{+/+}$ mice, $\beta 4^{-/-}$ mice exhibited decreased anxiety-like behavior in the light–dark box. Depression-like behavior in $\beta 4^{-/-}$ mice was decreased in the tail suspension test and increased in the forced swim test compared with $\beta 4^{+/+}$ mice. $\beta 4^{-/-}$ mice did not differ from $\beta 4^{+/+}$ mice in basal nociception but were less sensitive to the antinociceptive effect of nicotine in 2 tests of acute thermal pain.

Conclusions: Lack of $\beta 4$ -containing nAChRs resulted in small deficits in hippocampus- and amygdala-dependent memory retention functions. $\beta 4$ -containing nAChRs are involved in anxiety- and depression-like behaviors and contribute to the analgesic effects of nicotine.

Introduction

Nicotine contained in tobacco is considered to be the main ingredient that leads to addiction in human smokers (Mineur &

Piccio, 2008; Stolerman & Jarvis, 1995). In addition, nicotine induces diverse effects, including improvement of cognitive function (Herman & Sofuoglu, 2010; Levin, 2002; Levin et al., 2009; Sarter, Parikh, & Howe, 2009; Sofuoglu, 2010), amelioration of anxiety-like behavior (File, Cheeta, & Kenny, 2000), and analgesia (Marubio et al., 1999). Nicotine binds to nicotinic acetylcholine receptors (nAChRs) throughout the brain. To date, 12 subunits of nAChRs have been identified, nine α -type ($\alpha 2$ – $\alpha 10$) and three β -type ($\beta 2$ – $\beta 4$) that form structurally and functionally distinct hetero- and homopentameric receptors (Changeux, 2010; Collins, Salminen, Marks, Whiteaker, & Grady, 2009). Genetically modified mice with altered expression of various nAChR subunits have been a useful tool for determining the specific role of nAChR subtypes in neurobehavioral function (Fowler, Arends, & Kenny, 2008; Mineur & Piccio, 2008). The focus of the present studies was to evaluate the specific role of $\beta 4$ -containing nAChRs in cognitive function and nociception using mutant mice null for the $\beta 4$ nAChR subunit ($\beta 4^{-/-}$) and wild-type mice (WT, $\beta 4^{+/+}$).

$\beta 4$ -containing nAChRs are widely expressed in the peripheral nervous system (Xu et al., 1999). In the brain, high concentrations of $\beta 4$ -containing nAChRs are found in the olfactory bulb, medial habenula, pineal gland, interpeduncular nucleus, and inferior colliculus (Gahring, Persiyanov, & Rogers, 2004; Klink, de Kerchove d'Exaerde, Zoli, & Changeux, 2001; Quick, Ceballos, Kasten, McIntosh, & Lester, 1999; Salas, Pieri, Fung, Dani, & De Biasi, 2003). Considering that the habenulo–peduncular pathway is implicated in cognition (Klemm, 2004; Lecourtier & Kelly, 2007; Lecourtier, Neijt, & Kelly, 2004) and that $\beta 4$ nAChRs are also detectable in the hippocampus, amygdala, and cortex (Dineley-Miller & Patrick, 1992; Piccio, Caldarone, King, & Zachariou, 2000), $\beta 4$ -containing nAChRs may be involved in cognitive function. However, the specific role of $\beta 4$ -containing nAChRs in cognition has not been studied extensively. It has been shown that $\beta 4^{-/-}$ mice exhibited

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unaltered contextual learning compared with WT mice (Wehner et al., 2004). Other behavioral characteristics of $\beta 4$ KO mice known to date include decreased anxiety-like behavior, reflected in increased exploratory behavior in the elevated plus-maze and increased climbing activity in the staircase maze (Salas et al., 2003). In addition, $\beta 4^{-/-}$ mice were insensitive to nicotine-induced seizures and hypolocomotion, had an attenuated nicotine-induced hypothermic response, and did not show somatic signs of nicotine withdrawal (Kedmi, Beaudet, & Orr-Urtreger, 2004; Sack et al., 2005; Salas et al., 2003; Salas, Cook, Bassetto, & De Biasi, 2004a; Salas, Pieri, & De Biasi, 2004b). Furthermore, $\beta 4$ null mutation resulted in reduced hyperalgesia during nicotine withdrawal (Salas et al., 2004b), suggesting a role for $\beta 4$ -containing nAChRs in modulating pain sensitivity.

The current study explored the role of $\beta 4$ -containing nAChRs in regulating learning and memory processes by comparing the performance of $\beta 4^{+/+}$ and $\beta 4^{-/-}$ mice in a variety of cognitive tasks, such as the Barnes circular maze, contextual and cued fear conditioning, and spontaneous alternations in the Y maze. In addition, we evaluated anxiety-like behavior using the light–dark transfer test, compulsive-like behavior using marble burying, and depression-like behavior using the forced swim and tail suspension tests to further characterize the behavioral phenotype of $\beta 4^{-/-}$ mice. Furthermore, locomotor activity and exploratory behavior were also investigated because many of the cognitive tasks used in this study are based on exploration. Finally, the potential role of $\beta 4$ -containing nAChRs in mediating nicotine-induced analgesia was investigated by subjecting $\beta 4$ knockout mice to two standard assays of noxious heat sensitivity: the tail immersion and hot plate tests.

Materials and Methods

Animals

$\beta 4$ nAChR subunit knockout breeder mice were obtained from Drs. Al Collins and Michael Marks, University of Colorado at Boulder, CO, and these mice were backcrossed to C57BL/6J for at least 10 generations. Male and female WT and knockout littermates used in our study were generated by mating male and female heterozygous parents. Genotypes were determined by polymerase chain reaction. Mice were housed in groups of 2–4, with food and water available *ad libitum* except during testing. The animal holding room was maintained on a 12-h light/dark cycle (lights off at 07:00 hr), and behavioral testing occurred during the dark (active) phase. All experiments were conducted in accordance with the guidelines of the American Association for the Accreditation of Laboratory Animal Care and National Research Council's Guide for Care and Use of Laboratory Animals and were approved by the University of California San Diego and The Scripps Research Institute Institutional Animal Care and Use Committees.

Drugs

(–)-Nicotine hydrogen bitartrate salt (Sigma-Aldrich, St. Louis, MO) was dissolved in physiological saline (0.9%; Hospira, Lake Forest, IL) at a concentration of 0.3 mg/ml. The pH was adjusted to 7.2 ± 0.2 using sodium hydroxide, and the solution was sterilized through a 0.22 μm syringe filter. All nicotine doses are expressed as free base concentrations. Saline or nicotine

was injected subcutaneously (s.c., 0.05 ml/10 g body weight) 5 min before the test.

Experimental Design

Cognitive, Affective, and Locomotor Behaviors

All behavioral procedures are described in detail in the Supplementary material online. Two cohorts of mice were tested, one (15 $\beta 4^{+/+}$ and 14 $\beta 4^{-/-}$ females, 10 $\beta 4^{+/+}$ and 11 $\beta 4^{-/-}$ males) in a battery of cognitive tasks and the other (9 $\beta 4^{+/+}$ and 9 $\beta 4^{-/-}$ females, 8 $\beta 4^{+/+}$ and 11 $\beta 4^{-/-}$ males) to replicate the cognitive tests and examine locomotor activity and affective-like behaviors. For behavioral testing, a within-subjects design was used so that mice of each genotype were subjected to testing in the following order: Y maze, Barnes maze training, Barnes maze probe test, cue- and context-induced conditioned fear, Barnes maze retention, Barnes maze reversal, conditioned fear retention. After the completion of the cognitive test battery, the second mouse cohort was tested in additional procedures assessing locomotor activity and affective-like behavior. Again, a within-subjects design was employed such that mice of each genotype were subjected to testing in the following order: light–dark transfer test, marble burying test, locomotor activity test, tail suspension test, and forced swim test.

Nicotine-Induced Analgesia

Two additional cohorts of naïve $\beta 4^{+/+}$ and $\beta 4^{-/-}$ mice were tested for nicotine-induced analgesia using the tail immersion and hot plate tests (see Supplementary material online for details). The first cohort contained 21 $\beta 4^{+/+}$ and 21 $\beta 4^{-/-}$ females, and the second cohort contained 12 $\beta 4^{+/+}$ and 15 $\beta 4^{-/-}$ females, as well as 17 $\beta 4^{+/+}$ and 28 $\beta 4^{-/-}$ males. In each cohort, mice were first tested in the tail immersion test. Nicotine (0, 0.5, 1, 2, and 3 mg/kg, s.c., 5 min before test) was administered according to a within-subjects Latin square design during 5 consecutive days. One week later, the same mice were tested in the hot plate test. For each sex and genotype, mice were split into three subgroups, and nicotine (0, 1, and 3 mg/kg, s.c., 5 min before test) was administered according to a between-subjects design. Each mouse was only tested once in the hot plate test.

Statistical Analyses

Statistical analyses were performed using the Biomedical Computer Programs for Personal Computers Statistical Package (BMDP, Los Angeles, CA), the GraphPad Prism statistical package (GraphPad, San Diego, CA), and Statview v. 5.0 (SAS Institute Inc., Cary, NC). Two-way repeated-measures analysis of variance (ANOVA) was used to analyze the data collected from the cognitive test battery. The between-subject factors were Genotype and Sex, and the within-subject factors were Time Intervals (cued and contextual conditioning) and Trials/Blocks (the Barnes maze test). Data from the nicotine dose-response functions in the tail immersion and hot plate tests were analyzed using three-way ANOVAs, with Nicotine Dose as a within-subjects factor (tail immersion test) or between-subjects factor (hot plate test) and Genotype and Sex as the between-subjects factors. Data were then analyzed separately for each sex using two-way ANOVAs. When appropriate, post-hoc comparisons were carried out using the Newman–Keuls test. Nonparametric tests (exact Fisher's test) were used to compare the percentage of mice using the spatial strategy. All values are expressed as mean \pm SEM. Differences were considered statistically significant at $p < .05$.

Results

Cognitive Function in $\beta 4^{+/+}$ and $\beta 4^{-/-}$ mice

Y Maze

Spontaneous alternation behavior did not differ between $\beta 4^{+/+}$ and $\beta 4^{-/-}$ mice (data not shown). Analyses of the percentage of spontaneous alternations and total number of arm entries revealed no effect of Genotype or Genotype by Sex interaction.

Barnes Maze

Task acquisition. Both $\beta 4^{+/+}$ and $\beta 4^{-/-}$ mice acquired the Barnes maze task during 12 daily training trials (Supplementary Figure 1). The ANOVA revealed that the number of errors (Supplementary Figure 1A; main effect of Trials: $F(11, 913) = 21.15, p < .00001$) and time to find the escape tunnel (Supplementary Figure 1B; main effect of Trials: $F(11, 913) = 19.80, p < .0001$) were significantly decreased across training trials. There was a significant interaction between the factors Trial and Sex for the number of errors ($F(11, 913) = 3.42, p < .01$) and time to find the escape tunnel ($F(11, 913) = 2.77, p < .01$), with females learning faster than males (data not shown). There was no significant main effect of the factors Sex or Genotype and no interaction effects.

A comparison of strategies used by $\beta 4^{+/+}$ and $\beta 4^{-/-}$ mice during task acquisition revealed no Genotype differences (Supplementary Figure 2 in Supplementary material online). Across three 4-trial blocks, all mice progressively increased the use of spatial (main effect of Block; $F(2, 166) = 34.7, p < .0001$; Supplementary Figure 1C) and serial (main effect of Block; $F(2, 166) = 7.3, p < .001$; Supplementary Figure 1B) strategies and decreased the use of the random strategy (main effect of Block; $F(2, 166) = 62.17, p < .0001$; Supplementary Figure 1A). By the end of task acquisition (Block 3), independent of genotype, female mice were using the random search strategy significantly less (main effect of Sex; $F(3, 83) = 5.8, p < .05$) and the serial strategy significantly more (Block by Sex interaction; $F(2, 166) = 4.72, p < .01$) compared with male mice (data not shown). Additional analyses were performed to compare the use of a spatial strategy to find the target defined as the percentage of mice using spatial memory in at least three of the four last training trials in Block 3. Male $\beta 4^{-/-}$ mice tended to use the spatial strategy less than $\beta 4^{+/+}$ males or females of either genotype (Figure 1A).

Probe test. The percentage of time spent in the quadrant associated with the escape tunnel did not differ between genotypes and sexes (Supplementary Table 1A in Supplementary material online).

Memory retention test. The Memory retention test was performed on Day 73 of the experiment. The number of errors or the latency to find the escape tunnel did not differ between genotypes and sexes (Supplementary Table 1B). However, both male and female $\beta 4^{-/-}$ mice were using the spatial strategy less than $\beta 4^{+/+}$ mice, but the difference between genotypes was significant only in males (Figure 1B; Fisher's exact test, $p < .05$).

Reversal learning test. The reversal learning test was performed on Day 74 of the experiment. The number of

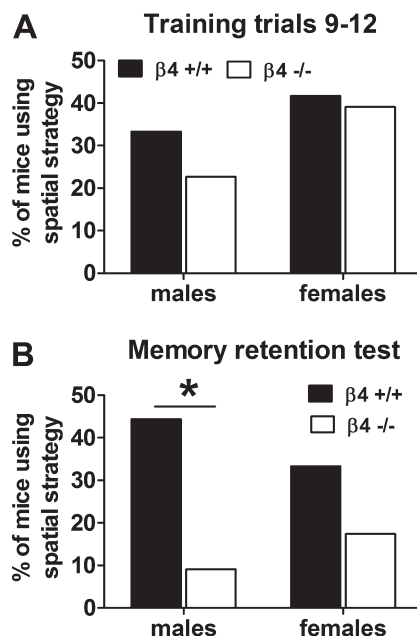


Figure 1. Use of spatial strategy to find the escape tunnel in the Barnes maze. Data (mean \pm SEM) are presented as the percentage of mice using a spatial strategy in at least three of the four trials during the final training trials 9–12 (A) and the memory retention test (B). *Significant difference between genotypes (Fisher's exact test).

errors did not differ between genotypes and sexes (Supplementary Table 1C). In the reversal test, female mice were faster than males to find the escape tunnel, independent of genotype (Supplementary Table 1C; Sex [$F(1, 83) = 9.52, p < .01$]).

Contextual Fear Conditioning

In a context previously associated with footshocks, both $\beta 4^{+/+}$ and $\beta 4^{-/-}$ mice, independent of sex, increased their freezing behavior during the test and retest of memory retention compared with freezing exhibited during the habituation period (Figure 2A). ANOVAs indicated a significant effect of Exposure to the context previously associated with footshocks during the test ($F(1, 83) = 80.55, p < .00001$) and memory retention retest ($F(1, 81) = 67.57, p < .00001$) but no effect of Genotype or Sex and no Genotype \times Sex \times Exposure interaction.

Cued Fear Conditioning

Initial presentation of a cue (CS+) previously associated with foot shocks increased freezing in all mice (Figure 2B). ANOVAs revealed significant main effects of Exposure ($F(1, 83) = 115.15, p < .00001$) and Genotype ($F(1, 83) = 6.95, p < .01$), and a significant Genotype \times Exposure interaction ($F(1, 83) = 4.64, p < .05$) but no three-way interaction. Post-hoc comparisons showed that both $\beta 4^{+/+}$ and $\beta 4^{-/-}$ female and male mice exhibited increased freezing during the initial cue-induced fear test (Figure 2B). Interestingly, however, the cue-induced conditioned fear response in $\beta 4^{-/-}$ male mice was significantly attenuated compared with $\beta 4^{+/+}$ male mice ($p < .01$, Newman–Keuls test; Figure 2B).

During the cue-induced fear conditioning retest, there were significant main effects of Exposure ($F(1, 81) = 73.81,$

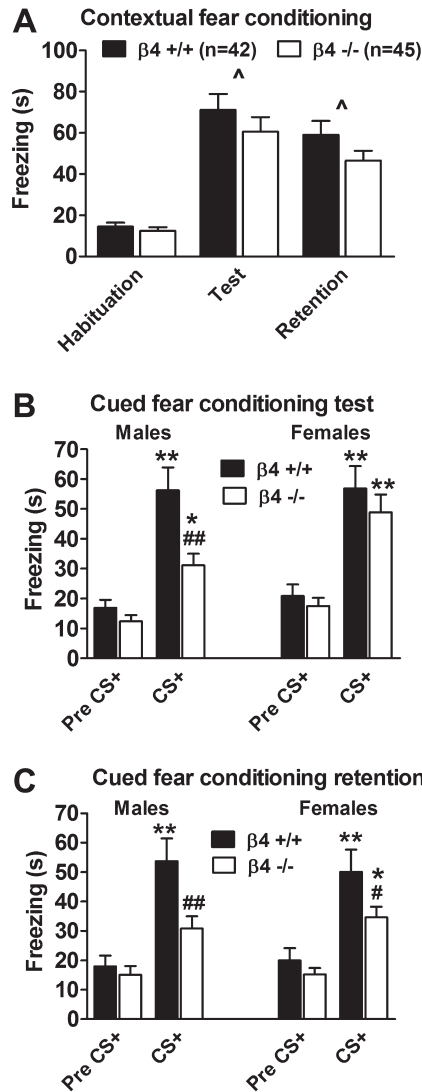


Figure 2. Context-induced fear conditioning during the test and retest (A) and cue-induced fear conditioning during test (B) and retest (C). Data are expressed as the number of seconds of freezing (mean \pm SEM). [^] $p < .05$, significant effect of exposure to the context previously associated with footshocks indicated in the ANOVA. ^{*} $p < .05$, ^{**} $p < .01$, significant difference between habituation (pre-CS+) and CS+ presentation conditions (Newman-Keuls test). [#] $p < .05$, ^{##} $p < .01$, significant difference between $\beta 4^{+/+}$ and $\beta 4^{-/-}$ mice (Newman-Keuls test).

$p < .00001$) and Genotype ($F(1, 81) = 9.59, p < .01$) and a significant Genotype \times Exposure interaction ($F(1, 81) = 6.88, p < .05$) but no effect of Sex or three-way interaction. Post-hoc comparisons showed that both $\beta 4^{+/+}$ and $\beta 4^{-/-}$ female mice exhibited increased freezing during the retest of cue-induced fear (Figure 2C). The cue-induced conditioned fear response in $\beta 4^{-/-}$ female mice was significantly attenuated compared with $\beta 4^{+/+}$ female mice ($p < .05$, Newman-Keuls test; Figure 2C). During the retest, $\beta 4^{+/+}$ male mice exhibited significantly increased freezing (Figure 2C). By contrast, freezing in $\beta 4^{-/-}$ male mice was not significantly different from the pre-CS+ level and was significantly lower than the CS+ level in $\beta 4^{+/+}$ male mice (Figure 2C).

Activity and Affective-Like Behavior in $\beta 4^{+/+}$ and $\beta 4^{-/-}$ Mice

Locomotor Activity Test

Overall, there were no significant differences between mouse genotypes and sexes in total ambulation, total activity in the center, and total horizontal activity (Supplementary Table 3 in Supplementary material online). From the total number of rearings, the ANOVA detected a significant effect of Sex ($F(1, 35) = 17.24, p < .0001$), with males rearing more than females, independent of genotype (data not shown).

Light-Dark Box

There was a significant main effect of Genotype ($F(1, 35) = 4.1, p < .05$) and Sex ($F(1, 35) = 4.3, p < .05$) but no Sex \times Genotype interaction for the number of transitions. Decreased anxiety-like behavior was observed in $\beta 4^{-/-}$ mice compared with $\beta 4^{+/+}$ mice, and this effect was attributable to $\beta 4^{-/-}$ male mice (Figure 3A and B). For the time spent in the light compartment, ANOVAs revealed no effect of Sex or Genotype and no Sex \times Genotype interaction, although the direction of the effects was the same as the number of transitions.

Marble Burying Test

Both $\beta 4^{+/+}$ and $\beta 4^{-/-}$ mice exhibited similar compulsive-like behavior in the marble burying test (data not shown).

Tail Suspension Test

In this experiment, six mice (one $\beta 4^{+/+}$ male, one $\beta 4^{-/-}$ male, one $\beta 4^{+/+}$ female, and three $\beta 4^{-/-}$ females) were excluded from the analyses because they exhibited excessive tail climbing behavior or fell off. There were significant effects of Genotype ($F(1, 24) = 4.8, p < .05$) and Sex ($F(1, 24) = 6.8, p < .05$) but no interaction between the two factors for immobility time. Visual inspection of the data indicated that $\beta 4^{-/-}$ mice spent less time immobile than $\beta 4^{+/+}$ mice, and this effect was attributable to $\beta 4^{-/-}$ females (Figure 3C).

Forced Swim Test

There was a significant effect of Genotype ($F(1, 35) = 7.1, p < .01$) but no effect of Sex and no interaction for immobility time. $\beta 4^{-/-}$ mice exhibited increased depression-like behavior compared with $\beta 4^{+/+}$ mice, reflected by increased immobility time (Figure 3D). In addition, there was a significant effect of Genotype on swimming time ($F(1, 35) = 5.4, p < .05$) but no effect of Sex and no interaction (data not shown). Climbing time did not differ between genotypes and sexes (data not shown).

Nicotine-Induced Analgesia

In the tail immersion test, baseline measures revealed an effect of Sex ($F(1, 110) = 17.0, p < .001$), with females having lower nociceptive thresholds than males, independent of genotype (Figure 4A). Deletion of the $\beta 4$ subunit, however, did not affect basal thermal pain sensitivity, with no main effect of Genotype and no interaction between Sex and Genotype. Nicotine had a dose-dependent antinociceptive effect in both males (Figure 4B) and females (Figure 4C). A three-way repeated-measures ANOVA revealed a significant effect of Sex ($F(1, 110) = 40.9, p < .001$), consistent with the sex difference in basal nociception. None of the two- or three-way interactions between Sex and the other factors were significant. Data from each sex were analyzed separately

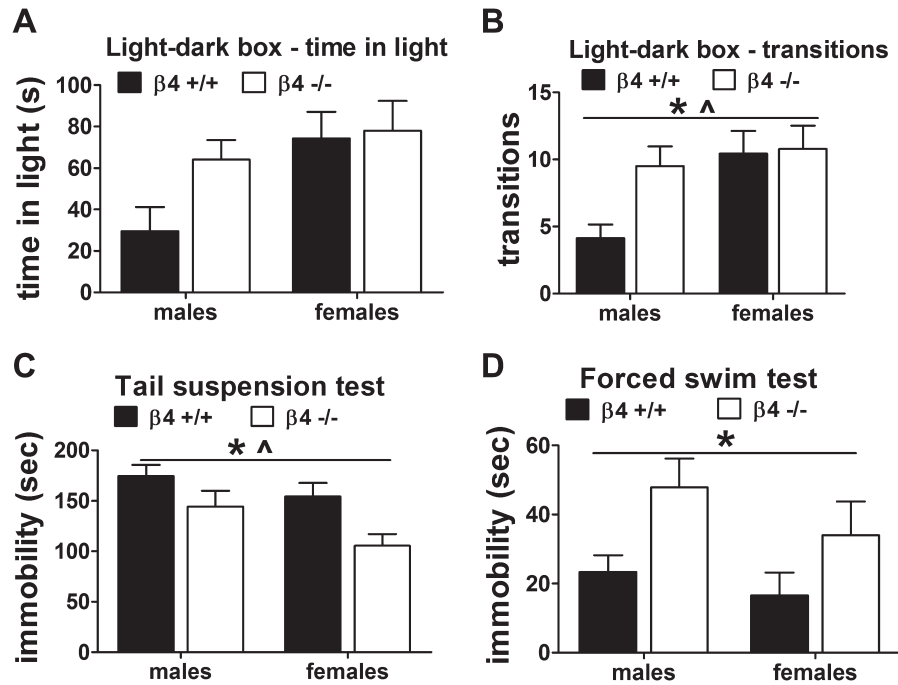


Figure 3. Anxiety-like behavior measured as time spent in the light compartment (A) and number of transitions (B) in the light–dark box test. Immobility measured in the tail suspension test (C) and forced swim test (D). All data are expressed as mean \pm SEM. * $p < .05$, significant main effect of Genotype in the ANOVA. $\wedge p < .05$, significant main effect of Sex in the ANOVA.

using two-way repeated-measures ANOVAs. There was a significant main effect of Nicotine in both sexes (males: $F(1, 43) = 17.3$, $p < .001$, Figure 4B; females: $F(1, 67) = 13.5$, $p < .001$, Figure 4C). A

main effect of Genotype (males: $F(4, 172) = 48.9$, $p < .001$; females: $F(4, 268) = 86.0$, $p < .001$) and a significant interaction between Nicotine and Genotype (males: $F(4, 172) = 11.8$, $p < .001$;

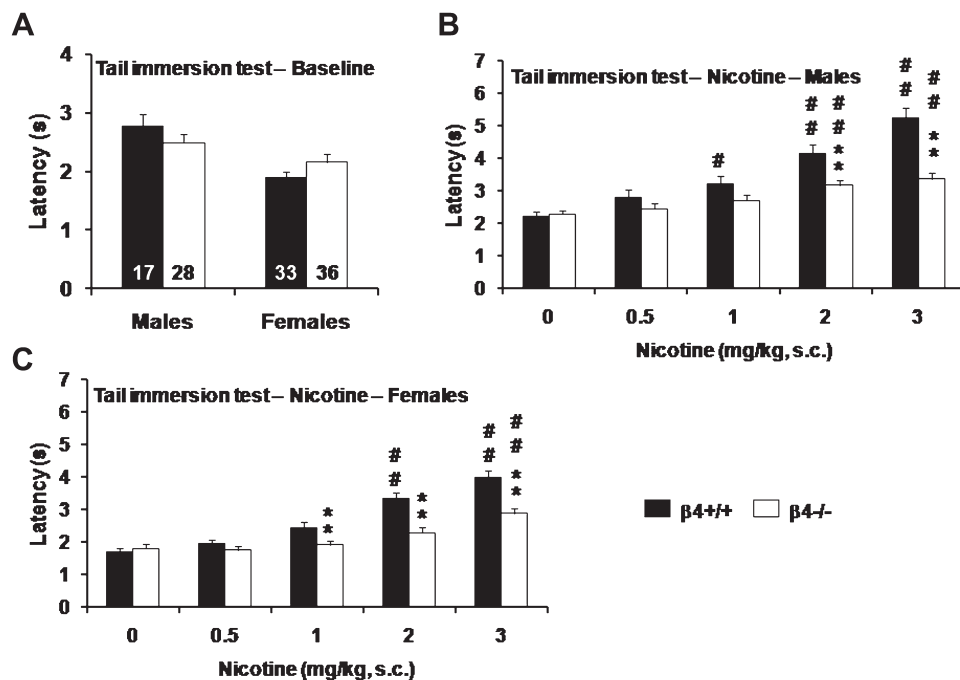


Figure 4. (A) Basal nociceptive thresholds in the tail immersion test. Tail withdrawal latencies (mean \pm SEM) were measured in naïve male and female $\beta 4^{+/+}$ (black bars) and $\beta 4^{-/-}$ (white bars) mice. The numbers of mice used in each group are indicated in the bars. Data were subjected to two-way ANOVA, followed by post-hoc comparisons (** $p < .01$, male vs. female, Newman–Keuls test). (B and C) Antinociceptive effect of nicotine in the tail immersion test. Tail withdrawal latencies (mean \pm SEM) were measured in male (B) and female (C) $\beta 4^{+/+}$ (black bars) and $\beta 4^{-/-}$ (white bars) mice 5 min after s.c. injection of nicotine. The numbers of mice used in each group are identical to (A). Data were subjected to repeated-measures ANOVA, followed by post-hoc comparisons (** $p < .01$, wild-type vs. knockout; # $p < .05$, ## $p < .01$, nicotine vs. saline, Newman–Keuls test).

females: $F(4, 268) = 10.0, p < .001$) were also detected in males and females, indicating that nicotine-induced analgesia was reduced in $\beta 4^{-/-}$ mice (Figure 4B–C).

A similar pattern of results emerged in the hot plate test (Figure 5). Nicotine increased the latencies to display the first nocifensive response to noxious heat (males, Figure 5A; females, Figure 5D), hindpaw licking or flinching (males, Figure 5B; females, Figure 5E), and jumping (males, Figure 5C; females, Figure 5F). Three-way ANOVAs revealed a significant effect of Sex for all responses (first sign: $F(1, 102) = 16.5, p < .001$; hindpaw sign: $F(1, 102) = 5.2, p < .05$; jumping: $F(1, 102) = 4.7, p < .05$), with females having longer latencies than males. None of the two- or three-way interactions between Sex and the other factors were significant, with the exception of the Sex \times Nicotine interaction for jumping ($F(2, 102) = 5.7, p < .05$), which reflected the higher sensitivity of females to the antinociceptive effect of nicotine for that sign. Data from each sex were then analyzed separately using two-way ANOVAs. There was a significant effect of Nicotine on the latency to display first sign (males: $F(2, 39) = 15.3, p < .001$, Figure 5A; females: $F(2, 63) = 52.9, p < .001$, Figure 5D), hindpaw sign (males: $F(2, 39) = 10.7, p < .001$, Figure 5B; females: $F(2, 63) = 13.8, p < .001$, Figure 5E), and jumping (males: $F(2, 39) = 3.9, p = .056$, Figure 5C; females: $F(2, 63) = 48.0, p < .001$, Figure 5F). A significant effect of Genotype was detected for all responses, with $\beta 4^{-/-}$ mice displaying shorter latencies than their WT counterparts overall (first sign: males, $F(1, 39) = 6.2, p < .05$; females, $F(1, 63) = 9.1, p < .05$; first hindpaw sign: males, $F(1, 39) = 6.3, p < .05$; females, $F(1, 63) = 8.7, p < .01$; jumping: males, $F(1, 39) = 4.8, p < .05$;

females, $F(1, 63) = 8.9, p < .01$). Finally, the interaction between Nicotine and Genotype was also significant for the three measures (first sign: males, $F(2, 39) = 4.4, p < .05$; females, $F(2, 63) = 6.9, p < .01$; first hindpaw sign: males, $F(2, 39) = 8.6, p < .001$; females, $F(2, 63) = 5.3, p < .01$; jumping: males, $F(2, 39) = 6.3, p < .01$; females, $F(2, 63) = 10.3, p < .001$).

Discussion

The present studies demonstrated that the lack of $\beta 4$ -containing nAChRs induced no pronounced learning and memory deficits in $\beta 4^{-/-}$ mice compared with $\beta 4^{+/+}$ mice in simple spatial working memory in the Y maze, during the acquisition of the Barnes maze, and contextual fear conditioning. In the Barnes maze memory retention test, male $\beta 4^{-/-}$ mice showed a reduction in the use of the spatial search strategy, indicating small spatial memory deficits compared with $\beta 4^{+/+}$ mice. In the cue-induced fear conditioning memory retention test, both male and female $\beta 4^{-/-}$ mice exhibited decreased memory retention, while during task acquisition memory deficits were observed only in male $\beta 4^{-/-}$ mice. Compared with $\beta 4^{+/+}$ mice, $\beta 4^{-/-}$ mice exhibited decreased anxiety-like behavior in the light–dark box. Depression-like behavior in $\beta 4^{-/-}$ mice was decreased in the tail suspension test and increased in the forced swim test compared with $\beta 4^{+/+}$ mice. Locomotor activity and exploratory behavior in the Y maze was similar in both genotypes. Finally, male and female $\beta 4^{-/-}$ mice did not differ from their $\beta 4^{+/+}$ counterparts in terms of basal nociception but were less sensitive to the antinociceptive effect of nicotine in two tests of acute thermal pain, indicating that

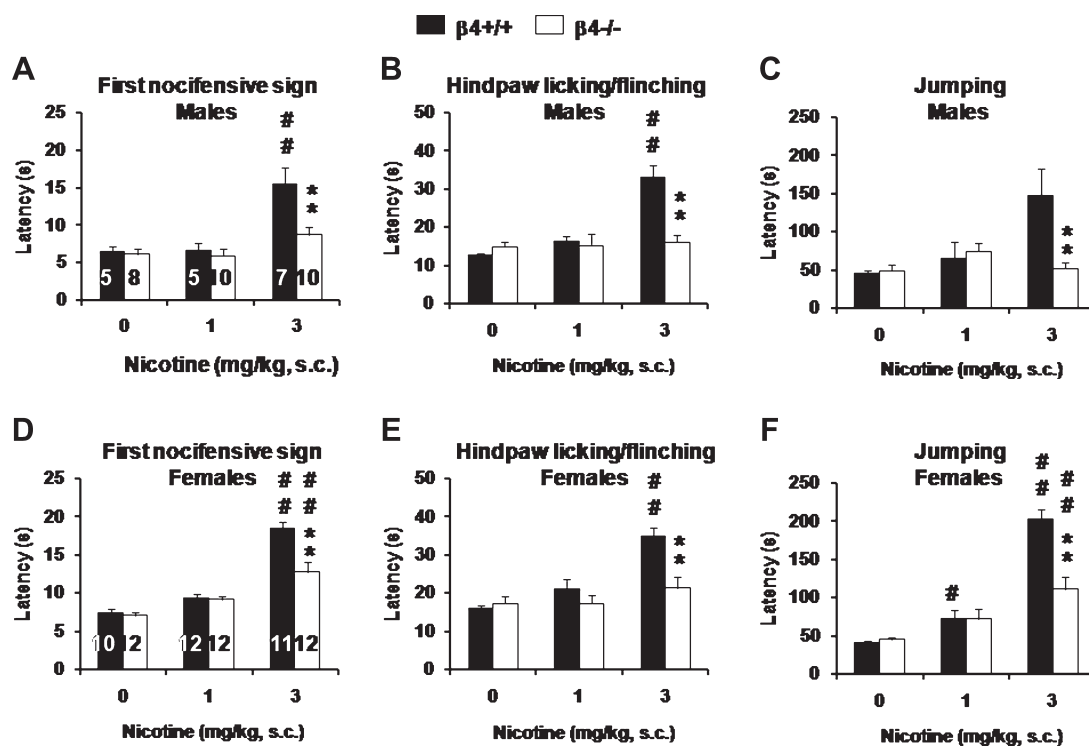


Figure 5. Antinociceptive effect of nicotine in the hot plate test. Latencies to show the first nocifensive sign (A and D), hindpaw licking or flinching (B and E), and jumping (C and F) were measured in male (A, B, and C) and female (D, E, and F) $\beta 4^{+/+}$ (black bars) and $\beta 4^{-/-}$ (white bars) mice 5 min after s.c. injection of nicotine. The numbers of mice used in each group are indicated in the bars in (A) and (D). Data were subjected to repeated-measures ANOVA, followed by post-hoc comparisons (** $p < .01$, wild-type vs. knockout; # $p < .05$, ## $p < .01$, nicotine vs. saline, Newman–Keuls test).

$\beta 4$ -containing nAChRs are involved in the modulation of nicotine-induced analgesia.

$\beta 4$ -Containing nAChRs in Cognitive Function

In the Y maze and locomotor activity tests, $\beta 4^{-/-}$ and $\beta 4^{+/+}$ male and female mice had similar levels of exploratory behavior and reactivity to novelty. Therefore, the observed changes in cognitive function and affective behavior in nAChR $\beta 4$ knockout mice discussed below cannot be attributed to the differences in general locomotor or exploratory activity.

In the Barnes maze, there were no performance differences between $\beta 4^{-/-}$ and $\beta 4^{+/+}$ mice during task acquisition, the probe test, or the reversal learning test. The transition from the use of hippocampus-independent nonspatial escape strategies (random and serial strategies) to the use of the hippocampus-dependent spatial escape strategy, which requires the use of external visuospatial cues to locate the escape tunnel (Harrison, Reiserer, Tomarken, & McDonald, 2006; O'Leary & Brown, 2012), was similar in both $\beta 4^{-/-}$ and $\beta 4^{+/+}$ mice during the acquisition of the Barnes maze task. The most pronounced differences between genotypes were observed during the memory retention test, during which $\beta 4^{-/-}$ male mice showed a significant reduction in the use of the spatial strategy, indicating deficits in spatial memory retention. The medial habenula receives inputs from the septal nuclei, which are themselves targeted by afferents from the hippocampus involved in spatial learning (Bannerman et al., 2003; Moser, Moser, & Andersen, 1993; Paylor, Zhao, Libbey, Westphal, & Crawley, 2001; Richmond et al., 1999). The habenular complex has been hypothesized to integrate spatial memories with outcome incentive value and thereby contribute to the selection of appropriate coping strategies in stressful situations (for review, see Lecourtier & Kelly, 2007). Accordingly, lesions of the habenular complex resulted in marked spatial memory impairment during acquisition and retrieval in the Morris water maze, a spatial memory task that is more anxiogenic and stressful than the Barnes maze (Harrison, Hosseini, & McDonald, 2009; Lecourtier et al., 2004). Although $\beta 4$ -containing nAChRs are expressed in the hippocampus and medial habenula (Dineley-Miller & Patrick, 1992; Gahring, Persiyanov, & Rogers, 2004; Picciotto et al., 2000), the genetic ablation of the $\beta 4$ nAChR subunit induced only limited deficits in spatial memory retention and did not affect general performance in the Barnes maze. Furthermore, $\beta 4^{-/-}$ mice tended to exhibit decreased anxiety-like behavior in the light–dark box compared with $\beta 4^{+/+}$ mice, suggesting that the observed small deficit in spatial memory retention in $\beta 4^{-/-}$ may not be anxiety related, although being in a brightly lit, open space in the Barnes maze is considered anxiogenic for mice.

In agreement with previously published findings (Wehner et al., 2004), contextual learning was similar in both $\beta 4^{-/-}$ and $\beta 4^{+/+}$ mice as measured by freezing during the contextual fear conditioning test and retest of memory retention. Furthermore, both $\beta 4^{-/-}$ and $\beta 4^{+/+}$ mice acquired the cued fear conditioning task and showed significant increases in freezing in response to the cue previously associated with footshocks. Interestingly, however, $\beta 4^{-/-}$ male but not $\beta 4^{-/-}$ female mice exhibited reduced freezing during the cue-induced fear conditioning test, indicating memory deficits in this task. In contrast, previously published findings reported no deficits in cue-induced fear conditioning

in $\beta 4^{-/-}$ mice (Wehner et al., 2004). During memory retention tests, both $\beta 4^{-/-}$ male and female mice exhibited reduced freezing indicating memory deficits in cue-induced fear conditioning. The differential effect of nAChR $\beta 4$ subunit deletion on context- and cue-induced learning may be explained by the distinct mechanisms underlying context and cue fear conditioning. Both the hippocampus and amygdala are involved in contextual fear conditioning, whereas only the amygdala is involved in cued fear conditioning (Frankland, Cestari, Filipkowski, McDonald, & Silva, 1998; Maren, Aharonov, & Fanselow, 1997; Phillips & LeDoux, 1992; Rudy, Huff, & Matus-Amat, 2004; Wehner & Radcliffe, 2004). Thus, amygdala-dependent learning was likely impaired in $\beta 4$ knockout mice to a larger extent than hippocampus-dependent learning.

$\beta 4$ -Containing nAChRs in Affective Behaviors

Our results showed that $\beta 4^{-/-}$ mice tended to exhibit less anxiety-like behavior compared with $\beta 4^{+/+}$ mice in the light–dark box as measured by the number of transitions but not time spent in the light compartment. Similarly, previous findings indicated that $\beta 4^{-/-}$ mice exhibited less anxiety-like behavior compared with $\beta 4^{+/+}$ mice on the elevated plus and staircase mazes, with no differences between genotypes in the light–dark box, open field, or mirrored chamber (Salas et al., 2003). Compulsive-like behavior measured in the marble burying test did not differ between $\beta 4^{-/-}$ and $\beta 4^{+/+}$ mice.

Depression-like behavior was measured in the tail suspension and forced swim tests, and conflicting results were obtained in these two tests. In the forced swim test, $\beta 4^{-/-}$ mice exhibited increased immobility compared with $\beta 4^{+/+}$ mice, indicating a tendency for increased depression-like behavior. In contrast, in the tail suspension test, antidepressant-like behavior was evident in $\beta 4^{-/-}$ mice. It is postulated that the biological substrates that underlie the depression-like behavior measured in these tests are different (Bai, Li, Clay, Lindstrom, & Skolnick, 2001). In the forced swim test, climbing behavior is indicative of norepinephrine neurotransmitter function (Cryan, Markou, & Lucki, 2002), whereas swimming behavior is indicative of serotonin neurotransmitter function (Cryan et al., 2002). Swimming was significantly decreased in $\beta 4^{-/-}$ mice compared with $\beta 4^{+/+}$ mice. This finding suggests that the lack of the nAChR $\beta 4$ subunit alters serotonin but not norepinephrine transmission. On the other hand, $\beta 4$ -containing nAChRs in the medial habenula and interpeduncular nucleus pathway may regulate glutamate release (Girod & Role, 2001; McGehee & Role, 1995) involved in both depression and anxiety (Krystal et al., 2002; Skolnick, 2002; Stewart & Reid, 2002). Therefore, the lack of the $\beta 4$ subunit may alter glutamatergic tone in the habenulographic pathway and mediate affective behavior in mice lacking $\beta 4$ -containing nAChRs. Finally, potential developmental compensatory changes in $\beta 4$ nAChR knockout mice cannot be ruled out in the expression of depression-like behavior. Specifically, the $\beta 2$ subunit of nAChRs, which is involved in depression-like behavior, may partially substitute for the loss of the $\beta 4$ subunit as it has been shown in the autonomic ganglia (Xu et al., 1999). It should be emphasized, however, that both the forced swim test and tail suspension test were developed and validated as animal models predictive of antidepressant activity (Cryan et al., 2002). Thus, it may not necessarily be the case that the opposite behavior to that induced by antidepressant treatments

(i.e., decreased immobility) necessarily reflects a depression-like state.

β 4-Containing nAChRs in Nociception

Our data show a role for β 4-containing nAChRs in the analgesic effects of nicotine. β 4 subunits contributing to nicotine-induced analgesia may be located in the brain and spinal cord because deletion of β 4 subunits in the knockout mice affected both reflex and integrated responses to noxious heat. β 4 subunits have a restricted pattern of expression in the brain, with notable enrichment in the habenulo–peduncular tract (Gahring et al., 2004). Interestingly, nicotine microinjection into the interpeduncular nucleus produces analgesia in a rat model of acute thermal pain (Hamann & Martin, 1992). At the spinal level, β 4 subunits are expressed presynaptically on primary afferents in the superficial layer of the dorsal horn (Khan et al., 2003), but nAChRs expressed on primary afferent terminals have been implicated in the pronociceptive, rather than antinociceptive, effect of nicotinic agonists (Khan et al., 2004).

The β 4 subunit belongs to a genomic cluster together with the α 3 and α 5 subunits, and these three subunits co-assemble into functional receptors (Boulter et al., 1990; Wang et al., 1996). Interestingly, deletion of α 5 subunits also decreases the antinociceptive effects of nicotine in models of acute thermal pain, and the α 3 and α 5 subunits also display strikingly high expression in the habenulo–peduncular pathway (Boulter et al., 1990; Jackson et al., 2010; Salas et al., 2004a; Wada et al., 1989). It can be hypothesized that activation of α 3 α 5 β 4 nAChRs expressed in the habenula or interpeduncular nucleus produces analgesia because some habenular neurons respond to noxious input (Dafny & Qiao, 1990), and habenular stimulation has analgesic effects (Cohen & Melzack, 1986).

While acute administration of nicotine produces analgesia, spontaneous or antagonist-precipitated withdrawal from chronic nicotine exposure induces hyperalgesia (Damaj, Kao, & Martin, 2003). Interestingly, nicotine withdrawal-induced hyperalgesia is also abolished in β 4^{-/-} mice (Salas et al., 2004b). The β 4 subunit therefore plays a central role in the modulation of nociceptive thresholds by nicotine, contributing to both nicotine-induced analgesia and the counter adaptive increase in pain sensitivity during nicotine withdrawal.

Conclusions

Overall, our findings provide insights into the role of β 4-containing nAChRs in cognitive function, affective behaviors, and nociception. The results indicate that: (a) lack of the β 4-containing nAChRs resulted in deficits in hippocampus- and amygdala-dependent memory function; (b) β 4-containing nAChRs are involved in anxiety- and perhaps depression-like behaviors; and (c) β 4-containing nAChRs contribute to the analgesic effects of nicotine.

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Declaration of Interests

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