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Decreased anxiety-like behavior in beta3 nicotinic receptor subunit knockout mice

T.K. Booker [a,*], Christopher M. Butt [b], Jeanne M. Wehner [c], Stephen F. Heinemann [a], Allan C. Collins [c]

[a] Salk Institute for Biological Studies, La Jolla, CA 92037 U.S.A.
[b] West Virginia School of Osteopathic Medicine, Lewisburg, WV 24901 U.S.A.
[c] Institute for Behavioral Genetics, University of Colorado, Boulder, CO 80309 U.S.A.

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Abstract

Nicotine, via a family of nicotinic acetylcholine receptors, elicits many physiological responses, including alterations in anxiety. Studies suggest that the effects of nicotine on anxiety may support smoking behaviors. We reported previously that mice lacking the beta3 nicotinic receptor subunit demonstrate increased activity in the open field arena. Open field activity has been shown to be a composite of anxiety and locomotor activity, behaviors that are both altered by nicotine. We therefore sought to differentiate the role(s) of beta3-containing receptors in anxiety and locomotor activity. Anxiety behaviors were examined in the elevated plus maze, the black/white box and the mirrored chamber. Beta3 null mutant mice demonstrated decreased anxiety with more time spent on the open arm of the elevated plus maze than their wildtype littermates. No significant differences were observed with the black/white box or the mirrored chamber. Levels of the stress hormone, corticosterone, were significantly higher in the beta3 null mutant mice at baseline and following exposure to stress. Increased locomotor activity in the Y-maze was also observed for the beta3 null mutant mice, but only following exposure to stress. These findings strongly suggest that beta3-containing nicotinic receptors influence anxiety and may be critical for the continuation of smoking behaviors.

Keywords: Nicotine; Nicotinic receptor; Knockout mouse; Beta3; Anxiety; Stress; Corticosterone; Locomotor

Despite major efforts to promote smoking cessation, millions of Americans continue to smoke tobacco, resulting in an epidemic of lung cancer and heart disease. One factor that may contribute to the persistence of smoking behaviors is the effect of nicotine on anxiety. Smoking increases during stressful periods, with smokers reporting decreased levels of anxiety following tobacco consumption (Gilbert et al., 1989; O'Neill and Parrott, 1992; Pomerleau and Pomerleau, 1984; Pomerleau et al., 1984b). Conversely, increased anxiety is a symptom of smoking cessation (Giannakoulas et al., 2003; Hughes et al., 1991; Jorenby et al., 1996; Tate et al., 1993), and studies suggest that smoking may continue in an effort to avoid this withdrawal symptom (Brown et al., 2001; Doherty et al., 1995; Pomerleau et al., 2000). In addition, those who have quit smoking often relapse during periods of heightened stress and anxiety (Daughton et al., 1990). Although cigarette smoking is a complex addiction influenced by multiple factors, if the mechanism underlying the effects of smoking on anxiety can be elucidated, it may provide a tangible target to aid in smoking cessation.

In the search for such a mechanism, an extensive, but seemingly contradictory, literature has developed that indicates that nicotinic cholinergic systems modulate anxiety. Some argue that chronic nicotine plays a causative role in the development of anxiety (McCabe et al., 2004; Zvolensky et al., 2003), as smoking is more common in individuals who suffer from anxiety disorders (Amering et al., 1999; Breslau et al., 1991). This is consistent with the findings that nicotine, when given via an inhaler, increases anxiety (File et al., 2001; Perkins et al., 1994) and that anxiety decreases approximately one week following smoking cessation in concert with the elimination of nicotine (Gross and Stitzer,

* Corresponding author.
E-mail address: booker@salk.edu (T.K. Booker).

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1989; West and Hajek, 1997). Others (Brown et al., 2001), however, argue that some people smoke as a means of self-medicating for negative mood states because many studies indicate that smoking decreases anxiety in chronic smokers (Gilbert et al., 1989; O'Neill and Parrott, 1992; Pomerleau and Pomerleau, 1984; Pomerleau et al., 1984). The anxiolytic effect of smoking is presumably induced by nicotine as this effect was not observed when nicotine-free tobacco was tested (Gilbert et al., 1989; Juliano and Brandon, 2002). The observations that the number of cigarettes smoked increases after modestly stressful conditions (Niaura et al., 2002; Todd, 2004) and that smoking increases (number of cigarettes smoked, number of smokers) after traumatic experiences (Boos and Croft, 2004) support the interpretation that some smokers use tobacco for the anxiolytic properties of nicotine.

Animal studies investigating the putative effects of nicotine on anxiety indicate that nicotine may have anxiolytic properties, but only within a limited dose range (Brioni et al., 1993; Cao et al., 1993). Lower doses of nicotine may elicit anxiolytic actions, but higher doses are clearly anxiogenic (Cheeta et al., 2001a,c; Irvine et al., 1999, 2001; Ouagazzal et al., 1999). These findings may explain the conflicting results obtained in human studies.

The opposing actions of nicotine on anxiety might be explained if the anxiolytic and anxiogenic properties are modulated by different nicotinic receptor subtypes. Eleven nicotinic receptor subunits have been identified in mammalian brain (alpha2–7, 9, 10 and beta2–4 (Hogg et al., 2003)). Different combinations of subunits assemble into distinct subtypes of nicotinic receptors, each with unique physiological characteristics and distributions that predict diverse roles across the brain. Although many roles have yet to be elucidated, some receptor subtypes modulate the release of neurotransmitters known to be involved in anxiety, such as GABA (Alkondon et al., 1997; Bertolino et al., 1997; Guo et al., 1998; Lena et al., 1993; Lu et al., 1998) and serotonin (Hery et al., 1977; Kenny et al., 2000; Ribeiro et al., 1993; Westfall et al., 1983). In addition, altered anxiety has been observed in several lines of mice with mutations in nicotinic receptor subunits. Nicotinic receptors containing alpha4 subunits likely modulate anxiety, as increased anxiety was observed in mice lacking the alpha4 subunit (Ross et al., 2000) and in mice expressing the Leucine9'Serine mutation in the alpha4 subunit (Labarca et al., 2001). A decrease in anxiety was observed in mice lacking the beta4 subunit (Salas et al., 2003). The opposing effects of nicotinic subunit deletion on anxiety support the possibility that the divergent effects of nicotine on anxiety may be mediated by different populations of nicotinic receptors.

Recent studies have demonstrated that two classes of beta3-containing nicotinic receptors are expressed in dopaminergic nerve terminals. One class consists of alpha4/alpha6/beta2/beta3 subunits, and the other consists of alpha6/beta2/beta3 subunits (Gotti et al., 2005; Salminen et al., 2004). The beta3-containing receptors are very sensitive to inhibition by alpha-conotoxin MII and are the highest affinity nicotinic receptors studied, to date, when agonist sensitivities are measured (Salminen et al., 2004). Deletion of the beta3 subunit eliminates these high affinity sites. However, maximal nicotine-stimulated striatal dopamine release increases in beta3 null mutants (Cui et al., 2003; Salminen et al., 2004), presumably due to compensatory activities of alpha-conotoxin MII insensitive nicotinic receptors that contain alpha4/beta2 subunits or alpha4/alpha5/beta2 subunits (Salminen et al., 2004). Deletion of the beta3 nicotinic receptor subunit also results in an increase in activity in an illuminated open field arena (Cui et al., 2003). The increase in the open field activity of beta3 null mutant mice compared to their wildtype littermates might be explained by the increased maximal dopamine release observed in the mutant animals (Cui et al., 2003). This explanation is consistent with the reported association between higher striatal dopamine release and increased locomotor activity (Ikemoto and Panksepp, 1999). However, open field activity is a complex behavior that is influenced by at least two factors.

Two major components of open field activity are anxiety, which reduces activity, and novelty seeking, which increases activity (Montgomery and Monkman, 1955; Rodgers et al., 1997). Nicotine has been shown to alter both anxiety and activity (Brioni et al., 1993; Cao et al., 1993; Cheeta et al., 2001a,c; Irvine et al., 1999, 2001; Marks et al., 1986, 1985; Ouagazzal et al., 1999). We undertook the following experiments to differentiate the role of beta3-containing nicotinic receptors in these behaviors using the beta3 null mutant mice. Our findings indicate that nicotinic receptors containing the beta3 subunit influence anxiety and suggest that these receptors may be critical for the continuation of smoking behaviors.

1. Methods

1.1. Generation of beta3 null mutant mice

Beta3 null mutant mice were generated as described previously (Cui et al., 2003). Mice were maintained on a mixed genetic background (129Svj × C57BL/6) by heterozygous matings. Genotype was determined by PCR using isolated tail DNA and the following primers which amplify DNA from the beta3 gene and the lacZ gene: *Beta3 exon 5 5′*: GGGCTCTCTCATGACCAAGG; *Beta3 exon 5 3′*: GTATCTGATGGACTCAGAGGCC; *LacZ 5′*:CACTACGTCTGAACGTCGAAAACCCG; *LacZ 3′*: CGGGCAAATAATATCGGTGGCCGTGG.

1.2. Animal care/handling

For the initial activity experiments, mice were bred at the Salk Institute for Biological Studies (La Jolla, CA) and then shipped at 32–53 days of age to the Institute for Behavioral Genetics (IBG) at the University of Colorado (Boulder, CO) where they were housed for four weeks prior to behavioral testing. For later activity experiments and for all anxiety tests, mice were born and tested in the same facility. Mice were maintained at both the Salk Institute and IBG with two to five animals per cage on a 12 h light/dark cycle (6 am–6 pm) in the vivarium with *ad libitum* access to food and water. Mice were tested at 60–90 days of age. Animals were transferred to clean cages during weekly cage changing but were otherwise not handled prior to behavioral

testing. Housing and handling conditions were identical for behavioral and neurochemical studies. All procedures were approved by the Animal Care and Utilization Committees at the Salk Institute and the University of Colorado.

1.3. Behavioral tests

For all behavioral tests, mice were acclimated to the testing room at least 4 h prior to testing. The testing room was maintained on the same light/dark cycle as the main colony. The experiments were designed such that only one mouse per cage was tested per day and each session of testing included representatives from each genotype. Experiments were conducted between 0800 and 1400 h.

1.4. Anxiety tests

1.4.1. Elevated plus maze

The elevated plus maze consists of two open arms (30 × 5 cm) and two closed arms (30 × 5 cm) constructed of black Plexiglas that extend from a center platform. The closed arms have sides of clear Plexiglas (15 cm tall, 0.5 cm thick). The entire apparatus is mounted 33 cm above the surface of the floor. Each test was begun by placing the mouse in a cylindrical starting tube (5 cm diameter, 15 cm tall) in the center of the maze for 20 s. The tube was removed and the subjects were observed on the maze for 10 min. Subjects were scored for the amount of time spent and the number of entries into the open and closed arms, determined by placement of all four paws into that area. Entries into the closed arms were assessed as a measure of activity. Data represent combined results from the Salk Institute and the Institute for Behavioral Genetics using mice bred and tested in the same facility.

1.4.2. Black/white box

The black/white apparatus was constructed as described previously (Costall et al., 1989; Crawley and Goodwin, 1980). The box (48 × 28 × 27 cm) is divided into two compartments, with one third of the box constructed from black Plexiglas with red illumination while the other two thirds of the box is constructed of white Plexiglas with bright fluorescent lighting. Mice can transit between compartments through an opening (7.5 × 7.5 cm) located at floor level in the center of the partition between compartments. Testing is initiated by placing the mouse in the white, aversive compartment to increase aversion to the light compartment and to increase the sensitivity of measuring anxiety behaviors (Chaouloff et al., 1997) and movement in the testing apparatus was monitored for 10 min. Four behaviors were assessed: 1) total number of transitions between compartments, with a transition scored when all four paws were placed in a compartment, 2) latency to enter the black compartment, i.e. the first transition into the black compartment, 3) latency to reenter the white compartment and 4) percent of time spent in the white chamber (seconds spent in the white chamber/600 s × 100). Data represent combined results from the Salk Institute and the Institute for Behavioral Genetics using mice bred and tested in the same facility.

1.4.3. Mirrored chamber

The mirrored chamber, designed by Toubas et al (Toubas et al., 1990), consists of an open ended cube (30 cm on each side) with mirrors on all interior sides including the floor and ceiling. The cube is placed inside a larger square box (40 × 40 × 30.5 cm) made of black Plexiglas, creating a 5 cm corridor around the mirrored cube. A mirror is also mounted on the interior face of the outer box that faces the open end of the cube, creating a reflective corridor in front of the mirrored chamber. Testing began by placing the mouse in the back corner of the box behind the mirrored chamber and movement in the apparatus was scored for 10 min. Four parameters were scored 1) latency to enter the mirrored chamber (all four paws in the chamber), 2) number of entries into the chamber, 3) percent of time spent in the chamber (seconds spent in mirrored chamber/600 s × 100) and 4) number of entries into the corridor in front of the mirrored chamber (all four paws in corridor). Data represent results obtained from mice bred and tested at the Institute for Behavioral Genetics.

1.4.4. Y-maze

Line crosses and rearing activity were assessed using an automated symmetrical Y-maze (Marks et al., 1985) constructed of red translucent Plexiglas with three covered arms measuring 26 cm long, 6.1 cm wide and 10.2 cm high. Movement between arms and rearing activity were recorded for a 3 min period. The initial set of mice tested in the Y-maze was bred at the Salk Institute and shipped to the Institute for Behavioral Genetics. Mice were then housed for four weeks prior to behavioral testing. Subsequent sets of mice were bred at both facilities and tested in the same facility in which they were bred.

1.4.5. Corticosterone levels

Mice were brought into the testing room the night before testing and were housed with at least one littermate. The animals and experiments were arranged so that only one mouse from each cage was tested per day. All experiments were performed between 0800 and 1100 h. Blood was taken by puncture of the retro-orbital sinus from animals that were unstressed (immediately after removal from their homecage) or stressed by restriction to the open arm of the elevated plus-maze for 20 min. The blood was collected in heparinized capillary tubes (40 µl capacity), and the plasma was separated by centrifugation for 10 min on a table top centrifuge. Plasma samples were analyzed for corticosterone levels with a standard radioimmunoassay kit (ICN Diagnostics). Data were obtained from mice bred and tested at the Institute for Behavioral Genetics.

1.4.6. Simulation of shipping stress

For experiments examining the effects of stress prior to activity or anxiety testing, stress was induced by strapping cages of mice to a rotating shaker (Orbitron Rotator II, Boekel Industries, Inc., Feasterville, PA) at an oscillation frequency of approximately 30 times per minute for 1 h to simulate the movements encountered during shipping. Mice were then

returned to the colony room and Y-maze activity or anxiety in the elevated plus maze was assessed the following morning. Data represent combined results from the Salk Institute and the Institute for Behavioral Genetics using mice bred and tested in the same facility.

1.5. Statistical analysis

The data were analyzed using two-way ANOVA to evaluate possible effects of genotype and gender (SPSS, Chicago, Illinois) with significance set at $p<0.05$. Data were collapsed across gender because no statistical difference was observed for this factor for any of the tests conducted. Significant differences between genotypes were assessed using Tukey's posthoc test. Power values are reported as (1-β), the difference between 1 and the probability of Type II error.

2. Results

2.1. Anxiety

We investigated the potential role of beta3-containing nicotinic receptors in anxiety by examining the beta3 null mutants and their wildtype and heterozygous littermates on several behavioral tests commonly used to assess anxiety. These three tests were selected as they present unique anxiogenic stimuli to elicit anxiety.

2.2. Elevated plus maze

This test assesses the willingness of the mouse to enter the unprotected open arms versus the enclosed, more protected closed arms of the elevated maze and is a potential model of panic disorder and generalized anxiety disorder in humans (Picciotto et al., 2002). A significant effect of beta3 genotype was detected for the percentage of time spent in the open arms ($F_{2,48}=3.349$, $p=0.044$; Fig. 1A) and the number of entries into the open arms ($F_{2,48}=4.480$, $p=0.016$; Fig. 1B). Beta3 null mutant mice entered the open arms more frequently and spent significantly more time in the open arms as compared to wildtype littermates ($p<0.05$, Tukey post hoc test). Although the beta3 null mutants tended to have higher number of entries into the closed arms, a common index of locomotor activity, the effect of genotype was not significant (Fig. 1C). Further study by analysis of covariance (ANCOVA) indicated that the number of entries into the open arm are independent of those into the closed arm ($F_{1,47}=0.586$, $p=0.448$), further supporting an effect of beta3 deletion on anxiety independent of locomotor activity.

2.3. Black/white box

In the black/white box, exploratory behavior between a white, brightly lit, aversive compartment and a dark, less aversive compartment is examined (Crawley and Goodwin, 1980) and may serve as a model for generalized anxiety disorder (Picciotto et al., 2002). The beta3 null mutant mice did not demonstrate any difference from heterozygous or wildtype littermates for the percent of time spent in the white compartment, the number of entries into the white chamber, or the latency to enter the black compartment (Fig. 2A–C). Factor analysis has identified the latency to reenter the white compartment as an activity dependent factor (Chaouloff et al., 1997). Although beta3 null mutants tended to reenter the white chamber faster than wildtype littermates, suggesting increased locomotor activity, no significant difference was observed for this measure (Fig. 2D).

2.4. Mirrored chamber

The mirrored chamber consists of a large Plexiglas box containing an open-sided cube, whose interior is lined with mirrors on all sides, including the floor and ceiling. Upon entering the internal chamber, the mouse is faced with what appears to be other animals from the multiple reflections, as well as a distortion of the environment from the reflections,

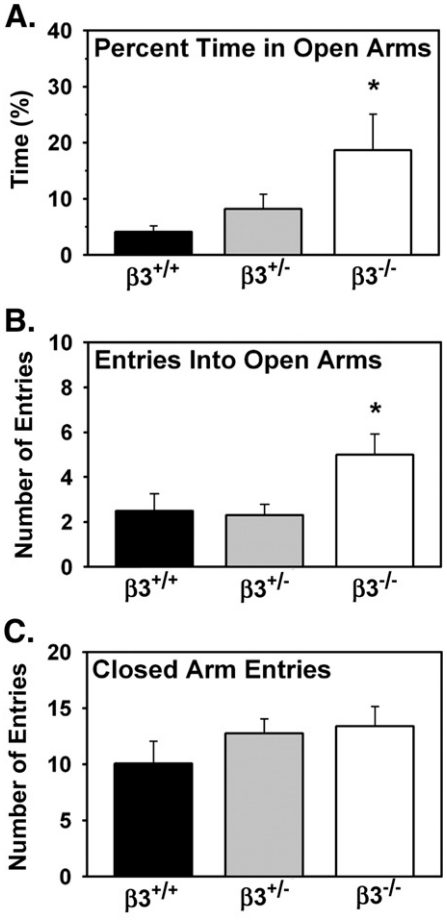

Fig. 1. Anxiety behaviors in the elevated plus maze. Mice of all genotypes were tested for 10 min on the elevated plus maze and were scored for (A) the percent time spent in the open arm, (B) the number of entries into the open arm and (C) the number of entries into the closed arms. Data are presented as mean±sem. (*=$p<.05$) (n: beta3 wildtype (β3+/+)=14 (8 male/6 female); beta3 heterozygous (β3+/−)=22 (15 male/7 female); beta3 homozyous null mutant (β3−/−)=15 (8 male/7 female)).

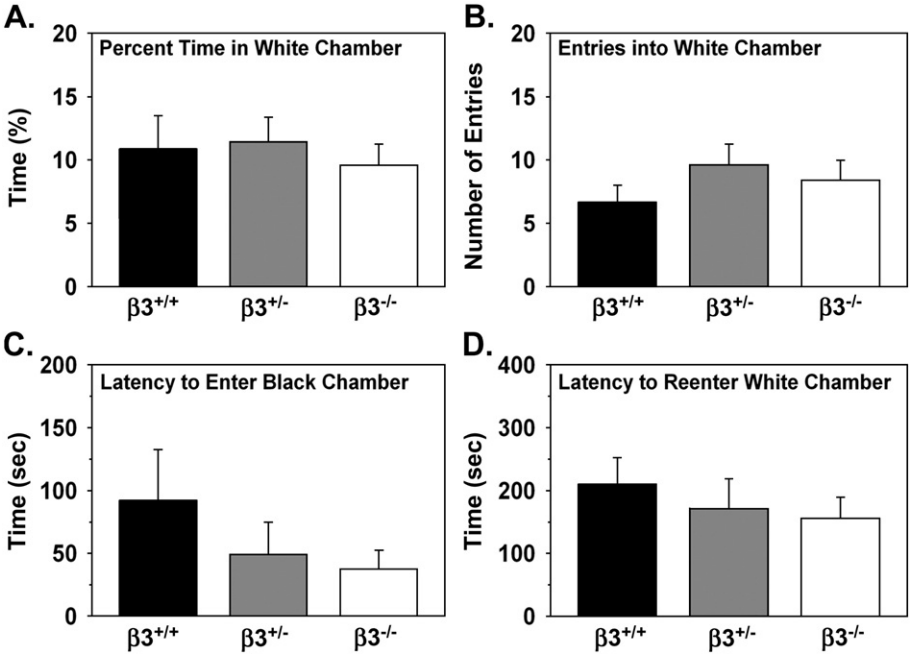

Fig. 2. Anxiety behaviors in the black/white box. Mice of all beta3 genotypes were tested for 10 min in the black/white box and assessed for (A) the percentage of time spent in white compartment, (B) the number of entries into the white compartment, (C) the latency to enter the black compartment and (D) the latency to reenter the white compartment. Data are presented as mean ± sem. (*n*: $\beta3^{+/+}$ = 24 (12 male/12 female); $\beta3^{+/-}$ = 24 (13 male/11 female); $\beta3^{-/-}$ = 23 (12 male/11 female)).

serving as a possible model of social anxiety as well as generalized anxiety disorder (Picciotto et al., 2002). Although beta3 null mutant mice displayed a provocative trend towards lower levels of anxiety with decreased latency to enter the mirrored chamber, increased entries, longer total time and greater than average time per entry into the chamber compared to wildtype littermates (Fig. 3A–D), the apparent differences were not statistically significant.

2.5. Corticosterone levels

A rapid increase in the concentration of circulating stress hormones is a common physiological response to stress or anxiety. Previous studies in animal models have demonstrated that exposure to stressful or anxiogenic situations produces a robust increase in the levels of the adrenocortical hormone corticosterone. We hypothesized that the decrease in anxiety

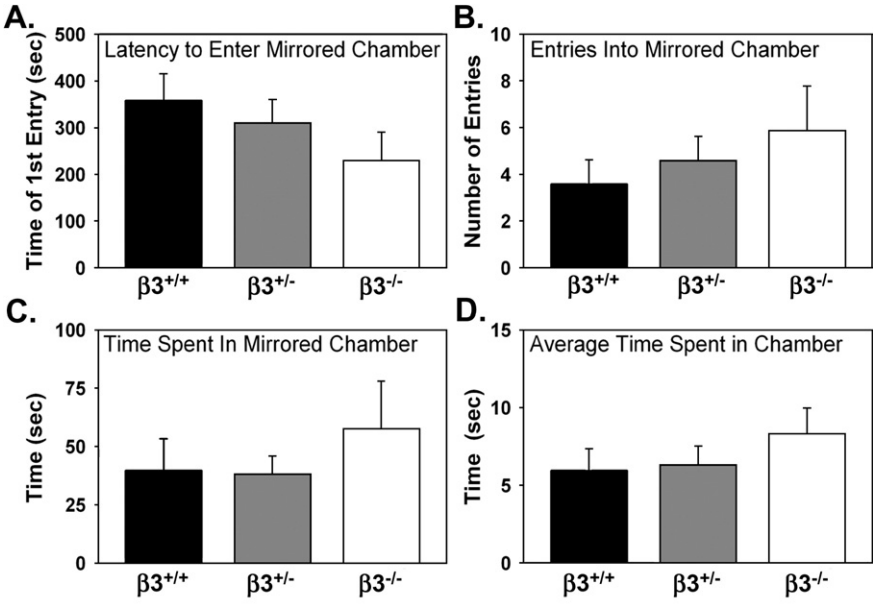

Fig. 3. Anxiety behaviors in the mirrored chamber. Mice of all beta3 genotypes were tested for 10 min and assessed for (A) latency to enter the mirrored chamber, (B) the number of entries into the chamber, (C) the total amount of time spent in the chamber and (D) the average time spent per entry. Data are presented as mean ± sem. (*n*: $\beta3^{+/+}$ = 19 (8 male/11 female); $\beta3^{+/-}$ = 24 (13 male/11 female); $\beta3^{-/-}$ = 16 (8 male/8 female)).

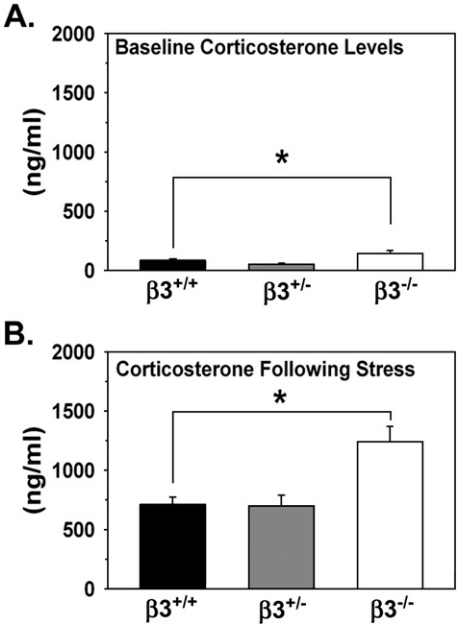

Fig. 4. Stress hormone levels. A. Basal levels of circulating corticosterone. (*$p<0.05$) (n: $\beta3^{+/+}=15$ (8 male/7 female); $\beta3^{+/-}=19$ (10 male/9 female); $\beta3^{-/-}=16$ (8 male/8 female). B. Corticosterone levels following induction of stress by 20 min exposure to the open arm of the elevated plus maze. (*$p<0.05$) (n $\beta3^{+/+}=5$ (3 male/2 female); $\beta3^{+/-}=12$ (6 male/6 female); $\beta3^{-/-}=5$ (3 male/2 female)). Data are presented as mean±sem.

behaviors observed in the beta3 null mutants may coincide with a reduction in corticosterone levels. To investigate this potential mechanism, we determined the levels of corticosterone at baseline and following exposure to stress. Basal levels of corticosterone were significantly higher in beta3 null mutant mice than in wildtype littermates ($F_{2,47}=9.24$, $p<0.001$, Tukey's $p<0.05$) (Fig. 4A). Using a previously established protocol in which corticosterone levels are significantly elevated following restriction to the open arm of an elevated plus maze (Minnick et al., 1995), we found that exposure to this stressor elicited significant increases in corticosterone levels in mice of all genotypes, with a significant effect of genotype on corticosterone levels following stress ($F_{2,20}=5.06$, $p=0.017$) (Fig. 4B). Beta3 null mutant mice exhibited a larger increase in corticosterone following stress than wildtype littermates ($p<.05$). These data demonstrate that beta3 null mutant mice have elevated basal levels of corticosterone and are able to respond to stress with a further increase in corticosterone.

2.6. Activity in the Y-maze

The data presented above reveal a role for beta3-containing nicotinic receptors in anxiety. To investigate their role in locomotor activity, we assessed activity levels in the symmetrical Y-maze chamber. Mice are placed in an enclosed, Y-shaped chamber made of red Plexiglas with no internal illumination, thereby reducing the anxiogenic stimuli experienced in a brightly lit open field arena. As shown in Fig. 5A, a significant effect of genotype was observed on Y-maze activity as measured by the number of line crosses ($F_{2,36}=4.470$, $p=.019$ one-way ANOVA). Beta3 null mutant mice displayed a robust increase in Y-maze crosses as compared to their wildtype littermates ($p<.05$, Tukey's). No significant difference was observed between genotypes for rearing activity. These data replicate the increases in activity observed in the beta3 null mutant mice in the open field arena (Cui et al., 2003).

Another set of mice were tested to confirm these findings with a greater number of animals. Much to our surprise, no significant effect of genotype in Y-maze activity could be detected (Fig. 5B). Similar experiments conducted at the Salk Institute produced similar findings. Overall activity recorded at the Salk Institute was greater than that observed at IBG, but no significant effect of genotype on Y-maze activity was detected (Fig. 5C).

Given the robust increase in locomotor activity observed in the initial group of beta3 mice and the loss of the phenotype in

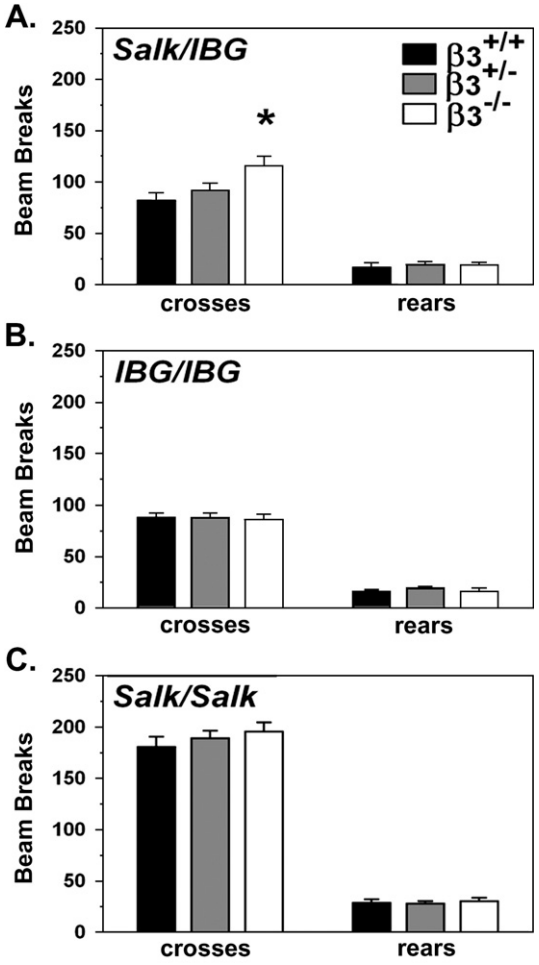

Fig. 5. Activity in the Y-Maze. Activity (crosses and rears) was assessed for 3 min in the Y-maze in mice that had been (A) bred at the Salk Institute and then shipped to the Institute for Behavioral Genetics (Salk/IBG) for testing (n: $\beta3^{+/+}=13$ (8 male/5 female); $\beta3^{+/-}=13$ (7 male/6 female); $\beta3^{-/-}=13$ (8 male/5 female)), (B) bred and tested at IBG (IBG/IBG) (n: $\beta3^{+/+}=57$ (30 male/27 female); $\beta3^{+/-}=93$ (59 male/34 female); $\beta3^{-/-}=45$ (22 male/23 female)) and (C) bred and tested at the Salk Institute (Salk/Salk) (n: $\beta3^{+/+}=23$ (13 male/10 female); $\beta3^{+/-}=44$ (21 male/23 female); $\beta3^{-/-}=17$ (6 male/11 female)). (*$p<.05$) Data are expressed as mean±sem.

the following two groups of animals, we attempted to identify any factor that may have influenced the results of the tests, including care and handling, food, time of day that the test was conducted, etc. After careful consideration, the only factor identified was that the first batch of mice had been shipped from the Salk Institute to IBG, whereas latter batches of mice had been born, reared and tested in the same facility. This led us to hypothesize that perhaps stress encountered during the shipping process produced long term alterations in activity via a mechanism involving beta3 nicotinic receptors.

2.7. Simulation of shipping stress

The effects of stress prior to testing in the Y-maze were evaluated to test this hypothesis. We attempted to recreate some of the stress of shipping by placing the mouse cages on a slowly rotating shaker for 1 h. Mice were then housed overnight under standard conditions and tested for performance in the Y-maze the following morning. Beta3 null mutant mice stressed prior to testing demonstrated significantly more Y-maze crosses ($F_{2,33}$= 5.47 p=0.009, Tukey's p<0.01) and rears ($F_{2,33}$=7.12 p= 0.003, Tukey's p<0.01) than wildtype littermates (Fig. 6). The increase in line crosses in the beta3 null mutant mice resembled that observed in the mice that had been shipped prior to testing (Fig. 5A), suggesting that beta3 nicotinic receptors mediate responses to anxiogenic stimuli including changes in locomotor activity.

Although no anxiety testing was performed on the initial set of mice shipped to IBG, the above finding that locomotor activity was altered in the beta3 null mutants in response to stress indicated that anxiety behaviors may also be affected. We therefore examined the effect of pre-exposure to stress on anxiety behaviors in the elevated plus maze in a different set of animals. As described above, stress was induced by placing mouse cages on a slowly rotating shaker for one hour. Mice were then housed overnight under standard conditions and tested for performance in the elevated plus maze the following morning. Exposure to stress prior to testing produced high levels of anxiety, with very few entries or percent time spent in the open arms of the maze (Fig. 7A and B) as opposed to unstressed animals (Fig. 1). This effect occurred in all groups, with no significant effect of beta3 genotype. However, consistent with the above findings that prior stress produces increased activity in the beta3 null mutants, a significant increase in the number of closed arm entries (a parameter measuring activity) was observed in the mutant mice as compared to wildtype littermates ($F_{2,47}$=3.381, p=0.043, Tukey's p<0.05) (Fig. 7C).

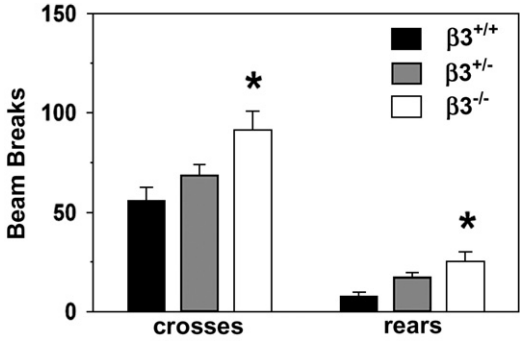

Fig. 6. The effects of stress prior to Y-maze testing. Mice of all genotypes were placed in cages on a rotating shaker for an hour and returned to the colony room. The following day, Y-maze crosses and rears were examined for 3 min. Data are presented as mean±sem. (*p<0.05) (n: $\beta3^{+/+}$=11 (6 male/5 female); $\beta3^{+/-}$=17 (12 male/5 female); $\beta3^{-/-}$=8 (4 male/4 female).

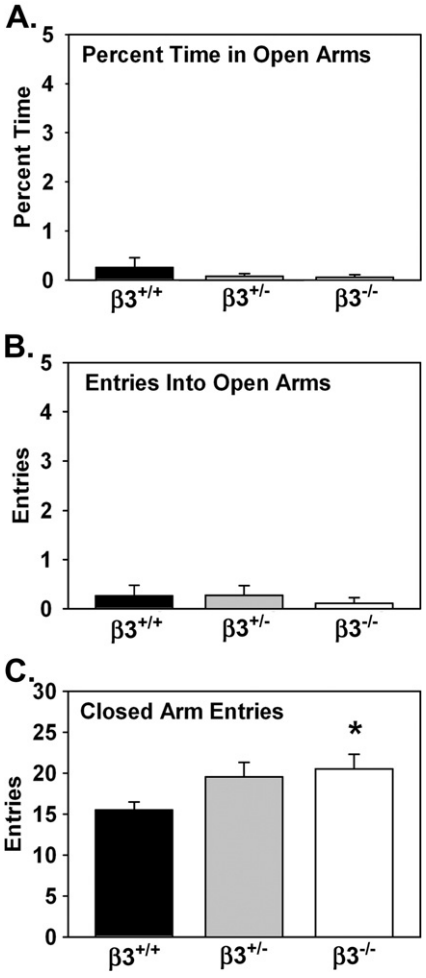

Fig. 7. The effects of stress prior to anxiety testing in the elevated plus maze. Mice of all genotypes were placed in cages on a rotating shaker for an hour and returned to the colony room. The following day, activity in the elevated plus maze was examined for 10 min. Data are expressed as mean±sem. (p<0.05) (n: $\beta3^{+/+}$=19 (9 male/10 female); $\beta3^{+/-}$=11 (5 male/6 female); $\beta3^{-/-}$=18 (7 male/ 11 female).

3. Discussion

The conditions under which we first observed increased activity in the beta3 null mutant mice in the illuminated open field arena most likely included an anxiety component as well as a locomotor activity component (reviewed in (Montgomery and Monkman, 1955; Rodgers et al., 1997)). We therefore performed the experiments presented here to differentiate between the possible roles of beta3-containing nicotinic receptors in both activity and anxiety.

We first investigated the role of beta3 receptors in anxiety by examining the beta3 null mutants in three established behavioral tests for anxiety. Beta3 null mutant mice displayed lower levels of anxiety in the elevated plus maze than their wildtype littermates. No significant effect of genotype was observed in the black/white box or the mirrored chamber, although the beta3 null mutants displayed a trend towards lower anxiety in the mirrored chamber. A power analysis indicated that testing a minimum of 100 more mice would be necessary to detect a significant effect in the mirrored chamber test (for example, time in chamber $(1\text{-}\beta)=0.159$) and more testing was therefore not pursued. The results from the anxiety tests indicate that at least some forms of anxiety are modulated by nicotinic receptors containing the beta3 subunit.

Evidence is emerging from quantitative trait loci (QTL) analyses that anxiety is a complex trait regulated by multiple gene products. Although a small number of identified QTLs affect all of the anxiety tests we employed, many anxiety-related QTLs have been found to affect only a single anxiety test (Conti et al., 2004; Fernandez-Teruel et al., 2002; Turri et al., 2001, 2004). These findings indicate that anxiety is likely governed by multiple genes and may have subclasses regulated by unique sets of genes. Similarly in humans, five different anxiety disorders have been delineated, including panic disorder (with or without agoraphobia), obsessive–compulsive disorder, social phobia, post traumatic stress disorder and generalized anxiety disorder (DSM-IV, 1994). Given the complexity of anxiety in both humans and animal models, it is not surprising that deletion of beta3 did not affect all of the examined phenotypes. While caution must be exercised when allocating anxiety traits observed in a mouse model directly to a human condition, the altered anxiety observed in the beta3 null mutants in a test that may model aspects of panic disorder or generalized anxiety disorder (Picciotto et al., 2002) indicates a potential role for beta3 in these conditions.

Locomotor activity was then examined under conditions of reduced anxiogenic stimuli in the Y-maze. Beta3 null mutant mice initially exhibited higher activity than the wildtype littermates. However, we were unable to detect this difference in subsequent experiments. The only variable identified between the groups of mice tested was that the initial set of mice had been shipped prior to behavioral testing. Given the results of the anxiety tests suggesting that beta3-containing receptors play a role in anxiety, we hypothesized that the alterations observed in Y-maze activity in the first group of mice may have been a result of exposure to stress during shipping. We simulated the stress of shipping by exposing naïve mice to movements mimicking those that might be experienced if they were packaged freight prior to testing in the Y-maze. Simulated shipping had no effect on the wildtype mice, however, a significant increase in Y-maze activity was observed in the beta3 null mutant mice. The increase in activity was reminiscent of that observed in beta3 null mutants that had actually been shipped prior to Y-maze testing. Correspondingly, greater activity as measured by entries into the closed arms of the elevated plus maze was observed in the beta3 null mutants stressed prior to testing. No effect of genotype was observed on anxiety behaviors, however, with all mice demonstrating high levels of anxiety.

A previous study reported that shipping has no consistent influence on multiple behaviors, including locomotor activity in the open field arena and anxiety in the elevated plus maze. These phenotypes were assessed in seven wildtype strains and one line of mutant mice that were shipped and then housed five weeks prior to testing (Crabbe et al., 1999). Our results for activity levels in wildtype mice were consistent with their findings, as activity in the Y-maze did not differ between wildtype mice that underwent shipping prior to testing or those bred and tested in the same facility. However, our data indicate that the stress of shipping had a robust effect on the activity levels of the beta3 null mutant mice. The discrepancy between the dramatic increase in anxiety across genotypes observed in our studies and those of Crabbe et al may be due to the time elapsed between stressor and testing or to the fact that we studied animals with a different null mutation. Our data strongly suggest that beta3-containing nicotinic receptors play a role in behavioral responses to anxiety and that prior stress can influence some of these anxiety-related responses.

The age at which stress is experienced can alter behavioral consequences in animal models and humans, with some studies showing altered response to stress in adolescent subjects as compared to adults (for example, see (Cheeta et al., 2001b; Doremus et al., 2003; Genn et al., 2003; Imhof et al., 1993; Spear, 2000). In mice, however, there is no clear consensus from the data, with increased, decreased or no change in adolescent responses to stress compared to adults (Adriani and Laviola, 2000; Hefner and Holmes, 2007; Macri et al., 2002; Stone and Quartermain, 1997). Comparison of these data sets is also difficult due to variations in maintenance/handling of the mice that are known to induce stress responses, such as individual housing and shipping prior to testing. In our study, similar increases in locomotor activity in response to stress were observed in mice that underwent shipping at 32–53 days of age (ranging from adolescence to young adulthood) and those that were exposed to simulated shipping stress as adults (60–90 days of age). Thus, the effects reported here do not appear to be a result of unique responses to stress experienced during adolescence.

A common physiological response to anxiogenic stimuli is a rapid rise in stress hormones such as corticosterone. We hypothesized that the beta3 null mutants might show a diminished rise in corticosterone levels because they demonstrate decreased anxiety-like behaviors when confronted with an anxiogenic stimulus. However, the null mutants responded with an increase in corticosterone, demonstrating that the physiological response to stress through the hypothalamus/pituitary/adrenal (HPA) axis is intact in the beta3 null mutant mice. The beta3 null mutant mice also had significantly higher basal levels of corticosterone than wildtype littermates prior to exposure to stress. Thus, it may be that the decreased levels of anxiety observed in the null mutants are a result of a negative feedback mechanism initiated by continuously high levels of corticosterone. Also, acute administration of corticosterone has been shown to have anxiolytic properties, however, chronic

administration did not alter anxiety levels (Andreatini and Leite, 1994a,b; File et al., 1979). As beta3 null mutant mice have chronically higher levels of corticosterone, the anxiolytic effects reported earlier for acute corticosterone are not likely to have influenced the results of the anxiety testing.

We and others have demonstrated that nicotine administration stimulates the release of corticosterone (Andersson et al., 1988; Balfour et al., 1975; Cam et al., 1979; Conte-Devoix et al., 1981; Pauly et al., 1992; Porcu et al., 2003; Sharp and Beyer, 1986) and this may occur via several possible mechanisms. Noradrenergic systems regulating the HPA axis and also the 'extrahypothalamic' pathways including the hippocampus and amygdala are regulated by nicotinic receptors. The major source of noradrenergic input to the paraventricular nucleus (PVN) of the hypothalamus is the nucleus tractus solitarius (NTS) and nicotinic receptors in the NTS regulate norepinephrine release from the PVN (Fu et al., 1997; Okada et al., 2003). High levels of beta3 mRNA have not been detected in the NTS by in situ hybridization. Beta3 subunits are, however, highly expressed in the locus coeruleus (Cui et al., 2003; Lena et al., 1999) and as this area provides another source of noradrenergic input to the PVN (for review see (Swanson and Sawchenko, 1983)), beta3 may alter norepinephrine release via this pathway. Noradrenergic neurons from the locus coeruleus also project to the hippocampus as part of the 'extrahypothalamic' pathway through which corticosterone is increased in response to stress (Feldman et al., 1995; Feldman et al., 1987). Nicotine stimulates the release of norepinephrine from these hippocampal neurons (Fu et al., 1999). α-Conotoxin MII inhibits nicotine-evoked norepinephrine release (Azam and McIntosh, 2006; Fu et al., 1999) and beta3 is now considered a critical component of α-conotoxin MII-sensitive nicotinic receptors (Azam and McIntosh, 2006; Cui et al., 2003). Furthermore, deletion of the beta3 subunit significantly reduces (<10% remaining in beta3 null mutant mice) nicotinic modulation of hippocampal norepinephrine release (Azam and McIntosh, 2006). Therefore, beta3-containing nicotinic receptors in the locus coeruleus–hippocampal pathway may mediate the observed changes in corticosterone. Similarly, nicotine stimulates the release of norepinephrine from neurons projecting from the locus coeruleus to the amygdala, another portion of the 'extrahypothalamic' pathway, however, the involvement of the beta3 subunit has not been investigated.

Alternatively, dopaminergic neurons project from the ventral tegmental area/substantia nigra to the central nucleus of the amygdala (Fallon and Moore, 1978) and specifically target CRF neurons (Eliava et al., 2003), suggesting that dopaminergic transmission may modulate the HPA axis via CRF release from amygdala. Beta3 is highly expressed in the ventral tegmental area/substantia nigra and we demonstrated previously that beta3-containing nicotinic receptors modulate the release of dopamine in the nigrostriatal pathway (Cui et al., 2003). It is possible that beta3-containing receptors modulate dopamine release in the amygdala in a similar manner and subsequently regulate corticosterone levels via CRF release.

Deletion of the alpha4 nicotinic subunit resulted in an increase in anxiety (Ross et al., 2000) and a decrease in nicotine-stimulated [^{3}H]dopamine release (Salminen et al., 2004). Similarly, mice expressing the Leucine9'Serine mutation in the alpha4 subunit demonstrate increased anxiety and decreased expression of dopaminergic neurons (Labarca et al., 2001), which likely results in a decrease in dopamine release in vivo. These effects are opposite the decreased anxiety and increased levels of nicotine-stimulated [^{3}H]dopamine release observed in the beta3 null mutants (Cui et al., 2003). Given the negative correlation between levels of dopamine release and anxiety in several lines of nicotinic mutant mice, it is tempting to speculate that nicotine may alter anxiety via a dopaminergic mechanism.

Similar to the findings reported here, mice lacking the beta4 subunit also demonstrated decreased levels of anxiety (Salas et al., 2003), however, it is unlikely that a population of nicotinic receptors containing both beta3 and beta4 mediate these effects. Both beta3 and beta4 subunits have limited expression in mouse brain, with overlap occurring only in medial habenula (Cui et al., 2003; Salas et al., 2003). Although anxiety can be induced by administration of nicotine to the dorsal raphe nucleus (Cheeta et al., 2001a), which receives innervation from medial habenula (Morris et al., 1999; Tomizawa et al., 2001), pharmacological evidence suggests that beta3 and beta4 are expressed in different populations of nicotinic receptors originating from medial habenula (Grady et al., 2001). Alpha-conotoxin MII, a nicotinic antagonist with high affinity for beta3-containing receptors (Cui et al., 2003), had no effect on nicotine-stimulated [^{3}H]acetylcholine release from intrapeduncular nucleus, whereas alpha-conotoxin AuIB, an antagonist with high affinity for beta4-containing receptors (Luo et al., 1998), inhibited 50% of release. These data support separate populations of nicotinic receptors that mediate anxiety and illustrate the potentially complex nature of the effect of nicotine on this behavior.

The results presented here were obtained using null mutant mice in which the beta3 gene was disrupted throughout the lifetime of the mouse and we cannot therefore disregard the potential for developmental effects due to the absence of the gene product during development. It is possible that compensatory changes may contribute to the phenotypic characterizations reported here. However, even phenotypes occurring due to compensatory mechanisms identify potential pathways, systems and roles for a subunit about which we knew little before. The potential for developmental abnormalities cannot be ignored, but taken as a possible caveat in interpreting the data obtained using one of the few tools available for dissecting the roles of this subunit.

With respect to the role of beta3 subunits in modulating anxiety and corticosterone levels, the recent report from Azam et al. indicated that in wildtype mice, nicotinic receptors robustly stimulate hippocampal norepinephrine release in two to three week old pups, but release is much less robust in adult mice (Azam and McIntosh, 2006). Deletion of beta3 nearly eliminates hippocampal nicotine-evoked norepinephrine release, indicating not only a role for beta3-containing receptors, but also that compensation by another nicotinic subunit did not occur. It is not yet known if this pathway underlies the effects of beta3-containing receptors on anxiety, but it is a valid candidate.

Given the robust response from beta3-containing receptors in young animals, we cannot, however, discount possible compensation with associated proteins involved in this system.

Our results demonstrate that some forms of anxiety are mediated by beta3-containing nicotinic receptors. Since smoking-related behaviors may continue to maintain the effects of nicotine on anxiety in humans (File et al., 2001; Hughes et al., 1984; Sonntag et al., 2000), our findings illuminate at least one site of action that likely mediates these effects. Elucidating the mechanism(s) through which nicotine alters anxiety may contribute to a better understanding of the many aspects of nicotine addiction and thereby lead to more effective measures for smoking cessation.

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