Altered Anxiety-Related Responses in Mutant Mice Lacking the \( \beta 4 \) Subunit of the Nicotinic Receptor

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Nicotine, acting at nicotinic acetylcholine receptors (nAChRs), is the primary addictive component of tobacco. Smokers often report an anxiolytic effect of cigarettes. This relief of anxiety, attributed to nicotine, is an important contributor to relapse when smokers try to quit. Hence, the study of the anxiolytic effects of nicotine is important for understanding the mechanisms underlying nicotine addiction. Mammalian nAChRs are pentameric ion channels usually composed of \( \alpha \) and \( \beta \) subunits. Taking advantage of \( \beta 4 \)-homozygous-null mice (\( \beta 4^{-/-} \)), we examined the role of the nAChR \( \beta 4 \) subunit in anxiety-related behaviors. The \( \beta 4^{-/-} \) mice behaved as though they were less anxious than wild-type littermates on the elevated-plus and staircase mazes, tests that measure anxiety-related behaviors. To obtain an independent, physiological indication of the stress produced by several tests, we measured changes in heart rate using telemetry. Consistently with the behavioral phenotype, \( \beta 4^{-/-} \) mice had a smaller heart rate increase in the elevated-plus maze than did wild-type littermates. In contrast, during social isolation, a separate test for anxiety, \( \beta 4^{-/-} \) mice exhibited a greater increase in heart rate than did wild-type littermates. Finally, \( \beta 4^{-/-} \) mice were indistinguishable from their wild-type littermates in the open field, the light/dark box, and the mirrored chamber. The overall results demonstrate that \( \beta 4 \)-containing (\( \beta 4^* \)) nAChRs influence behavioral responses during anxiety-related tests, and that this effect depends on the type of anxiety-provoking experience. Through their influence on anxiety-related behavior, \( \beta 4^* \) nAChRs might influence both tobacco consumption and smoking relapse.

Key words: nicotinic acetylcholine receptors; \( \beta 4 \) subunit; anxiety; elevated-plus maze; open field; light/dark box; telemetry; heart rate

Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) are pentameric ligand-gated cation channels that mediate the addictive properties of nicotine (Dani and De Biasi, 2001). In mammals, eight \( \alpha \) (\( \alpha 2 \) to \( \alpha 7 \), \( \alpha 9 \), and \( \alpha 10 \)) and three \( \beta \) (\( \beta 2 \) to \( \beta 4 \)) subunits have been cloned (Sargent, 1993; Elgoyhen et al., 1994, 2001; McGehee and Role, 1995; Lindstrom et al., 1996; Tassonyi et al., 2002). Most nAChRs are composed of \( \alpha \) and \( \beta \) subunits. \( \alpha 7 \) can form either homopentamers or \( \alpha \beta \) heteropentamers (Seguela et al., 1993; Khirogu et al., 2002), and \( \alpha 9 \) and \( \alpha 10 \) are believed to assemble as \( \alpha \)-only homopentamers (Elgoyhen et al., 2001; Sgard et al., 2002). Neuronal nAChRs have been implicated in processes such as memory (Levin and Simon, 1998), anxiety (File et al., 2000; Ross et al., 2000), sleep control (Domino and Yamamoto, 1965; Salin-Pascual et al., 1999), antinociception (Marubio et al., 1999), and autonomic nervous system function (Xu et al., 1999a,b; De Biasi, 2002). Nicotinic receptors may also be involved in neurological disorders such as nocturnal epilepsy, schizophrenia, Parkinson’s disease, and Alzheimer’s disease (Levin and Simon, 1998; Rusted et al., 2000; Dani and De Biasi, 2001; Leonard et al., 2001; Mouland, 2001).

A number of nicotinic receptor-mutant mice have been generated by various groups, and their analysis has shed light on the functions of different nAChR subunits: \( \alpha 3 \) (Xu et al., 1999a; Yu et al., 2000), \( \alpha 4 \) (Marubio et al., 1999; Ross et al., 2000; Labarca et al., 2001), \( \alpha 5 \) (N. Wang et al., 2002; Salas et al., 2003), \( \alpha 6 \) (Champaitaux et al., 2002), \( \alpha 7 \) (Orr-Urtreger et al., 1997; Paylor et al., 1998; Franceschini et al., 2000, 2002; Broide et al., 2002), \( \beta 2 \) (Picciotto, 1995; Zoli et al., 1999; Cohen et al., 2002; Shoabi et al., 2002), \( \beta 2/\beta 4 \) (Xu et al., 1999b), and \( \beta 3 \) (Booker et al., 2000). A recurrent behavioral phenotype among nAChR-mutant mice is a difference in anxiety-like responses on the elevated-plus maze with little if any effect on other anxiety tests, as revealed by the analysis of \( \beta 3 \)- and \( \alpha 4 \)-mutant mice (Booker et al., 2000; Ross et al., 2000; Labarca et al., 2001).

Nicotine has been shown to affect anxiety in different ways (Picciotto et al., 2002). In rodents, nicotine can be anxiolytic, anxiogenic, or have no effect on anxiety, depending on the dose used and the route of administration, even when the same behavioral test is performed (Cheeta et al., 2000; File et al., 2000). In humans, according to smokers’ accounts, smoking may have anxiolytic effects, which are likely attributable to the nicotine contained in cigarettes (Kassel and Unrod, 2000). However, despite the reported anxiolytic effect of cigarettes, smokers display higher scores in anxiety-related tests than nonsmokers and smokers who quit (Parrott, 1995).

The \( \beta 4 \) subunit of nicotinic receptors is widely expressed in the peripheral nervous system, but in the rat CNS, it is restricted to a few regions (Duvoisin et al., 1989; Dineley-Miller and Patrick, 1992; Poth et al., 1997; Xu et al., 1999b). We show here that, in the mouse, the CNS expression pattern of \( \beta 4 \) is even more restricted than in the rat, with its expression significantly detected only in the olfactory bulb (Olf), medial habenula (MHB), pineal...
gland (Pin), interpeduncular nucleus (IPN), and inferior colliculus (IC). To date, no specific function for CNS β4-containing (β4*) nAChRs has been reported. Our data show that β4+/− mice display behavioral differences when compared with their wild-type (β4+/+) littermates on anxiety-related tests. The β4−/− mice showed increased exploratory behavior in the elevated-plus maze and increased climbing activity in the staircase maze, suggesting decreased anxiety-like behavior. Conversely, social isolation was more anxiogenic in the β4−/− mice. The behavior in the open field, light/dark box, and mirror chamber was unchanged.

Materials and Methods

Animals. β4−/− mice were generated as described previously (Xu et al., 1999b). Experiments were performed on mice backcrossed for six generations into the C57BL/6J background. All of the tests were performed on 2- to 7-month-old animals of both sexes. Mice were housed (three to five per cage) under a 12 hr light/dark cycle with ad libitum access to food and water. Behavioral experiments were performed during the light phase. The experimenters were blind to the genotypes until data were gathered. β4+/+, β4−/+ and, β4−/− mice were generated by crossing heterozygous mice. We used all of the β4+/+ and β4−/− and some of the β4−/+ littermates from each litter. As reported previously, β4−/− mice do not show any obvious physical or neurological deficits (Xu et al., 1999b). In the behavioral experiments, a minimum of 15 mice per genotype was used on each replication, and the experiments were done in two separate batches of mice. For clarity and space, the data from both replicates of the behavioral experiments were pooled after confirming that both groups showed the same results (including statistical significance in the elevated-plus and staircase mazes). Groups of 14–21 mice were used in the telemetry experiments. All of the procedures were approved by the Baylor College of Medicine Animal Research Committee and followed the guidelines for animal intramural research from the National Insti-

in situ hybridization. Probes for in situ hybridization were cloned by reverse transcription-PCR using total RNA isolated from a mouse sepal neuroblastoma cell line (SN56 cells) (Blustajn et al., 1992). The primers were designed from the published rat sequences to encompass the third intracellular loop of each subunit. The template DNA sequences and sizes were as follows: arx (1046–1789; 733 bp), β4 (1056–1428; 372 bp), and β3 (1119–1566; 447 bp). PCR fragments were subcloned into pBluescript II (Stratagene, La Jolla, CA) and sequenced (Franceschini et al., 2002). In situ hybridization with [35S]UTP-labeled DNA as a riboprobe was performed as described previously (Broide et al., 1996). Quantification of in situ signals was performed as described previously (Broide et al., 2002).

Locomotor activity in the open field. Mice were placed in a clear Plexiglas arena (40 × 40 × 40 cm), and locomotor activity was measured over a 30 min session using a computer-operated Ethovision system (Noldus, Wageningen, The Netherlands). This system was also used in all of the other behavioral experiments. The total distance moved in the arena and the distance moved in a center square (20 × 20 cm) were recorded. The ratio of the distance moved in the center to the total distance moved was calculated and used as a measure of anxiety-related behavior (Paylor et al., 1998).

Light/dark exploration. The light/dark exploration test, which is believed to measure anxiety-related behavior (Crawley, 1980) was performed by placing the mouse in a cage (44 × 21 × 21 cm) that has two chambers, one bigger and bright, and the other smaller and dark. The animal was initially placed in the lighted side, and transitions between sides and the time spent in each division were recorded for 10 min.

Elevated-plus maze. Mice were placed for 5 min on an elevated-plus maze consisting of four arms (25 × 7 cm), two with high, black walls (15 cm high), and two without walls. Mice were placed in the intersection between the arms (7 × 7 cm), and the number of entries into, and the time spent in, the open and closed arms were recorded. These two parameters were taken as measures of anxiety-related behavior (Pellow et al., 1985).

For some animals, the experiment was repeated 1 d later (trial 2).

Mirror chamber. For the mirror chamber test (Toubas et al., 1999), mice were placed in a black chamber (30 × 30 × 40 cm) located inside another chamber (40 × 40 × 40 cm). The interior walls, floor, and ceiling of the inside chamber are mirrors, and there is also a mirror in the internal wall of the bigger chamber that is facing the entrance of the mirrored box. Mice were placed on a corner opposite to the entrance, and their movement was monitored for 5 min. Entrances into the mirrored chamber, as well as the time spent in the mirrored chamber, were recorded. Entrances into the two lateral corridors were recorded and used as control for total activity.

Staircase maze. The staircase maze test, which is also sensitive to anxiety-related drug treatments and behaviors, was performed as described previously (Simiand et al., 1984; Weizman et al., 1999). Briefly, mice were placed for 3 min in the staircase, a rectangular (45 × 10 cm) maze that has six steps (10 × 7.5 cm, 2.5 cm high). We recorded rearing and step climbing (defined as forepaws on the next step), because these behaviors have been shown to be decreased in mice showing anxious-like behavior (Simiand et al., 1984; Weizman et al., 1999).

Telemetry. Radiotelemetry implants (TAEA-F20; Data Sciences, St. Paul, MN) were used to monitor heart rate (HR) in conscious, freely moving animals. The body of the implants was inserted into the abdominal cavity of anesthetized animals, and the leads were positioned in a lead II electrocardiogram configuration. Animals were allowed a 9 d recovery period during which weight and food intake were monitored. After recovery from surgery, an implanted mouse was housed with at least two same-sex littermates (home cage), and its HR was monitored for 48 hr, taking one measurement every 2 or 10 min. The animal was then transferred to another cage in which it was kept in isolation for 48 hr (single cage). While HR was monitored. After the isolation period, the mouse was returned to its home cage, and HR was monitored until it returned to basal levels (usually within 24 hr).

Light/dark box and elevated-plus tests on telemetry-implanted mice. Behavioral tests were conducted at least 2 d after the social isolation experiment was finished, and the heart rate returned to pretest levels. For the light/dark box test, basal heart rate was measured for 30 min before the beginning of the experiment. The animals were then allowed to freely explore the box for 20 min. During this time, the behavior of the animals was recorded on tape. After the test, mice were returned to their cage, and HR was monitored for another hour. The average HR for the 20 min the mouse spent in the light/dark box was compared with the average HR during baseline. In addition, we compared average HR in the box with the average HR obtained when the mouse was exploring the lighted chamber. At least 1 d after the light/dark box experiment, the implanted mice were tested in the elevated-plus maze. Basal HR was recorded for 30 min before placing the mice in an elevated-plus maze. The receiver for telemetry was placed above the maze. Because of spatial constraints of the receiver, the closed arms of the maze were 7 cm shorter than in the previous experiments. Mice were placed into the elevated-plus maze and allowed to explore it for 20 min while the HR was monitored. The animals were subsequently returned to their home cage, and HR was monitored for 1 additional hr.

Data acquisition and analysis. Behavioral data were analyzed using Excel (Microsoft; Redmond, WA) or Statistica (StatSoft, Tulsa, OK), and unpaired Student’s t test or ANOVA and Newman–Keuls post hoc comparisons. Differences were considered significant when p < 0.05. The HR data were collected using the Datquest ART version 1.10 system (Data Sciences). The HR parameter files were exported and analyzed using SigmaPlot 4.1 (SPSS, Chicago, IL) and ANOVA.

Results

Expression pattern of the β4 nAChR subunit mRNA in the CNS

The expression pattern of β4 mRNA in the mouse brain is shown in Figure 1. From rostral to caudal, the regions of expression were as follows. Relatively high levels of β4 expression were found on the olf (Fig. 1A). The β4 signal was restricted to mitral cells, which are the main input–output cells of the olfactory bulb. We found very high levels of β4 mRNA in the MHb (Fig. 1D), whereas the IPN showed lower levels of β4 subunit mRNA (G). Along with the MHb, the pineal gland (Fig. 1J) expressed the...
highest levels of β4 mRNA, in agreement with previous results in rat tissue (Duvoisin et al., 1989; Dineley-Miller and Patrick, 1992; Zoli et al., 1995). Detectable, low levels of β4 subunit mRNA were also found in the inferior colliculus (Fig. 1M), which is the main target for MHB neurons. In β4−/− mice, no β4 mRNA was detected (Fig. 1 B,E,H,K,N). Sense controls showed no specific signal (Fig. 1 G,F,I,L,O). Three β4+/+ and three β4−/− mouse brains were used for these experiments. Representative brain sections are shown for each genotype. The sense probe was used on β4+/+ brains only.

Several anxiety-related tests show no effect of the β4 genotype
In the open-field test, the total distance traveled by the mouse is a measure of locomotion and activity, and the ratio of the distance traveled in the center to the total distance is a measure of exploration and anxiety-like behavior. In this experiment, β4−/− mice and littermate controls exhibited similar activity and center distance/total distance ratios. Therefore, in the open-field test, β4−/− mice revealed normal exploratory activity and anxiety-related behavior (Fig. 2 A,B).

In the light/dark exploration test, the number of transitions between the dark and lighted compartments and the time spent in the light are measures of anxiety-like behavior (Crawley, 1980). The β4−/− mice and their β4+/+ littermates showed no statistically significant differences in the number of transitions between the lighted and the dark compartments, or in the time spent in the dark compartment (Fig. 2 C,D).

In the mirror chamber test, the animal chooses between staying in a black corridor and entering a chamber with mirrored walls, floor, and ceiling (Toubas et al., 1990). The number of entries and the time spent in the anxiety-provoking mirror chamber are recorded. The β4−/− mice and littermate controls showed no statistically significant differences in entries and time spent in the mirror chamber (Fig. 2 E,F).

β4−/− mice show anxiety-related differences in the elevated-plus maze and the staircase maze
The elevated-plus maze is used to analyze anxiety-related behavior on the basis of the hypothesis that there is greater stress from being in the open arms versus the closed arms of an elevated maze. The number of entrances into the open arms and the time spent in the open arms provide indications of anxiety-like behavior, and the total number of entrances into all of the arms is a measure of total activity (Pellow et al., 1985). β4−/− mice showed a significant increase in both the time spent and the number of entrances into the open arms (Fig. 3), with no differences in total number of entrances. The β4−/− mice not only entered the open arms more often (Fig. 3 A), but also stayed longer after entry (Fig. 3 B). This result was the first behavioral phenotype found for β4−/− mice, and it suggests that these animals have lower levels of anxiety-like behavior as measured in this particular test. We also tested β4+/+ mice in the elevated-plus maze and analyzed the data using ANOVA and Newman–Keuls post hoc comparisons (open time, F(2,0.05) = 9.35; entry ratio, F(2,0.05) = 4.31). The results on heterozygous mice were similar to those of the β4−/− animals (Fig. 3).

Previous research has shown that, when nicotine is injected directly into the hippocampus, it does not affect performance in the elevated-plus maze on the first trial, but nicotine has anxiolytic effects on a second trial performed at least 1 d after the first trial (Cheeta et al., 2000). On the basis of that observation, we repeated the elevated-plus experiment 1 d after the first trial in the maze. The results showed that mice made fewer entries and stayed for shorter times in the open arms on trial 2, but the β4 genotype still had the same influence as on trial 1. The time spent in the open arms dropped from 25 ± 5 sec (mean ± SEM) on trial 1 to 6 ± 2 sec on trial 2 for β4+/+ mice, and from 66 ± 6 sec to 23 ± 4 sec for β4−/− mice. A similar result was observed in the percentage of entries in the open arms, which dropped from 41% ± 7 to 14% ± 3 for β4+/+ mice, and from 63% ± 6 to 30% ± 4 for β4−/− animals. These data were analyzed using Student’s t test. The percent change in arm entries and time between days 1 and 2 was not statistically different between β4+/+ and β4−/− mice. These results indicate that mice explore less the second day, but this difference is similar in β4+/+ and β4−/− mice. Hence, habituation to the maze is normal in β4-
played significantly more steps up and rearing events than did the mutant mice, and the behavior observed is likely to be attributable to decreased anxiety-like behavior and not to abnormal learning or habituation to the maze.

The staircase maze (Simiand et al., 1984) is another test for anxiety-related behavior that has been validated using different anxiotolitics (Pick et al., 1997; Weizman et al., 1999). In this test, mice explore a rectangular maze that has six steps. The number of steps climbed and the rearing behavior of the mice are recorded as measures of anxiety-related behavior. The β4−/− mice displayed significantly more steps up and rearing events than did the β4+/+ littermates (Fig. 4). The data were analyzed using ANOVA and Newman–Keuls post hoc comparisons (steps, \( F_{(2,0.05)} = 7.41 \); rearing \( F_{(2,0.05)} = 3.69 \)). This result is consistent with our experiments in the elevated-plus maze.

Heart rate changes confirm the behavioral results

There is a large body of literature in humans that correlates emotions and anxiety with the activation of the autonomic nervous system (Berntson et al., 1998; Friedman and Thayer, 1998; Johansen-Berg and Walsh, 2001; O’Connor et al., 2002; Watkins et al., 2002). Such activation is reflected by changes in heart rate and blood pressure, among other factors. To obtain an independent measurement of anxiety-related effects, we monitored HR changes in maze tests and blood pressure while animals were placed in the light/dark box. By directly measuring HR without the confounds of handling or restraint, telemetry gives a quantitative, nonsubjective measure of a parameter (HR) that is linked to changes in anxiety-related behavior (Tornatzky and Miczek, 1995; Southwick et al., 1999; Bouwknecht et al., 2000). As shown in Figure 5A, basal HR was statistically the same for β4+/+ and β4−/− mice, and HR increases were observed when the animals were placed in the light/dark maze were comparable. We also measured the mean HR of the mice when they were in the lighted region of the maze, and compared that with the average HR in the maze. Both β4+/+ and β4−/− mice had increased HR while they explored the lighted chamber, and these increases were not statistically different. For β4+/+ mice, the average HR in the lighted chamber was \( 21 \pm 7 \) beats per minute (bpm) higher than the average HR during the total time mice spent in the light/dark box. For the β4−/− mice, the average HR increase in the lighted chamber was \( 30 \pm 15 \) bpm. Increases in HR were also observed in the elevated-plus maze, but the increase in HR was significantly lower for β4−/− mice than for β4+/+ mice (\( p < 0.001 \) (Fig. 5B)). Differences in basal heart rate across genotypes or experiments were not statistically significant (β4+/+, light/dark box, \( \text{HR}_{\text{basal}} = 485 \pm 26 \) bpm; β4+/+, elevated plus, \( \text{HR}_{\text{basal}} = 517 \pm 40 \) bpm; β4−/−, light/dark box, \( \text{HR}_{\text{basal}} = 529 \pm 36 \) bpm).
Figure 5. Telemetric measurement of HR during behavior. A, HR (± SEM; black bars) of β4 +/+ and β4 −/− (white bars) mice in the light/dark (L/D) box (β4 +/+; n = 9; β4 −/−; n = 5). No statistically significant differences were seen between β4 +/+ and β4 −/− mice in average basal HR or HR during maze exploration. B, HR of β4 +/+ and β4 −/− mice in the elevated-plus (El. plus) maze. Statistically significant differences were observed in HR during maze exploration but not in basal HR. No statistically significant differences were found among any of the four basal HRs from A and B. C, HR of β4 +/+ and β4 −/− mice during social isolation (β4 +/+; n = 12; β4 −/−; n = 9). D, Average increase (± SEM) in heart rate after social isolation for β4 +/+ and β4 −/− mice. **p < 0.001; *p < 0.05.

Stress during social isolation increases heart rate

Single-cage housing has been shown to produce HR increases in rats (Gardiner, 1977; Naranjo, 1985), probably because of changes in anxiety-like responses (Guidotti et al., 2001). We examined whether social isolation produces the same effect in mice, and whether that effect is dependent on the β4 genotype. The HR of β4 +/+ and β4 −/− mice were normal during normal housing conditions (three to five littermates per cage) and under social isolation (one mouse per cage). Animals were monitored for much longer periods of time than in any of the other behavioral tests: 48 hr in the home cage and the single cage, respectively. Over these long periods of time, β4 +/+ mice (on average) had statistically the same heart rate as β4 −/− animals when housed with littermates: 543 ± 18 bpm for β4 +/+ and 451 ± 21 bpm for β4 −/− mice, respectively. Under isolation, the heart rates were 524 ± 16 bpm for β4 +/+ and 567 ± 17 bpm for β4 −/− mice (p > 0.05). However, although during isolation the HR increased for both genotypes (Fig. 5D), the HR of β4 +/+ mice increased significantly less (68 ± 10 bpm) than the HR of β4 −/− mice (115 ± 13 bpm; p < 0.05). The increase in heart rate was calculated by subtracting the HR in the home cage from the HR in β4 −/− mice, the expression pattern of α4 remains the same, which argues against the possibility of subunit compensation (Fig. 6B, E, H, K, N). No specific signal was observed when sections were probed with sense α4 probe (Fig. 6C, F, I, L, O).

Figure 7 shows the expression pattern of β3 mRNA in the brain areas in which β4 was found (Fig. 1). No α4 was found in the mitral cells of the Olf (Fig. 7B, E, H, K, N), excluding the occurrence of subunit compensation in β4 mutant mice, at least at the level of mRNA. Sense controls showed no signal (Fig. 7C, F, I, L, O).

The results indicate that the only areas in which α4, β3, and β4 are significantly coexpressed are the MHb and the IPN. To verify that the expression of β3 and α4 was unchanged in β4 −/− mice, we performed quantitative in situ hybridization. Sections from three to four brains from β4 +/+ and β4 −/− mice were exposed below saturation of signal, and specific areas were chosen for densitometry. Table 1 shows that β4 +/+ and β4 −/− mice express similar amounts of β3 and α4 mRNA in the areas of the brain selected for the study.

Discussion

Our investigation of the anxiety-related behaviors in β4 nAChR-mutant mice was motivated by two observations. First, human smokers often mention the anxiolytic effect of cigarettes, likely arising from nicotine, as a driving force to continue smoking.
(Parrott, 1995; Kassel and Shiffman, 1997; Stewart et al., 1997; Kassel and Unrod, 2000). Second, recent experiments on different nAChR-mutant mice suggested a link between nAChRs and anxiety-related behavior. It has been reported that α4−/− mice behave as though they are more anxious than control mice in the elevated plus, whereas they are normal in a series of basic behavioral tests (Ross et al., 2000). In addition, a gain-of-function mutation in the α4 subunit that produces channels with higher conductance also alters anxiety-like behavior in the mutant mouse. Mice heterozygous for this mutation display increased anxiety-like behavior in the mutant with normal behavior in the light/dark box (Paylor et al., 1998). That report did not explore the elevated-plus maze. The fact that anxiety-related phenotypes are consistently found on the elevated-plus maze and not on open field or light/dark box should not be surprising. Factor analysis of behaviors on anxiety-related experiments in animals has shown that different tests reflect different underlying factors (File, 1992; Belzung and Le Pape, 1994; Ramos et al., 1997). Therefore, the fact that β4−/− and other nicotinic mutants show such a specific phenotype on the elevated-plus and not on other mazes might simply reflect the fact that these tests measure different dimensions of anxiety-like behaviors. Evidence that the measures of anxiety-like behaviors assessed in different tasks may reflect different aspects of anxiety-like responses comes from not only the nAChR-mutant mice but also other animal models (Griebel et al., 1996, 1997, 2000; Andreatini et al., 2001), including mice overexpressing the corticotropin-releasing hormone (van Gaalen et al., 2002). Another possible explanation is that the effects of β4* nAChRs on anxiety appear only under the most stressful situations. For example, we demonstrated that, in wild-type mice, the heart rate increase in the elevated plus is even higher than that in the light/dark box, suggesting that the elevated plus is a more stressful experience. There could be a threshold of stress beyond which β4* nAChRs become important for the behavioral and physiological expression of anxiety. The existence of such a threshold would explain why there are differences in the elevated-plus maze but not in the open field or light/dark box.

Although the lack of the α4 subunit increases anxiety-like behavior on the same test in which absence of β3 or β4 decrease anxiety-like behavior, all three mutations are effective on the same test. This consistent specificity of the testing suggested that α4*, β3*, and β4* nAChRs are involved in the neuronal processing associated with the elevated-plus maze, possibly by influencing the same neuronal areas of the mouse brain. That reasoning led us to investigate where in the brain α4, β3, and β4 are coexpressed. The β4 subunit was only found (at the sensitivity levels of in situ hybridization) in the mitral cells of the Olf, MHB, Pin, and IC. Within the regions in which α4 was expressed, β3 and α4 subunits were detected at significant levels only in MHB and IPN. This finding suggests the possibility that the MHB and its main target, the IPN, could participate in the anxiety phenotype observed in α4, β3, and β4 mutant mice. Indeed, nicotine injections in the dorsal raphe nucleus, which receives innervation from the habenula (Morris et al., 1999; Tomizawa et al., 2001), have been demonstrated to be anxiolytic in the rat (Cheeta et al., 2001). It has also been shown that β4* nAChRs dominate function in MHB neurons (Quick, 1999). In addition, nicotine produces a shift between the excitatory and the inhibitory actions of acetylcholine in the MHB–IPN axis: long-term exposure to low levels of nicotine leaves the inhibitory effect of muscarinic activation unaffected while decreasing the excitatory effect of nicotinic activation (Girod and Role, 2001). This effect is probably mediated by functional inactivation of presynaptic nAChRs. Therefore, it is possible that β4−/− mice have a constitutive lower activation of α4 mRNA

IPN function. Heavy smokers tend to adjust their nicotine intake to obtain constant levels of circulating nicotine during wake hours (Russell, 1989). In that sense, it is tempting to speculate that the lack of $\beta 4^*$ receptors in the MHb could have, by decreasing IPN activity, one of the effects smokers seek: a decrease in anxiety levels.

There is a body of literature in humans that correlates emotions with the activation of the autonomic nervous system. This autonomic activation has been associated with changes in heart rate and blood pressure. There is also evidence in humans that different natural stressors can provoke unique configurations of cardiovascular activity. For example, fear produces a decrease in heart rate (Maschke et al., 2002), but stress-induced anxiety reaction to the maze consistent with less stress. In contrast, the heart rate responses to social isolation seem to be enhanced in mice, which suggests that different stressors may act through different neuronal systems.

Because the $\beta 4$ nAChR subunit is highly expressed in the autonomic nervous system (Rust et al., 1994), altered autonomic function might also influence the differences observed between wild-type and mutant mice. It is possible that the $\beta 4$-null mutation produces an overall dysregulation of the autonomic nervous system, and that heart rate responses could be attenuated or enhanced depending on the stress paradigm used. Additional experimentation will be needed to address autonomic function in $\beta 4$-/- mice in basal conditions and during stress.

In conclusion, we showed that the lack of the nAChR $\beta 4$ subunit alters the behavioral responses to certain anxiety-provoking experimental paradigms. The effect might be dependent on the

**Table 1. Densitometric analysis of $\alpha 4$ and $\beta 3$ mRNA expression in various regions of $\beta 4+/+$ and $\beta 4-/-$ mouse brains**

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Data represent the mean ± SEM for three to four animals and are presented as a percentage of wild-type controls.
MHb–IPN expression of the β4 subunit. The data on α4, β3, and β4 coexpression might help to explain how different nAChR types can exert their influence on anxiety-related behavioral tests.

A major long-term goal of the nicotine addiction field is to understand how the specific nAChR subtypes contribute to the addiction process. Identifying the type and location of the nAChRs involved in anxiety-related behaviors could lead to therapies that aid in smoking cessation and prevention.

References


