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Deficiency of Nicotinic Acetylcholine Receptor β4 Subunit Causes Autonomic Cardiac and Intestinal Dysfunction

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ABSTRACT

Neuronal nicotinic acetylcholine receptors (nAChR) are composed of 12 subunits (α2–α10 and β2–β4), which play the central role in autonomic transmission. β4 subunits are abundantly expressed in autonomic ganglia, forming acetylcholine binding sites and ion channels with α3 or α3 and α5 subunits as pentameric receptors. To investigate the physiological and pharmacological properties of β4 subunits in autonomic ganglia, we measured autonomic functions in knockout mice lacking nAChR subunit β4 ($\beta4^{-/-}$) and wild-type mice. $\beta4^{-/-}$ mice had an attenuated bradycardiac response to high frequency (60 pulse/s) vagal stimulation, as well as an increased sensitivity to hexamethonium blockade at low dose (3 mg/kg) and a reduced ileal contractile response to the nicotinic agonists cytisine, dimethylphenylpiperazinium iodide, nicotine (10 mg/kg each), and epibatidine (0.1 mg/kg). The results suggest that β4 subunits are important components of nAChRs in autonomic ganglia. Deficiency of β4 subunits altered ion channel properties, conductance, and sensitivity and affinity of receptors to agonists and antagonists, affecting ganglionic transmission.

Twelve distinct genes encoding neuronal nicotinic acetylcholine receptor (nAChR) subunits have been identified (α2–α10, β2–β4) (Sargent, 1993; Karlin, 2002). They encode various combinations of neurotransmitter receptors on neurons throughout the central nervous system and in peripheral autonomic nervous system (ANS) ganglia, in which they mediate fast excitatory postsynaptic transmission. In the ANS, dysfunction of neuronal nAChRs is implicated in many diseases. Recently, autoantibodies against ganglionic nAChRs, specifically against α3 subunits, were identified in patients with subacute autonomic neuropathy (Vernino et al., 2000). An autosomal recessive disease, megacystis-microcolon-intestinal hypoperistalsis syndrome is associated with absent gene expression of nAChR α3 subunits (Richardson et al., 2001). Thus, the study of physiological and pharmacological properties of nAChR subunits is important for understanding of the pathophysiology and clinical manifestations of such disorders, for developing therapeutic agents interacting with specific nAChR subtypes, and for expression of cloned nAChR subunits as possible therapeutic agents.

In view of the large number of subunits, there is a tremendous potential for nAChR diversity. However, only 5 of the 12 nAChR subunits (α3, α5, α7, β2, and β4) are known to exist in autonomic ganglia (Mandelzys et al., 1994; Zhou et al., 1998; Devay et al., 1999; Nelson and Lindstrom, 1999; Erkman et al., 2000), and they are not distributed homogenously (Covernton et al., 1994; Klimaschewski et al., 1994; Poth et al., 1997; Sivilotti et al., 1997). Different nAChR subunit combinations are spatially segregated from each other in discrete membrane microregions relative to synapses (Horch and Sargent, 1995; Poth et al., 1997; Shoop et al., 1999). These nAChRs segregate and assemble into two distinct classes. One class, distinguished by its sensitivity to α-bungarotoxin, is composed of homomeric α7 subunits (Cuevas et al., 2000). The second class, composed of α3-containing receptors coassembled with α5, β2, and β4 subunits (as α3β2, α3α5β2, α3β4, or α3α5β4), is believed to constitute the basic receptors that respond by ACh-induced fast excitatory postsynaptic transmission in the ANS (Wang et al., 1996; Nelson et al., 2001). Although β2 and β4 subunits are expressed in autonomic ganglia and can be interchanged to mediate ACh transmission with α3 subunits (Xu et al.,

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; ANS, autonomic nervous system; C_6, hexamethonium; DMPP, dimethylphenylpiperazinium iodide; HR, heart rate; HR_a, heart rate under anesthesia; HR_r, heart rate at rest; HR_s, heart rate after stress induced by shaking the cages; HR_{versus}, heart rate during cervical vagal stimulation; SCG, superior cervical ganglia; WT, wild-type; pps, pulses per second; ANOVA, analysis of variance; bpm, beats per minute; ACh, acetylcholine.

1999b; De Biasi et al., 2000), there are apparent differences between them in numerous properties. For example, during development, mRNA of $\beta4$ subunits occurs in high levels in superior cervical ganglia (SCG) of chick embryos until embryonic day 18, whereas mRNA of $\beta2$ subunits maintains low levels during development (Erkman et al., 2000). Postganglionic nerve transection of the adult rat SCG leads to a decrease in the transcript levels of $\beta4$ subunits in SCG neurons. In contrast, $\beta2$ transcripts remain almost at the control level and may even slightly increase 3 days after postganglionic axotomy (Zhou et al., 1998). β subunits contribute to the nAChR channels and ACh-binding sites, which are believed to be at interfaces between α and β subunits. In a heterologous expression system, the receptors show apparent differences in their activation kinetics, conductance, channel open time, and sensitivity to agonists and antagonists, depending on whether $\alpha3$ subunits combine, respectively, with $\beta2$ or $\beta4$ subunits (Papke and Heinemann, 1991; Covernton et al., 1994; Hussy et al., 1994; Sivilotti et al., 1997; Nelson and Lindstrom, 1999). For example, electrophysiological studies show that the longer burst duration of $\alpha3\beta4$ compared with $\alpha3\beta2$ channels reflects a slower rate of channel closure in combination with slower desensitization, resulting in longer activation during agonist binding to these nAChRs (Nelson and Lindstrom, 1999). In *Xenopus laevis* oocytes, $\beta4$ containing nAChRs (particularly $\alpha3\beta4$) had lower binding affinities for epibatidine (Parker et al., 1998) and higher binding affinities to cytisine (Covernton et al., 1994) compared with $\beta2$ containing homologs (particularly $\alpha3\beta2$). Isolated systems, however, have limitations because of overlapping expression of native receptors and lack of selective agonists and antagonists for each kind of subunit. The physiological and pharmacological properties of $\beta4$ subunits in autonomic ganglia are therefore largely unclear. To investigate the functional role and pharmacological properties of a specific nAChR subunit in the ANS, we examined autonomic functions in mice lacking either $\alpha5$ or $\beta4$ nAChR subunits. Our previous studies with $\alpha5$ knockout ($\alpha5^{-/-}$) mice showed supersensitivity to the ganglionic blocker hexamethonium (C_6) and significantly increased ileal contractile responses to the nicotinic agonists cytisine and epibatidine, but not dimethylphenylpiperazinium iodide (DMPP) and nicotine (Wang et al., 2002). In the present study, we investigated autonomic functions in $\beta4$ subunit knockout animals. The results show impaired heart rate responses during electric stimulation of the cervical vagus, a largely reduced nicotinic agonist-induced contractile response in ilea, and a strikingly increased sensitivity to C_6 blockade.

Materials and Methods

Congenic mice with $\beta4$ subunit deficiency ($\beta4^{-/-}$) that were backcrossed eight generations onto C57BL/6J background and wild-type (WT) control mice were used for these experiments. The mice were housed in group cages, with food and water freely available, in thermostable rooms (21°C). A light-dark schedule of 12:12 h was maintained. The animals used in this study were cared for in accordance to the guidelines published in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Experiments were performed in 2- to 4-month-old $\beta4^{-/-}$ and control mice. Only one kind of experiment was performed in each group of mice. The experiments were performed with the experimenter blinded to the mouse genotype. All mice were genotyped again after finishing the experiments as described previously (Xu et al., 1999b).

Thermoregulation. To investigate thermoregulation in $\beta4^{-/-}$ mice, rectal temperature was measured in an ambient temperature of 21°C and during exposure to a short-term cold stress (6°C). The mice were kept in individual cages, moving freely. The rectal probe (model MF-28; Yokogawa, Tokyo, Japan) was inserted to a depth of 1.5 cm. Rectal temperature was measured three times, and the highest temperature was recorded as the baseline temperature. The baseline rectal temperature was measured at 2:00 PM for 5 days in 21°C. During short-term cold stress, the rectal temperature was measured at half-hour intervals for 4.5 h. The mice were then immediately returned to ambient temperature of 21°C, at which the rectal temperature continued to be measured till its recovery. Body temperature was also measured for 210 min after injection of 30 mg/kg morphine (Adler et al., 1988), in an attempt to cause central rather than environmental hypothermia.

Pupil Size. Injection of morphine (30 mg/kg) induces mydriasis in small animals, such as mice and rats. The effect is primarily caused by a disruption of parasympathetic innervation of the iris (Korczyn et al., 1979; Murray et al., 1983). Pupillary diameters were measured using a binocular microscope (Olympus, Tokyo, Japan) with a magnification of 20X. One of the oculars was fitted with a divided 0.1-mm ruler. All of the measurements were done while the animals were nonanesthetized and held gently under the microscope in ambient temperature of 21°C. Total handling time was less than 5 s. Both pupils of each animal were always measured, and the average value was recorded. (−)-Morphine hydrochloride was injected subcutaneously at doses of 30 mg/kg to groups of mice (Korczyn and Maor, 1982). Pupillary diameter was measured before as well as 15, 30, 60, 90, 120, 150, and 180 min after drug administration.

Regulation of Heart Rate. To assess cardiac autonomic function, an ECG was performed using stainless-steel needle electrodes inserted subcutaneously on the backs of mice with use of an ECG machine (model 7P6B; Grass Instruments, Quincy, MA) having a paper speed of 30 mm/s. Heart rate (HR) was identified by visually inspecting *R*-waves in the ECG, and beat-to-beat interval was defined as the duration between successive *R*-waves in the ECG.

For testing HR at rest (HR_r) in awake mice, each mouse was transferred to a cage (30 × 22 × 13 cm) in which it could move freely. The cages were placed in a stable temperature (21°C) within a noise-free environment. If the mice seemed distressed by the placement of the recording electrodes, these were changed so that the mice seemed comfortable. Heart rates were measured at 30-min intervals for 4 h, and the lowest HR was recorded as HR_r. After establishing the HR_r, the stressful stimulation was applied the same groups of mice by strongly shaking the cage. The stressed heart rate (HR_s) was measured immediately after strongly shaking the cage for 1 min. In the separate experiment, the HR changes in groups of awake mice were recorded at 30-min intervals for 270 min during cold exposure.

To observe vagal cardiac parasympathetic transmission, under pentobarbital (30 mg/kg, i.p.) anesthesia, the right cervical vagus was exposed in the neck and placed on silver electrodes, connected with a stimulator (model SD9; Grass Instruments). For nerve stimulation, voltage was set at 2 V, and square wave pulses were delivered (duration, 0.2 ms). The stimulation frequency was gradually increased (5, 10, 20, 40, 60, and 100 pulses per second [pps]). Each train of vagal stimuli was given for 10 s with 2- to 5-min intervals. HR was recorded before, during the period of vagal stimulation (HR_{versus}), immediately after, and 30, 60, 90, and 120 s after each vagal stimulation subsequently until HR recovery. The effect of vagal stimulation on HR was defined as $(HR_{versus} - HR_a) \times 100/HR_a$, where HR_a indicates the HR recorded before each vagal stimulation with or without injection of C_6 (see below) under anesthesia. To maintain the depth of anesthesia, additional doses of pentobarbital (10 mg/kg) were administered at intervals of ~1 h.

To observe the effects of ganglionic blockade on vagal stimulation, C_6 (Sigma, St. Louis, MO) was injected intraperitoneally at 3, 15, and

30 mg/kg to groups of anesthetized mice. HR_a was measured 10 min after injection of each dose of C_6, repeating the vagal stimulations and measurement of HR_{versus} as described above.

Ileal Contractile Response to Nicotinic Agonists. To prepare the ilea, the mice were killed by cervical dislocation. The abdomens were opened, and the ileum was carefully removed immediately and kept in Krebs' solution with bubbling oxygen containing 5% CO_2. Four distal segments of ileum from the same animal, each 2 to 2.5 cm long, were cleaned from adhering tissue and were used freshly. Preparations were suspended with silk thread number 3 and attached to isometric force transducer FTO3C, which was connected to a polygraph (model 7B; Grass Instruments). The amplitude of response was calibrated so that each gram of tension equaled 3 cm in amplitude. Before drug administration, the ileum segments were allowed to equilibrate for at least 1 h at a resting tension of 1 g in a 10-ml organ bath filled with Krebs' solution that was kept at 37°C and constantly aerated with bubbling oxygen containing 5% CO_2; the Krebs' solution was replaced every 20 min.

The optimal concentrations to elicit contractile responses were determined in preliminary experiments (Wang et al., 2002). The nonspecific muscarinic agonist bethanechol and the nicotinic agonists cytisine, DMPP, and nicotine itself were used at concentrations of 0.1, 1, 3, 10, 30, and 100 μM. The nicotinic agonist epibatidine was applied at concentrations of 0.01, 0.1, 0.3, 1, 3, and 10 μM (all drugs were from Sigma). Log concentration-response curves were drawn in WT mice. For each drug, experiments were performed on six preparations from different mice with a 30-min interval between subsequent drug administrations. The results showed consistent concentration-response curves for these drugs. Maximal responses were induced by bethanechol at concentrations of 10 to 30 μM; by cytisine, DMPP, and nicotine at doses of 10 to 30 μM; and by epibatidine at doses of 0.1 to 0.3 μM. The responses to the four nicotinic agonists were independent of the order of administration. For subsequent studies, each agonist was used at a single concentration (10 μM cytisine, 10 μM DMPP, 10 μM nicotine, and 0.1 μM epibatidine), repeated three times in the same preparation. These doses evoked efficient and consistent responses, and no tachyphylaxis was observed.

To characterize the contractile responses to different nicotinic agonists, bethanechol was used as a reference agent that was applied in progressively increasing doses to give a final concentration of 1 to 10 μM. The agonists were injected as follows: bethanechol (1, 3, and 10 μM); cytisine (10 μM), DMPP (10 μM), epibatidine (0.1 μM), and nicotine (10 μM) at 40-min intervals with four washouts after each administration of drug. At the end of the test, bethanechol 3 μM was again applied to ensure that the preparations were still viable. Four preparations were examined from each mouse.

The contractile responses to ganglionic agonists were calculated as a percentage of the response to 10 μM bethanechol in the same preparation. The data of four preparations from the same mouse were averaged. One-way ANOVA with Dunnett's multiple comparison was used for comparing the responses of $\beta4^{-/-}$ mice and WT control mice.

Results

Under physiological unchallenged conditions, there was marked similarity between $\beta4^{-/-}$ and WT mice. All the $\beta4^{-/-}$ mice grew to normal size showing no obvious physical, neurological, or autonomic deficit. No difference in body weight was found between WT and $\beta4^{-/-}$ mice.

The rectal temperatures of the $\beta4^{-/-}$ ($n = 5$) and WT mice ($n = 26$) in ambient temperature of 21°C were 38.2 ± 0.2 and 38.4 ± 0.3°C (mean ± S.D.), respectively. During exposure to cold stress, the rectal temperature of the $\beta4^{-/-}$ ($n = 5$) and WT ($n = 17$) mice decreased gradually to 28.8 ± 4.3 and 28.6 ± 5.4°C, respectively, after 270 min, with similar recovery after being returned to an ambient temperature of 21°C.

After injection of 30 mg/kg morphine at 21°C ambient temperature, hypothermia developed within 30 min, reaching a nadir of 33.9 ± 1.3 and 34.4 ± 0.8°C in $\beta4^{-/-}$ ($n = 5$) and WT mice ($n = 16$), respectively. The rectal temperature recovered to baseline levels 240 min after injection of the drug. There was no difference in these measures between the two strains of mice.

All of the mice showed normal pupillary size. The mean pupil diameters at baseline were 0.44 ± 0.12 and 0.49 ± 0.9 mm in $\beta4^{-/-}$ ($n = 10$) and WT ($n = 25$) mice, respectively. Administration of (−)-morphine hydrochloride (30 mg/kg) caused mydriasis in both $\beta4^{-/-}$ ($n = 5$) and WT ($n = 24$) mice. However, the pupillary size changes after morphine in $\beta4^{-/-}$ mice were similar to those in WT mice (1.6 ± 0.3 and 1.6 ± 0.5 mm, respectively, reaching maximal response in $\beta4^{-/-}$ and WT mice 90 min after morphine administration).

The HR were similar between the $\beta4^{-/-}$ and WT mice at rest, when stressed by cage shaking, during exposure to cold stress, or when anesthetized (Fig. 1). In awake mice, the HR_r values were 478 ± 84 ($n = 5$) and 421 ± 82 ($n = 23$) beats per min (bpm), respectively; stressful stimulation by cage shaking induced extreme tachycardia, reaching 728 ± 18 ($n = 5$) and 723 ± 17 ($n = 23$) bpm, respectively (significantly higher than in resting, $p < 0.001$, paired t-test), in $\beta4^{-/-}$ and WT mice. Exposure to cold stress also induced extreme tachycardia, reaching 705 ± 13 in $\beta4^{-/-}$ ($n = 5$) and 710 ± 27 bpm in WT ($n = 13$) mice. Figure 1 illustrates the HR findings 30 min after exposure to 6°C in $\beta4^{-/-}$ and WT mice, which were similar to the effects of strongly shaking the cages and not significantly different in the two strains. The HR under pentobarbital anesthesia (HR_a) was also similar in $\beta4^{-/-}$ ($n = 7$) and WT ($n = 9$) mice: 423 ± 113 and 420 ± 92 bpm, respectively (Fig. 1).

Attenuation of Cardiac Vagal ACh Transmission in $\beta4^{-/-}$ Mice. Vagal stimulation caused a frequency-dependent bradycardia in both mutant ($n = 7$) and WT ($n = 9$) mice and finally cardiac arrest in all WT mice (Fig. 2A). At stim-

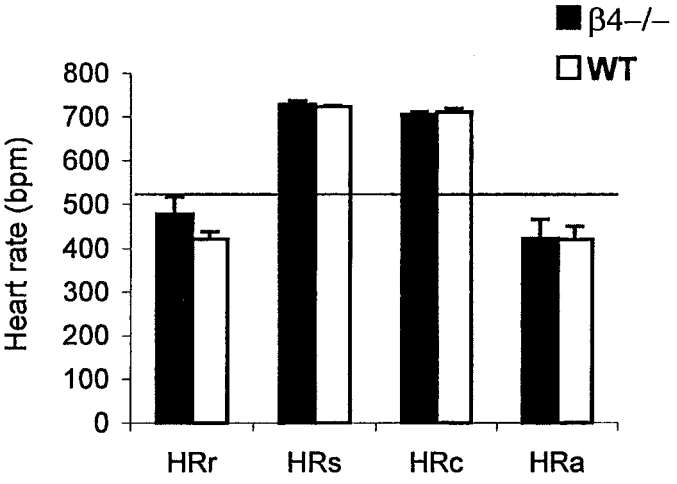

Fig. 1. Heart rate (in bpm) of $\beta4^{-/-}$ and WT mice. HR_r, the HR in awake ($\beta4^{-/-}$, $n = 5$ and WT, $n = 23$) mice at rest; HR_s, the HR after stressful stimulation induced by strongly shaking the cages; HR_c, the HR 30 min after exposure to cold stress in $\beta4^{-/-}$ ($n = 5$) and WT ($n = 13$) mice; HR_a, resting HR of $\beta4^{-/-}$ ($n = 5$) and WT ($n = 9$) mice under anesthesia. The HR did not show significant differences between $\beta4^{-/-}$ and WT mice. Vertical bars indicate S.E. of the mean.

ulation rates of 5 and 20 pps, the HR_{versus} of $\beta4^{-/-}$ mice was approximately 10 and 44% lower than at baseline, respectively, whereas in WT mice, the HR_{versus} values were, respectively, 5% and 60% lower than their baseline. However, at high-frequency stimulation, the bradycardic effect was significantly attenuated in $\beta4^{-/-}$ mice ($p < 0.05$ and $p < 0.01$ at 40 and 60 pps, respectively, one-way ANOVA with Dunnett's multiple comparisons) (Fig. 2A). Stimulation at 60 pps caused cardiac arrest in all nine WT mice but not in any of the $\beta4^{-/-}$ mice ($\chi^2 = 5.47$, $p < 0.01$) (Fig. 2A). Thus, deficiency of $\beta4$ subunits influences parasympathetic transmission to the heart.

Supersensitivity of Hexamethonium on Blockade of the Cardiac Responses to Vagal Stimulation. Ganglionic blockade using different concentrations of C_6 failed to alter the resting HR in both mutant and WT control mice (data not shown), probably because of equal sympathetic and parasympathetic contributions to HR_a. The effects of ganglionic blockade on HR responses to vagal stimulation are shown in Fig. 2, B, C, and D. The response to vagal stimulation was completely abolished by 30 mg/kg C_6 (Fig. 2D) in both $\beta4^{-/-}$ and WT mice, but lower concentrations showed a differential sensitivity of $\beta4^{-/-}$ mice to C_6. For example, whereas 3 mg/kg produced only a slight depression of the vagal response in WT mice, a nearly complete abolition of the response occurred in $\beta4^{-/-}$ mice (mean ± S.D. HRs were 15.1 ± 14.4% and 76.9 ± 27.0% below their baselines, respectively, in $\beta4^{-/-}$ and WT mice at 60 pps vagal stimulation; $p < 0.01$, one-way ANOVA with Dunnett's multiple comparisons) (Fig. 2B).

Reduced Ganglionic Agonist-Induced Ileal Transmission in $\beta4^{-/-}$ Mice. Preliminary experiments of ileal contractions in vitro revealed that final concentrations of 10 μM for cytisine, DMPP, and nicotine and 0.1 μM for epibatidine induced efficient submaximal responses. There was no tachyphylaxis in either $\beta4^{-/-}$ or WT mice.

Bethanechol induced a dose-dependent contractile response in ilea. There was no difference in the mean magnitude of contraction between mutant ($n = 5$) and WT ($n = 20$) mice at different doses ($p > 0.05$, t-test) (Table 1).

The nicotinic agonist-induced responses normalized to bethanechol (10 μM) in ilea of $\beta4^{-/-}$ ($n = 5$) and WT ($n = 20$) mice are illustrated in Fig. 3. In $\beta4^{-/-}$ mice, the responses to all four nicotinic agonists were reduced compared with the response obtained in WT mice. The percentages of responses relative to bethanechol in comparison between $\beta4^{-/-}$ and WT mice were 38.0 ± 6.8 and 75.4 ± 6.0% (mean ± S.D.; $p < 0.01$, one-way ANOVA with Dunnett's multiple comparisons) to 10 μM cytisine, 62.1 ± 9.9 and 72.0 ± 8.3% ($p < 0.05$) to 10 μM DMPP, 62.6 ± 14.8 and 82.6 ± 7.0% ($p < 0.01$) to 0.01 μM epibatidine, and 50.3 ± 14.5 and 75.9 ± 8.2% ($p < 0.01$) to 10 μM nicotine, respectively. To more closely understand these changes, the dose-response to cytisine was examined separately in five $\beta4^{-/-}$ and four WT mice. The results showed a right-shifted response curve in $\beta4^{-/-}$ mice. The maximal response to cytisine at a concentration of 10 μM was strikingly reduced; the results normalized to bethanechol response (10 μM) were 33.9 ± 17.2% in $\beta4^{-/-}$ and 76.4 ± 6.4% in WT mice (Fig. 4).

Discussion

This study and a previous one describing mice lacking $\alpha5$ subunits (Wang et al., 2002) show that neither $\alpha5$ nor $\beta4$ subunits are obligatory for normal development and survival. The knockout mice seem and behave normally, and their autonomic responses, e.g., tachycardia during stress, or the pupillary and temperature responses to morphine are apparently intact. Because it is well known that both subunits are integral parts of normal ganglionic nicotinic receptors (Mandelzys et al., 1994; Poth et al., 1997; Nelson and Lindstrom, 1999; Erkman et al., 2000), their lack must be well compensated for, presumably by the formation of other combinations (e.g., $\beta2$ replacing $\beta4$ subunits). However, these new receptors respond differently under some conditions from wild-type $\beta4$-containing receptors. For example, there is marked supersensitivity to C_6 in both $\alpha5^{-/-}$ and $\beta4^{-/-}$. Other similarities between the two knockout strains are the attenuation of heart rate responses to vagal transmission induced by direct cervical vagal stimulation. However, $\alpha5^{-/-}$ and $\beta4^{-/-}$ mice are also different from each other. Ileal contractile responses were greatly decreased to all four nicotinic agonists in $\beta4^{-/-}$ mice, mostly to cytisine and nicotine, but significantly increased to certain agonists (cytisine and epibatidine,

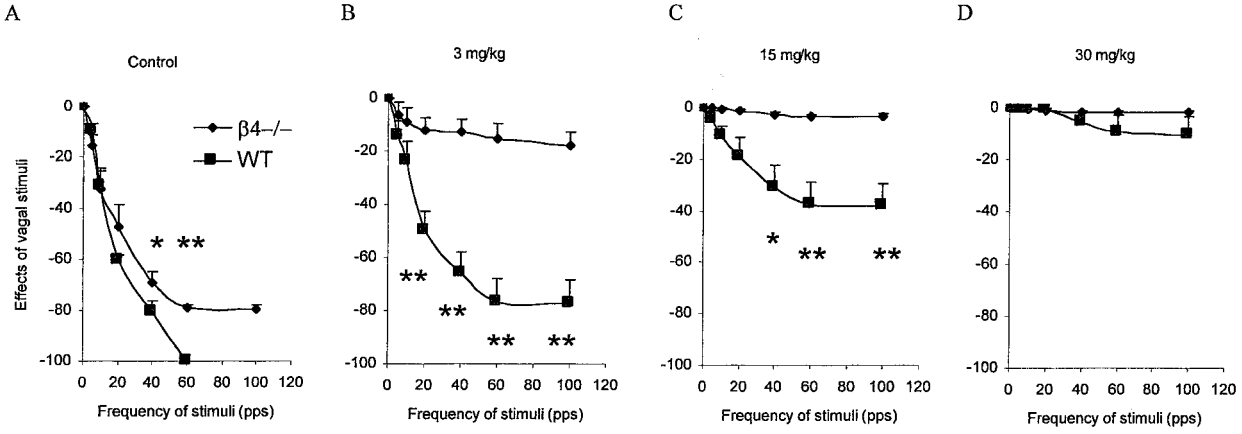

Fig. 2. Effects of vagal stimulation on HR and its blockade by C_6 in $\beta4^{-/-}$ ($n = 7$) and WT ($n = 9$) mice. A, effects of vagal stimulation on HR at baseline. B, blockade by 3 mg/kg of C_6. C, blockade by 15 mg/kg of C_6. D, blockade by 30 mg/kg of C_6. The effects of vagal stimulation on HR are presented as the percentage $(HR_{versus} - HR_a) \times 100/HR_a$. HR_{versus}, HR during vagal stimulation; HR_a, resting HR under anesthesia before each vagal stimulation. Each vagal stimulation was given for 10 s at 5-min intervals (2 V, 0.2 ms duration). *, $p < 0.05$, **, $p < 0.01$, one-way ANOVA with Dunnett's multiple comparisons in $\beta4^{-/-}$ mice compared with WT mice. The vertical bars indicate S.E. of the mean.

but not to DMPP and nicotine) in $\alpha5^{-/-}$ mice; the attenuation of heart rate responses to vagal stimulation in $\beta4^{-/-}$ mice was of a higher intensity than that in $\alpha5^{-/-}$ mice. Cardiac arrest developed in three of seven $\alpha5^{-/-}$ mice (mean HR was 84.5 ± 16.0% lower than their baseline) but not in any $\beta4^{-/-}$ mice (mean HR was 79.3 ± 4.0% lower than their baseline) at 60 pps vagal stimuli.

Modulation of ACh transmission via autonomic ganglia to different tissues, which is associated with intensity of signals needed by the end neurons (Devay et al., 1999), probably underlies the diversity of nAChRs in gene expression, subunit composition distribution, and functional properties. Currently, the distribution of subunits in different tissues is unknown. Nevertheless, $\alpha3$ subunits seem to be the predominant type of α subunits, as evidenced in a study in mice lacking $\alpha3$ subunits who suffer severe autonomic dysfunctions and die within 1 week after birth (Xu et al., 1999a). In vitro studies have shown that, to be functional, $\alpha3$ subunits must combine with β subunits, which construct the ACh binding sites and ion channels with α subunits. Without β subunits, the $\alpha3$-containing receptors would lose a "structural supporter" (Sargent, 1993), suggesting the importance of β subunits when coassembled with $\alpha3$ subunits.

$\beta4$ subunits are abundantly expressed in the ANS (Mandelzys et al., 1994; Poth et al., 1997; Zhou et al., 1998; Devay et al., 1999; Erkman et al., 2000). The normality of $\beta4^{-/-}$ mice in the regulation of certain autonomic functions under physiological conditions and after environmental manipulation may therefore reflect redundancy of gene expression. In $\beta4^{-/-}$ mice, the physiologically functional receptors in the ANS possibly are replaced by other β subunits, presumably $\beta2$. It is unclear whether there is reduction in the total number of ganglionic nicotinic receptors in knockout $\beta4^{-/-}$. If this is the case, the total number of missed receptors does not reach a critical level to block the transmission of autonomic signals to these target tissues under physiological conditions. However, under more demanding conditions, either by maximal nerve stimulation or through drug manipulations, the existing receptors may be insufficient to mediate fully the cholinergic transmission to cardiac and intestinal end organs. Alternatively, in the case of α/β compositions, the number of nicotinic receptors remains intact, but instead of the normal heterogeneity ($\alpha3\beta2$ and $\alpha3\beta4$), they now all consist of $\alpha3\beta2$ receptors, which differ in their pharmacological responses from native $\alpha3\beta4$ receptors. The striking pharmacological changes observed—supersensitivity to C_6 and great reduction of the responses to agonists, e.g., on the ileal contractile response to cytisine in $\beta4^{-/-}$ mice—could be explained by both mechanisms: either a decreased total number of nAChRs or an alteration in their affinity to agonists/antagonists.

$\alpha5$ subunits are coexpressed with approximately 70 to 80% of $\alpha3$-containing receptors in the chick ciliary ganglion neurons (Conroy and Berg, 1995) and human neuroblastoma cells (Wang et al., 1996), approximately 30% of rat cardiac parasympathetic neurons (Poth et al., 1997), and nearly all rat SCG neurons (Skok et al., 1999). It is well known that $\alpha5$ subunits participate in the formation of ion channels but do not form ACh binding sites (Ramirez-Latorre et al., 1996; Wang et al., 1996; Gerzanich et al., 1998; Nelson and Lindstrom, 1999). In our previous work on $\alpha5^{-/-}$ mice, we observed increased responses to both agonists and antagonists, suggesting pharmacological modulation effects of $\alpha5$ subunits in receptors. These effects of $\alpha5$ subunits altering pharmacological and physiological properties may be caused by their structural participation in functional receptor complexes and by the contributions of $\alpha5$ subunit M2 segment to the lining of the ion channels. Although they are not directly involved in the agonist binding sites, $\alpha5$ subunits may be responsible for the changes in the overall structure of the receptors, which influences their ability to make the concerted changes in subunit orientation needed for channel opening (Ramirez-Latorre et al., 1996; Wang et al., 1996; Gerzanich et al., 1998; Nelson and Lindstrom, 1999), resulting in the alteration of the EC_{50} or efficacy of some drugs to the receptors. In the $\beta4^{-/-}$ mice, however, the reduction of sensitivity to agonists such as those in this work compared with increased sensitivity to antagonists, as shown here, may imply a different mechanism of receptor activation or inhibition by agonists or antagonists. Previous studies suggest that both α and β subunits contribute to pharmacological properties of nAChRs (Tomaselli et al., 1991; Covernton et al., 1994; Hussy et al., 1994; Sivilotti et al., 1997; Parker et al., 1998; Webster et al., 1999). $\beta4$ subunits may act directly through their association with the α subunit to alter the agonist binding sites. The $\beta4$ subunits might also provide or modify allosteric modulatory sites or channel-blocking sites at which ligands could act (Luetje and Patrick, 1991). $\beta4$ subunits largely influence the agonist affinity and sensitivity in native receptors. Previous studies showed the differences of agonist and antagonist properties between $\beta2$ and $\beta4$ subunit-containing receptors. For example, in oocytes, the sensitivity to cytisine in $\beta4$-containing receptors (e.g., $\alpha3\beta4$) was greater than that of $\beta2$-containing receptors (e.g., $\alpha3\beta2$). In contrast, the sensitivity to epibatidine or DMPP in $\beta2$-containing receptors was greater than that in $\beta4$-containing receptors (Luetje and Patrick, 1991; Covernton et al., 1994; Parker et al., 1998). As noted above, we observed reduced ileal responses to all four agonists in the $\beta4^{-/-}$ mice, particularly to cytisine. The results provide evidence that the native $\beta4$ subunits selectively influence nAChR affinities and sensitivities to agonists.

Mice with double knockout of the genes encoding $\beta2$ and $\beta4$ ($\beta2^{-/-}\beta4^{-/-}$) show a severe phenotype reminiscent of the mice lacking $\alpha3$ subunits, consisting of impaired growth and increased postnatal mortality, mydriasis, no pupillary response to light, megabladder, etc. (Xu et al., 1999b). However, so far, no mutation has been identified in the $\beta4$ subunit

TABLE 1

Amplitude of contractile responses to bethanechol in $\beta\ 4^{-/-}$ ($n = 5$) and wild-type ($n = 20$) mice

Amplitudes of responses (grams) are given as mean ± S.D. There was no difference between $\beta\ 4^{-/-}$ and WT mice in different doses ($p > 0.05$, t test).

Dose	1 μM	3 μM	10 μM	3 μM[a]
$\beta4^{-/-}$ mice	0.52 ± 0.10	0.70 ± 0.08	1.05 ± 0.04	0.75 ± 0.11
WT mice	0.50 ± 0.12	0.69 ± 0.14	0.92 ± 0.16	0.69 ± 0.14

[a] Repeated administration of bethanechol at the end of the experiments.

gene of patients with megacystis-microcolon-intestinal hypoperistalsis syndrome (Lev-Lehman et al., 2001), the manifestations of which are similar to those observed in the $\alpha3^{-/-}$ mice (Xu et al., 1999a). Furthermore, mice with single knockout of the genes encoding $\beta2$ or $\beta4$ do not exhibit a similar phenotype. The autonomic dysfunctions shown in $\beta2^{-/-}\beta4^{-/-}$ mice (Xu et al., 1999b) are probably caused by a lack of structural support of $\alpha3$-containing receptors. Thus, $\alpha3$-containing receptors can be functional if coexpressed with either $\beta2$ or $\beta4$, but they lose ACh binding sites and cannot form effective ion channels when both β subunits are lost. Studies in $\beta2^{-/-}\beta4^{-/-}$ and $\beta4^{-/-}$ mice suggest that $\beta2$ subunits could play a compensatory role to maintain normal physiological function in the ANS when $\beta4$ is absent (De Biasi et al., 2000). Theoretically considering the pharmacological properties of $\alpha3\beta2$ AChRs shown in in vitro studies, in the ANS, $\beta2$ subunits should be an integral part of autonomic nAChRs. However, in $\beta2^{-/-}$ mice, nicotine-induced currents were not altered in SCG neurons, and bladder strips responded well to nicotine (Xu et al., 1999b). Similarly, no significant alterations were observed in the ileal contractile responses to all four agonists (N. Wang, R. Rabinowitz, J. Chapman, A. Orr-Urteger, and A. D. Korczyn, unpublished observations). We therefore suggest that the decreased responses to agonists and increased responses to antagonists of $\beta4^{-/-}$ mice may reflect the loss of agonist binding sites and altered channel conductance. The remaining receptors were not sufficient to open all channels by agonists, whereas for competitive antagonists, the remaining binding sites of receptors were easily saturated. Thus, $\beta4$ subunits may be more abundantly expressed in ganglia, and $\alpha3\beta4$ combinations are the critical AChRs mediating fast ganglionic transmission.

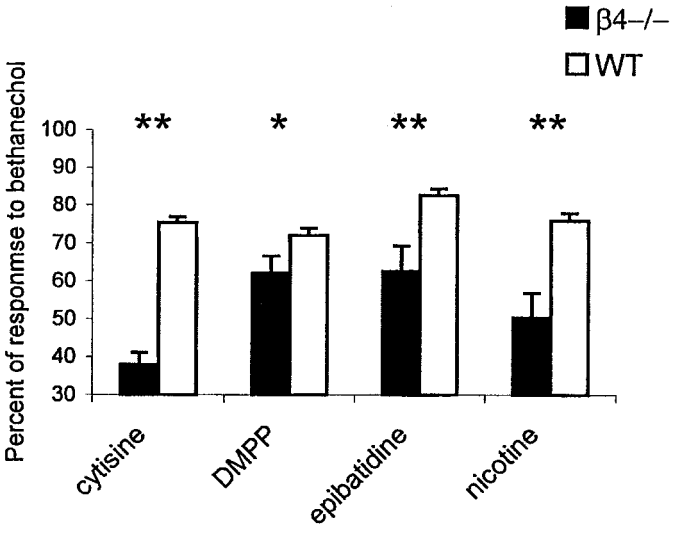

Fig. 3. Ileal contractile responses to nicotinic agonists in $\beta4^{-/-}$ ($n = 5$) and WT ($n = 20$) mice. Ileal contractile responses are represented as a percentage of response to bethanechol at a dose of 10 μM. The nicotinic agonists were used at doses of 10 μM for cytisine, DMPP, and nicotine and 0.1 μM for epibatidine. *, $p < 0.05$, **, $p < 0.01$, one-way ANOVA with Dunnett's multiple comparisons in $\beta4^{-/-}$ mice compared with WT mice. Vertical bars indicate S.E. of the mean.

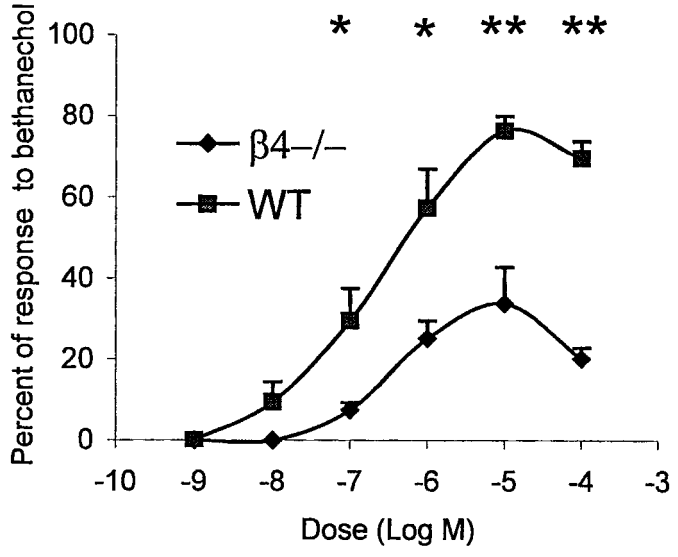

Fig. 4. Concentration-dependent contractile response curves to cytisine in ilea. The responses are represented as the percentage of response to bethanechol (10 μM). *, $p < 0.05$, **, $p < 0.01$, one-way ANOVA with Dunnett's multiple comparisons in $\beta4^{-/-}$ mice compared with WT mice. Vertical bars indicate S.E. of mean.

It is necessary to measure total number of $\alpha3$ and $\alpha3\beta2$ AChRs, as well as the mRNA expression of $\alpha3$, $\alpha5$, and $\beta2$ subunits, in ganglia of WT and $\beta4^{-/-}$ mice to determine whether the number of $\alpha3\beta2$ AChRs remains constant in the $\beta4^{-/-}$ mice or is up-regulated to compensate for the loss of $\alpha3\beta4$ AChRs and determine whether $\alpha5$ subunits are important for targeting or stabilizing the AChRs at the synapse. This may allow the determination of whether the increased sensitivity to inhibition by C_6 reflected simply a decrease in the number of AChRs or an increased susceptibility to channel block in AChRs lacking $\alpha5$ subunits. We plan to perform these experiments in the near future.

Taken together, the results obtained in this study demonstrating an impaired heart rate response to cervical vagal stimulation, strikingly increased sensitivity to C_6 in blockade of the effect of vagal stimulation, and greatly reduced ileal contractile responses to all four nicotinic agonists in $\beta4^{-/-}$ mice suggest that $\beta4$ subunits are critical in the ANS to construct functional receptors with $\alpha3$ subunits. The deficiency of $\beta4$ subunits adversely affects autonomic transmission, probably by the loss of ACh binding sites in ganglia and by the alteration of properties of ion channels and affinities of some drugs.

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