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Regulation of muscarinic acetylcholine receptor function in acetylcholinesterase knockout mice ☆

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Abstract

Acetylcholinesterase (AChE) hydrolyzes acetylcholine to terminate cholinergic neurotransmission. Overstimulation of cholinergic receptors by excess acetylcholine is known to be lethal. However, AChE knockout mice live to adulthood, although they have weak muscles, do not eat solid food, and die early from seizures. We wanted to know what compensatory factors allowed these mice to survive. We had previously shown that their butyrylcholinesterase activity was normal and had not increased. In this report, we tested the hypothesis that AChE −/− mice adapted to the absence of AChE by downregulating cholinergic receptors. Receptor downregulation is expected to reduce sensitivity to agonists and to increase sensitivity to antagonists. Physiological response to the muscarinic agonists, oxotremorine (OXO) and pilocarpine, showed that AChE −/− mice were resistant to OXO-induced hypothermia, tremor, salivation, and analgesia, and to pilocarpine-induced seizures. AChE+/− mice had an intermediate response. The muscarinic receptor binding sites measured with [^{3}H]quinuclinyl benzilate, as well as the protein levels of M1, M2, and M4 receptors measured with specific antibodies on Western blots, were reduced to be approximately 50% in AChE −/− brain. However, mRNA levels for muscarinic receptors were unchanged. These results indicate that one adaptation to the absence of AChE is downregulation of muscarinic receptors, thus reducing response to cholinergic stimulation.

Keywords: Acetylcholinesterase; Knockout mice; Muscarinic receptor; Oxotremorine; Pilocarpine; Seizures

1. Introduction

Acetylcholinesterase (AChE; EC 3.1.1.7) has a crucial role in cholinergic neurotransmission, hydrolyzing the neurotransmitter acetylcholine to terminate nerve impulse transmission. Acute AChE inhibition by nerve agents or organophosphorus pesticides may be lethal. No case of total AChE deficiency in the human population has ever been reported, leading to the speculation that inactive AChE mutations may be embryonically lethal. Therefore, it was a surprise to find out that AChE knockout mice without AChE activity survived to adulthood (Duysen et al., 2002; Li et al., 2000; Xie et al., 2000).

The absence of AChE activity most likely results in abnormally high levels of acetylcholine in the cholinergic synapses. AChE −/− mice have motor tremor and pinpoint pupils, signs indicative of the presence of excess acetylcholine. Although acute overstimulation of acetylcholine receptors by excess acetylcholine is known to be lethal (Mileson et al., 1998), surprisingly, AChE −/− mice live to adulthood when maintained on a liquid diet (Duysen et al., 2002) and many have survived for nearly 2 years. Death occurs from seizures. The cholinergic marker, choline acetyl transferase, showed normal anatomical distribution in AChE −/− brain, indicating that the absence of AChE does not affect the development of cholinergic

Abbreviations: AChE, acetylcholinesterase; BChE, butyrylcholinesterase; mAChR, muscarinic acetylcholine receptor; ERK2, extracellular signal-regulated kinase 2; OXO, oxotremorine; [^{3}H]QNB, [^{3}H]quinuclinyl benzilate; M1–M4, muscarinic receptors 1–4.

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pathways in the central nervous system (Mesulam et al., 2002).

The mice live despite the complete absence of AChE, suggesting that they have adapted to the absence of AChE, or that a backup enzyme substitutes for the missing AChE activity. Butyrylcholinesterase (BChE; EC 3.1.1.8) did not undergo compensatory increases in the AChE −/− mouse (Li et al., 2000). However, inhibition of BChE was lethal to AChE −/− mice (Xie et al., 2000). It is possible that the normal level of BChE plays a role in keeping AChE −/− mice alive by hydrolyzing acetylcholine (Li et al., 2000; Mesulam et al., 2002). The present study examined whether AChE knockout mice have adapted to the absence of AChE by downregulating muscarinic receptors. Several other adaptation mechanisms are possible, but have not yet been tested. For example, nicotinic receptors may be downregulated, and there may be changes in rates of acetylcholine synthesis and release.

The action of acetylcholine is mediated by nicotinic and muscarinic acetylcholine receptors (mAChRs). The mAChR family belongs to the G protein-coupled receptor gene superfamily and consists of five subtypes (M1–M5), which regulate numerous fundamental physiological processes, including motor control, temperature regulation, pain perception, learning, and memory (Messer et al., 1990; van der Zee and Luiten, 1999). In the peripheral nervous system, mAChR mediates smooth muscle contraction, glandular secretion, and cardiac function (Caulfield and Birdsall, 1998; Eglen, 2001). Acetylcholine is known to regulate the level of both nicotinic and muscarinic receptors in many systems. Chronic agonist stimulation of mAChR results in desensitization and downregulation of mAChR (Honda et al., 1995). Downregulation of muscarinic receptors is generally accompanied by decreased sensitivity to muscarinic agonists and increased sensitivity to muscarinic antagonists. Conversely, upregulation of mAChR is found after chronic administration of muscarinic antagonists (Ben-Barak and Dudai, 1980). Therefore, we tested whether mice without AChE have adapted to the absence of AChE by downregulation of cholinergic receptors. We found that AChE knockout mice have a significant reduction in the number and responsiveness of muscarinic receptors, indicating that the chronic absence of AChE produces marked changes in the cholinergic system. Downregulation of muscarinic receptors is one of the mechanisms that explains the survival of AChE −/− mice.

2. Materials and methods

2.1. Drugs

Oxotremorine (OXO) sesquifumarate salt, pilocarpine hydrochloride, and atropine sulfate salt were from Sigma (St. Louis, MO). OXO and atropine were dissolved in sterile water. Pilocarpine was dissolved in phosphate-buffered saline.

2.2. Pharmacological and behavioral studies

Animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institutes of Health. The AChE −/− mouse colony is maintained by breeding heterozygotes (Duysen et al., 2002; Xie et al., 2000). Two- to 4-month-old mice of both sexes were used. No significant differences in responses were found between male and female animals.

Mice from each genotype were injected subcutaneously with 1 or 0.2 mg/kg OXO, a nonselective muscarinic receptor agonist. Body temperature, salivation, and tremor were assessed immediately before and after injection. Body temperature was measured with a surface thermometer (Physitemp Instruments, Clifton, NJ). Salivation was scored as: 1 = *no salivation*; 2 = *moisture on face only*; and 3 = *moisture on the face and chest*. Tremor was scored on a scale of 1 = *no tremor*; 2 = *intermittent head and body tremor*; and 3 = *nearly continuous whole body tremor*. The data were expressed as a percent of the maximum possible score.

The antinociceptive effect of OXO was investigated using tail-flick and hot-plate tests. The tail-flick test was carried out by immersing approximately 1 cm of the tip of the mouse tail in a 55 °C water bath. The response latency for the animal to withdraw its tail was measured immediately before (baseline) and 30 min after drug injection. A 10-s maximum cut-off was imposed to prevent tissue damage. The hot-plate test was performed by using an electronically controlled hot plate set to 55 °C. The animal was placed on the hot plate and the time required for the animal to jump or lick its paws was measured before and 30 min after OXO injection. The cut-off time was 30 s.

Seizures were induced by injecting freshly dissolved pilocarpine at a dose of 200 mg/kg ip. Pilocarpine solutions, prepared the day before and stored at 4 °C, gave a decreased response. The mice were observed for 45 min for seizures after drug injection. Salivation and lacrimation were scored on the same scale as described above. Gastrointestinal mobility was observed as the amount of defecation.

To determine the LD_{50} of atropine in AChE+/+, +/−, and −/− mice, the "up-and-down" procedure for acute toxicity testing was employed (Bruce, 1987). This method requires the use of fewer animals relative to other methods of estimating the LD_{50}. Adult female mice (56–91 days) were injected intraperitonally with atropine. AChE+/+ ($n = 24$), +/− ($n = 27$), and −/− ($n = 15$) animals were monitored continuously throughout the treatment period. Moribund animals were euthanized immediately.

The LD_{50} values were estimated by a probit regression analysis of the data, using the PROBIT procedure of the SAS system.

2.3. Ligand binding assay

Mouse whole brains from wild type and nullizygous mice were flash frozen in liquid nitrogen, and stored at − 70 °C until use. Plasma membrane was prepared by the method of Gray and Whittaker (1962). Two wild type brains or two nullizygous brains were pooled and homogenized by hand with 20 strokes of a Dounce tissue homogenizer in 10 vol of ice-cold 50 mM Tris–Cl (pH 8.0) containing 0.32 M sucrose and protease inhibitor cocktail (Roche, Germany). The homogenate was centrifuged for 10 min at 1000 × *g*. The pellet (crude nuclear fraction) was discarded and the supernatant was centrifuged at 17,000 × *g* for 55 min. The membrane pellet was suspended in 2 ml of 50 mM Tris buffer (pH 7.4). Protein concentrations were determined by the BCA protein assay (Pierce Chemical, Rockford, IL).

The ligand binding properties of mAChR from wild type and AChE − / − brain extracts were determined by titration with [^{3}H]quinuclinyl benzilate ([^{3}H]QNB; 39 Ci/mmol; Perkin-Elmer, Boston, MA) (Peterson and Schimerlik, 1984). [^{3}H]QNB is a nonselective muscarinic antagonist, which labels all five muscarinic receptor subtypes. Briefly, membrane factions (100 μg of protein) were mixed with 2 ml of 50 mM potassium phosphate buffer (pH 7.4), containing various concentrations of [^{3}H]QNB, ranging from 10 to 400 pM. The mixture was incubated for 1.5 h at room temperature. Nonspecific binding was determined in the presence of 10 μM atropine. The reaction was terminated by addition of 3 ml of ice-cold 50 mM potassium phosphate buffer, then immediately filtered under vacuum through Whatman GF/B glass fiber filter, and washed three times with 3 ml of ice-cold 50 mM potassium phosphate buffer. The filters were placed in 7-ml plastic minivials and dried. Four milliliters of EcoLume scintillation cocktail (ICN) was added and radioactivity was measured using a Beckman liquid scintillation counter. Titration data were fit to the equation for a single dissociation process using nonlinear regression analysis (SigmaPlot, version 4.16). [Comp]= {[M][QNB]}/{K_d+[QNB]}, where [M] = total concentration of muscarinic receptor per milligram of protein; [QNB] = total concentration of QNB; K_d = dissociation constant; [Comp] = measured concentration of QNB receptor complex per milligram of protein.

Total QNB binding sites in brain plasma membranes were determined using a saturating concentration of [^{3}H]QNB (3 nM). Brain plasma membranes were prepared from three 23-day-old AChE+/+ and three 23-day-old AChE − / − mice.

2.4. Northern analysis

Total RNA was extracted from tissues of 20- to 40-day-old mice using Trizol reagent (GibcoBRL, Rockville, MD) according to the manufacturer's instructions. To prepare probes, DNA fragments corresponding to parts of the coding regions from M1, M3, and M4 muscarinic receptors were amplified by polymerase chain reaction (PCR) from 129SvJ mouse genomic DNA. The primers were: M1 receptor sense (5′-GCTACATCCAGTTCCTCTCCCAA C) and M1 receptor antisense (5′-TGCCTTCTTCTCCTTGACCAGTG); M3 receptor sense (5′-CAGAAGCGGAAGCAGAAAACTTTG) and M3 receptor antisense (5′-TTTGAAGGACAGAGGTAGAGCGGC); M4 receptor sense (5′-AGCCATTGCT GCCTTCTACCTG) and M4 receptor antisense (5′-TCACTGCCTGTCTGCTTTGTCAC). The Genbank accession numbers for the M1, M3, and M4 sequence are NM007698, AF264050, and NM007699, respectively. The PCR products were sequenced to check for correctness and then used directly as templates to synthesize ^{32}P-labeled single-stranded probes. The M2 receptor probe was a 519-bp *Bst*xI fragment from mM2-PCDPS vector (kindly provided by Dr. Jurgen Wess). Northern hybridization was performed using ExpressHyb hybridization solution (Clontech, Palo Alto, CA) according to the manufacturer's instructions. Blots were hybridized at 68 °C, and washed at 25 and 50 °C.

2.5. Western analysis

Total forebrains from two 33-day-old AChE+/+ and two 33-day-old AChE − / − mice were flash frozen in liquid nitrogen. Homogenization buffer (50 mM Tris–HCl, pH 7.5; 50 mM NaCl; 10 mM EGTA; 5 mM EDTA) and protease inhibitor cocktail (Roche) were added and the samples were sonicated. Samples were solubilized in 0.125 M Tris–Cl, pH 6.8, buffer containing 4% sodium dodecyl sulfate (SDS), 20% glycerol, and 10% mercaptoethanol. Fifty micrograms of each sample was loaded onto 8% polyacrylamide SDS gels and then transferred to PVDF membrane. The blots were incubated in primary antibody diluted in blocking buffer (M1, 1 μg/ml; M2, 1:500; M4, 1 μg/ml), followed by horseradish peroxidase-conjugated goat antirabbit secondary antibody, and detected by Renaissance Western Blot Chemiluminescence Reagent (Perkin-Elmer). The blots were reprobed for total extracellular signal-regulated kinase 2 (ERK2; 1:500) (Cell Signaling, Beverly, MA).

The M1, M2, and M4 are polyclonal antibodies prepared in the laboratory of Levey et al. (1991). A fusion protein of glutathione-*S*-transferase and the i3 loop of each human receptor were expressed in bacteria to make the antigen. The purified antigen was injected into rabbits. Negative controls to show the specificity of the M1, M2, and M4 receptor antibodies were brain extracts from muscarinic receptor knockout mice (Gerber et al., 2001; Gomeza et al., 1999a,b).

Commercially available antibodies did not give the same results as the antibodies from Levey et al. The mAChR M1 (C-20) goat polyclonal antibody raised against a carboxy terminal peptide of human M1 (catalog no. sc-7470; Santa Cruz Biotechnology, Santa Cruz, CA) gave a single strong band of 100 kDa. The rabbit polyclonal antibody against a

10-amino-acid peptide from the carboxyl terminus of human M2 receptor (catalog no. WR-3721; Research and Diagnostic Antibodies, Benicia, CA) hybridized with proteins that were larger than 65 kDa. The mouse monoclonal antibody against purified porcine cardiac M2 receptor (catalog no. MA3-044, clone 31-1D1; ABR, Golden, CO) recognized no proteins in the mouse brain. The goat polyclonal antibody against a peptide from the carboxyl terminus of human muscarinic M3 receptor (catalog no. sc-7474; Santa Cruz Biotechnology) gave many bands including a band of the expected size of 75 kDa. The mouse monoclonal antibody against the i3 loop of human M4 receptor, fused to GST (catalog no. MAB1576; Chemicon International, Temecula, CA), gave a single strong band of 70 kDa. None of the commercial antibodies showed a difference in band intensity between AChE+/+ and −/− muscarinic receptors.

3. Results

Behavioral studies were undertaken to test the status of muscarinic receptors in AChE knockout mice. It was expected that receptor downregulation will reduce sensitivity to agonists (pilocarpine and OXO), but will increase sensitivity to antagonists (atropine).

3.1. AChE−/− mice are resistant to pilocarpine-induced seizures

The administration of pilocarpine, the mAChR agonist, produces seizures in mice via activation of the M1 receptors (Hamilton et al., 1997). Pilocarpine was used to investigate the functional status of M1 receptors in AChE −/− mice. In response to pilocarpine, 200 mg/kg ip, all the wild type mice experienced multiple seizures and only 20% survived the treatment. AChE+/− mice, with 50% of normal AChE activity, showed intermediate sensitivity to pilocarpine-induced seizures, with 50% having seizures and 60% surviving. In contrast, none of the AChE −/− mice experienced seizures. All the AChE −/− mice survived (Table 1). The reduced sensitivity to pilocarpine-induced seizures in AChE −/− and +/− mice indicates that functional levels of M1 muscarinic receptors are reduced in both the complete and partial AChE deficiency states.

Table 1
AChE −/− mice are resistant to pilocarpine-induced seizures (200 mg/kg ip)

AChE genotype	Number of mice	Seizures [%]	Survival [%]
+/+	10	100	20
+/−	10	50	60
−/−	6	0	100

Mice of the indicated genotypes were injected with a fresh pilocarpine solution at a dose of 200 mg/kg ip. The animals were observed for seizures for 45 min after injection.

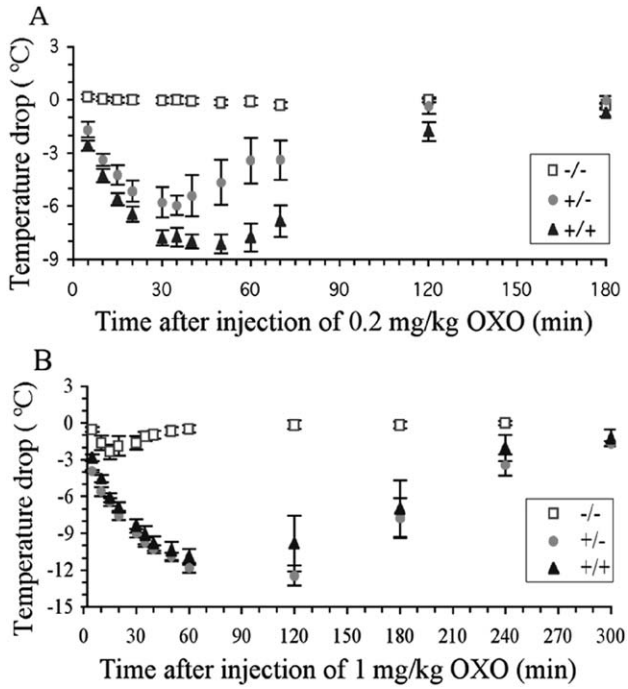

Fig. 1. Hypothermic response to OXO administration in mice. Mice of the indicated genotypes ($n=6$ for each treated group) were injected subcutaneously with a dose 0.2 mg/kg (Panel A) or 1 mg/kg (Panel B) of the nonselective muscarinic agonist, OXO. The data are presented as mean ± S.E. AChE −/− response to OXO-induced hypothermia was significantly reduced compared to AChE+/+ and +/− mice ($P<.00001$, t test).

3.2. AChE−/− mice are resistant to OXO-induced hypothermia, tremor, salivation, and analgesia

One of the effects of the administration of the nonselective mAChR agonist, OXO, in normal mice is induction of hypothermia (Gomeza et al., 1999a). Following administration of 0.2 mg/kg OXO, AChE+/+ mice showed an 8 °C decrease in body temperature 30–60 min after agonist injection. The body temperature of AChE+/+ mice recovered to baseline 37.1 °C approximately 2–3 h after agonist injection (Fig. 1A). In contrast, there was no effect of this dose of OXO on body temperature in the AChE −/− mice. The decrease in temperature was less pronounced in AChE+/− mice (6 °C) and began to recover to baseline sooner than in AChE+/+ mice. Following a higher dose of OXO (1 mg/kg), AChE −/− mice showed only a slight 2 °C decrease in temperature that returned to baseline 36.5 °C by 45 min, whereas AChE+/+ and +/− mice showed an equivalent 12 °C drop in temperature that recovered 5 h after agonist injection (Fig. 1B). These results indicate that AChE −/− and +/− mice had decreased hypothermic response, an effect mediated by central M2 receptors (Gomeza et al., 1999a).

OXO also induces motor tremor (Gomeza et al., 1999a). As expected, OXO induced massive whole body tremor in AChE+/+ mice. The tremorogenic effects of OXO were

significantly reduced in AChE −/− mice, even when the dose was increased to 1 mg/kg (Fig. 2A and B). The fact that OXO did induce some tremor in AChE −/− mice indicates the presence of some functional M2 receptors. Heterozygous mice showed an intermediate response (Fig. 2A), but only at low doses of OXO. Recovery from OXO-induced tremor was rapid in AChE −/−, intermediate in AChE+/−, and slowest in AChE+/+ mice (Fig. 2A). Because the OXO-induced tremor response is primarily mediated by central M2 receptors (Gomeza et al., 1999a), it can be concluded that AChE deficiency at the 100% level and, surprisingly, also at the 50% level reduces the number of functional M2 receptors.

Administration of 0.2 mg/kg OXO resulted in salivary secretion in wild type and heterozygous mice, but not in AChE −/− mice (Fig. 3A). The response was intermediate in AChE+/− mice. When the dose was increased to 1 mg/kg, the AChE −/− mice had a mild salivation response, indicating that they did have some functional receptors (Fig. 3B). At the higher dose, the response of AChE+/+ and +/− mice was indistinguishable. These results indicate that AChE −/− and +/− mice have decreased levels of functional M3 receptors because salivation is primarily mediated by M3 receptors (Matsui et al., 2000).

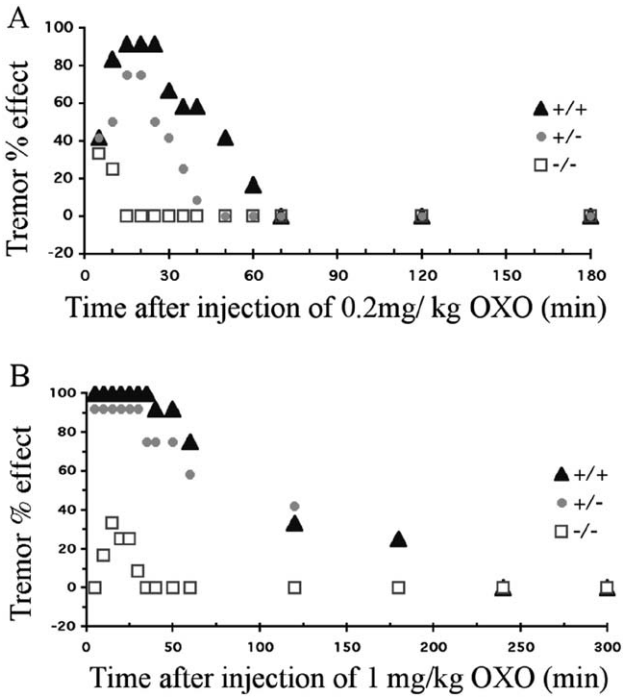

Fig. 2. Tremorogenic response to OXO administration in mice. Mice of the indicated genotypes ($n=6$ for each treated group) were injected subcutaneously with 0.2 mg/kg (Panel A) or 1 mg/kg (Panel B) of OXO. Data are presented as percent effect, where the score for each group was averaged and expressed as a percent of the maximum possible score. Standard error ranged from 0% to 16%. AChE −/− response to OXO-induced tremor was significantly reduced compared to AChE+/+ and +/− mice ($P<.0001$, AChE+/+ vs. AChE −/−; $P<.005$, AChE+/− vs. AChE −/−, by t test).

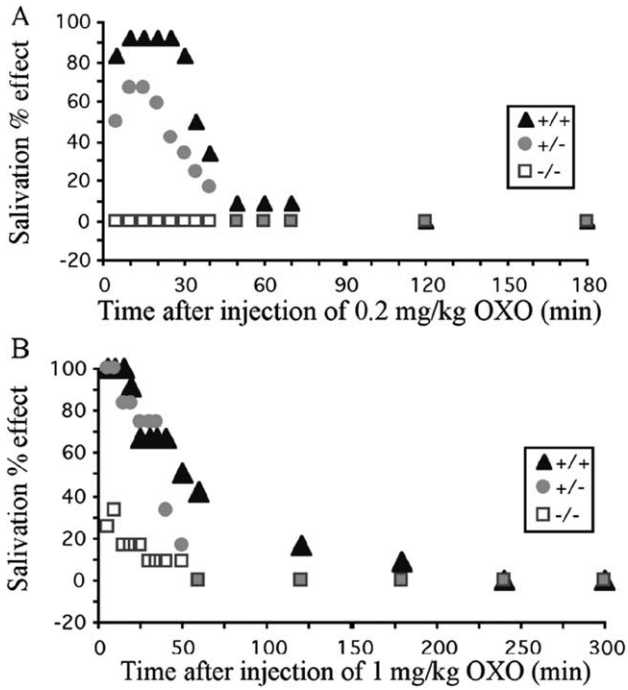

Fig. 3. Salivation response to OXO administration in mice. Mice of the indicated genotypes ($n=6$ for each treated group) were injected subcutaneously with a dose 0.2 mg/kg (Panel A) or 1 mg/kg (Panel B) of OXO. Data are presented as percent effect, where the score for each group was averaged and expressed as a percent of the maximum possible score. Standard error ranged from 0% to 20%. AChE −/− response to OXO-induced salivation was significantly reduced compared to AChE+/+ and +/− mice ($P<.00001$, AChE+/+ vs. AChE −/−; $P<.005$, AChE+/− vs. AChE −/−, by t test).

Muscarinic agonists can cause analgesic effects by activating the muscarinic receptors in the pain pathway (Eisenach, 1999). The analgesic effect of OXO was investigated by using the tail-flick and hot-plate tests. The tail-flick test assesses pain sensitivity primarily at the spinal level and the hot-plate test measures pain responses mediated by supraspinal mechanisms (Konig et al., 1996). Normal baseline response to thermal stimuli with the hot-plate test (5–6 s) and tail-flick test (1–2 s) did not differ significantly in AChE+/+, +/−, and −/− mice (Fig. 4A and B). In contrast, OXO administration (0.2 mg/kg) induced strong analgesic effects in AChE+/+ as well as AChE+/− mice in both tests. OXO-dependent analgesic responses in AChE −/− mice were significantly reduced compared to AChE+/+ and +/− mice. AChE+/− mice were almost as sensitive as wild type mice to OXO-induced analgesia in the tail-flick test. However, AChE+/− mice showed intermediate analgesic response to OXO in the hot-plate test. Reduced analgesic response to OXO in AChE −/− and +/− mice indicates that the functional M2 and M4 muscarinic receptors are reduced in AChE-deficient mice. Muscarinic analgesia is exclusively mediated by a combination of M2 and

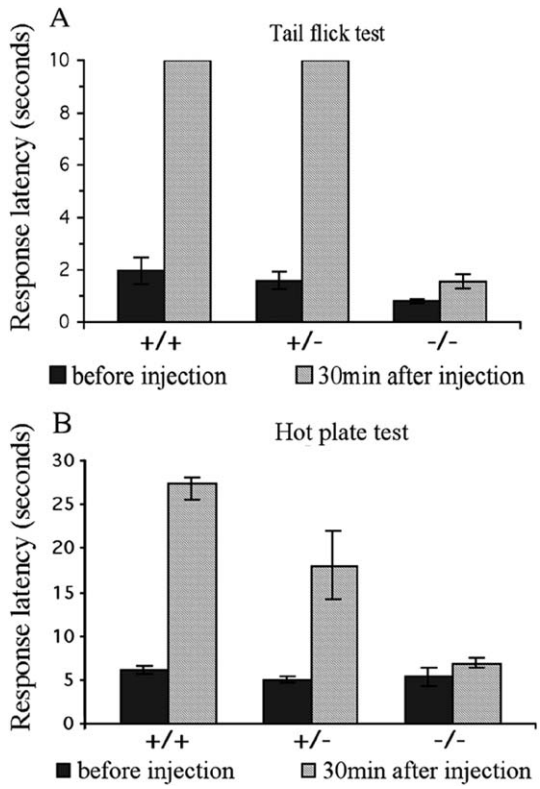

Fig. 4. Analgesic response to OXO administration in mice. Mice of the indicated genotypes ($n=6$ per group) were injected with OXO subcutaneously (0.2 mg/kg) and analgesic responses were measured by the tail-flick and hot-plate tests. Data are presented as mean ± S.E. In (A), AChE −/− response to OXO was significantly reduced compared to AChE+/+ and +/− mice in tail-flick test ($P<.00001$, t test). In (B), AChE −/− response to OXO was significantly reduced compared to AChE+/+ and +/− mice in hot-plate test ($P<.00001$, AChE+/+ vs. −/−; $P<.01$ AChE+/− vs. AChE −/−, by t test).

M4 receptors at both spinal and supraspinal sites (Duttaroy et al., 2002).

The intermediate response of AChE+/− mice to OXO-induced effects suggests that efficient M2, M3, and M4 receptor-induced effects require a relatively high fractional receptor occupancy.

3.3. AChE−/− mice have low LD_{50} for atropine

Atropine, a specific muscarinic receptor antagonist, was used to further confirm that AChE −/− mice have reduced functional muscarinic receptors. The toxicity of atropine was determined by measuring the LD_{50} of atropine. Animals, regardless of genotype, experienced severe whole body tremor, initial hyperlocomotion, and clonic convulsions prior to death. The median lethal dose of atropine was 251 mg/kg (95% CI = 233; 272) in AChE+/+ mice, 216 mg/kg (95% CI = 209; 226) in AChE+/− mice, and 97 mg/kg (95% CI = 72; 107) in AChE −/− mice. The lower LD_{50} values of atropine for AChE −/− and +/− mice further confirmed that functional muscarinic receptors are reduced in AChE-deficient mice.

3.4. Muscarinic receptor binding sites are reduced in AChE−/− mice

Reduced responsiveness to the behavioral effects of central mAChR stimulation could result from decreased levels of mAChR. Radioligand binding assays were performed to determine the total number of mAChR in mouse brain plasma membranes using [^{3}H]QNB, a muscarinic antagonist that binds to all muscarinic subtypes with high affinity and specificity. The total number of muscarinic receptor binding sites in brain plasma membrane of AChE −/− mice was approximately 50% of that in wild type mice (Fig. 5). Heterozygotes showed an intermediate value, corresponding to a 25% loss in total [^{3}H]QNB binding. Titration of receptors with [^{3}H]QNB indicated that the binding characteristics of muscarinic receptors in wild type and AChE −/− mice were not significantly different, within the limits of the standard deviation. The dissociation constant was 176 ± 33 pM for AChE+/+ and 98 ± 22 pM for AChE −/− (Fig. 6). These data suggest that it is the number of muscarinic receptors, not their reactivity, which is reduced in AChE −/− mice. Thus, loss of muscarinic receptors is a major factor responsible for loss of physiological responses to agonist stimulation in AChE −/− mice.

3.5. Muscarinic receptor mRNA levels are unchanged in AChE−/− mice

Reduced levels of mAChR binding sites in AChE −/− mice could result from enhanced receptor degradation or decreased receptor expression. To determine whether alteration in mRNA expression of muscarinic receptors was responsible for reduced muscarinic receptor level in AChE −/− mice, mRNA levels of the M1–M4 subtypes

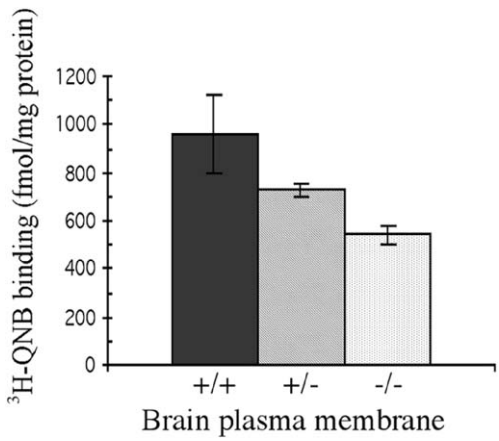

Fig. 5. Radioligand binding analysis of muscarinic receptor densities in AChE+/+, +/−, and −/− mice ($n=3$ for each group). Brain membranes were prepared from the indicated genotypes. Muscarinic receptor densities were determined by radioligand binding using a saturating concentration (3 nM) of the muscarinic antagonist, [^{3}H]QNB. Data are given as mean ± S.E.

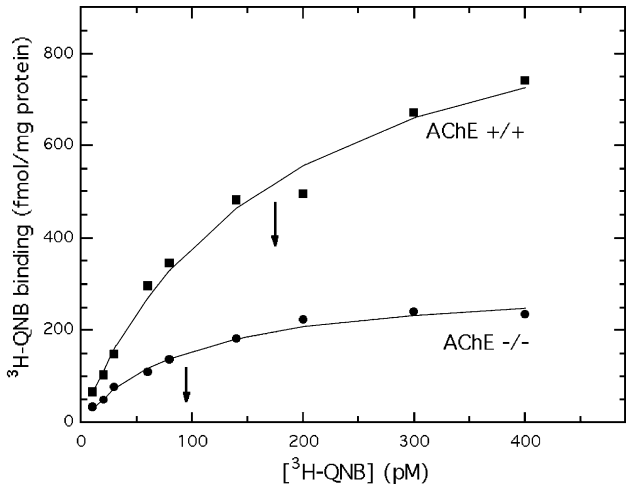

Fig. 6. Titration of [^{3}H]QNB binding to muscarinic receptors in brain plasma membranes. Brain plasma membranes were prepared from two 32-day-old AChE+/+ and two 32-day-old AChE −/− mice. Titration was performed as described in Materials and Methods. Square (■) represents AChE+/+, and circle (●) represents AChE −/−. The arrows indicate the position of dissociation constants. The lines are fitted to the data using the following parameters: AChE+/+, K_d = 176 pM, B_{max} = 1050 fmol/mg protein; AChE −/−, K_d = 98 pM, B_{max} = 306 fmol/mg protein. Each point was the average of three measurements. The titration experiment was performed twice with essentially the same results.

were determined by Northern blotting. As shown in Fig. 7, mRNA levels of M1–M4 receptors were unchanged in the brain, intestines, and heart of AChE −/− mice. It is concluded that downregulation of functional muscarinic receptors in AChE −/− mice is not associated with a decrease of muscarinic receptor mRNA levels.

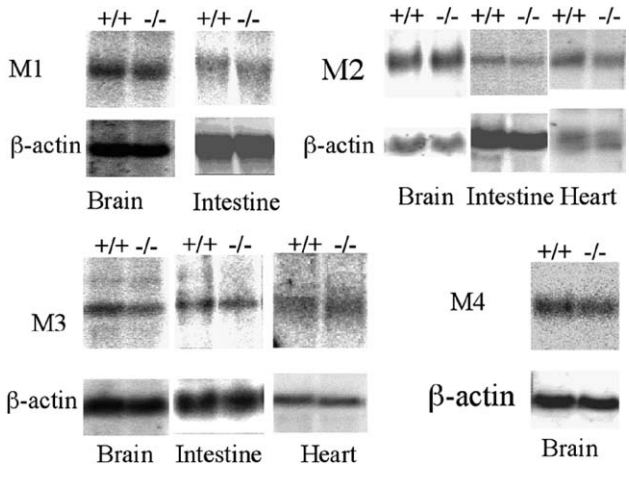

Fig. 7. Northern blot analysis of muscarinic receptor gene expression in AChE+/+ and −/− mice. Total RNA was extracted from the indicated tissues and hybridized with ^{32}P-labeled specific probes for muscarinic receptors M1–M4. Membranes were rehybridized with a β-actin probe as a loading control. Northern blotting was quantitated by ImageQuant software (Molecular Dynamics).

3.6. M1, M2, and M4 mAChR proteins are markedly reduced in AChE−/− mice

Binding assays demonstrated that total mAChR levels were reduced in the brains of AChE −/− mice. Because [^{3}H]QNB does not distinguish among individual mAChR subtypes, the reduction in binding sites might preferentially involve one or more of the subtypes. The protein levels of M1, M2, and M4 muscarinic receptors, which are the primary mAChR expressed in the brain, were determined by Western blotting with specific antibodies (Levey et al., 1991). Brains from two AChE+/+ and two AChE −/− mice were used for these studies; individual results are shown in Fig. 8. The protein levels for M1, M2, and M4 muscarinic receptors in AChE −/− mice were each reduced approximately by 50% (M1, 38%; M2, 57%; and M4, 52%) compared to wild type mice. These findings suggest that

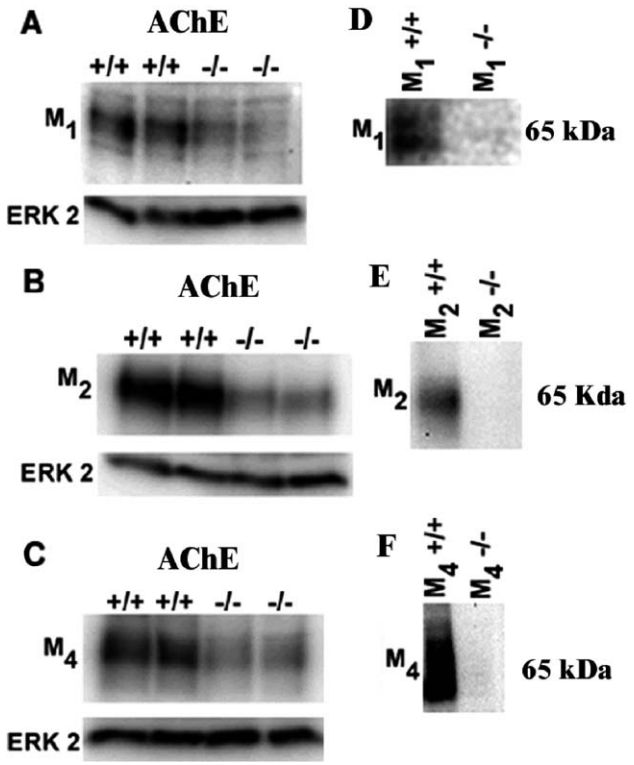

Fig. 8. Western blot analysis of muscarinic receptor proteins in AChE+/+ and −/− brains. Total forebrain homogenates from AChE+/+ and −/− mice were immunoblotted with M1 (Panel A), M2 (Panel B), and M4 (Panel C) antibodies. Each lane represents homogenates from individual AChE+/+ and −/− mice. Membranes were rehybridized with an ERK2 antibody as a loading control. The intensity of bands was quantitated by Kodak 1-D Image Analysis software and normalized using the intensities of total ERK2. Panels (D), (E), and (F) demonstrate the specificity of M1, M2, and M4 antibodies. Total brain homogenates from M1+/+ and −/− mice (Gerber et al., 2001), M2+/+ and −/− (Gomeza et al., 1999a), or M4+/+ and −/− (Gomeza et al., 1999b) knockout mice were immunoblotted with M1, M2, and M4 antibodies. The blots show M1, M2, and M4 immunoreactivity in the respective +/+ mice, but no immunoreactivity in the respective muscarinic receptor knockout mice.

the muscarinic receptor binding sites and proteins are downregulated in AChE −/− mice.

4. Discussion

The presence of tremor, pinpoint pupils, and seizures in AChE −/− mice implies a dysfunction of central and peripheral pathways. Such conditions are consistent with impaired function of the cholinergic receptors. The absence of AChE from cholinergic synapses would be expected to result in elevated levels of the cholinergic transmitter, acetylcholine. It is known that chronic agonist stimulation of muscarinic receptors induces downregulation of the receptors and results in loss of the cell response to further agonist stimulation in cultured cells (Fukamauchi et al., 1993; Lenz et al., 1994; Waugh et al., 1999). Myasthenia gravis patients treated chronically with the cholinesterase inhibitor, pyridostigmine, develop tolerance to the toxic effects of the drug (Johns and McQuillen, 1966). Similarly, rats and mice treated chronically with cholinesterase inhibitors develop tolerance. The tolerance is explained by downregulation of cholinergic receptors (Bushnell et al., 1994; Costa et al., 1982; Gupta et al., 1986; Russell et al., 1981; Schiller, 1979). AChE −/− mice can be regarded as an extreme example of tolerance to anticholinesterase agents. As such, they are expected to downregulate not only muscarinic but also nicotinic receptors.

4.1. Downregulation of multiple muscarinic receptor subtypes

Downregulation of receptors is generally associated with decreased sensitivity to agonists and hypersensitivity to antagonists. We have found that AChE −/− mice are remarkably insensitive to the muscarinic receptor agonists, OXO and pilocarpine. In addition, AChE −/− mice are supersensitive to the muscarinic receptor antagonist, atropine. Together, these results strongly suggest that muscarinic receptors have undergone a compensatory downregulation in AChE −/− mice.

Comparison of our functional studies on the AChE −/− mouse to similar studies with muscarinic receptor knockout mice makes this conclusion more compelling. For example, it is known that the M1 receptor is required for the initiation of seizures in the pilocarpine model of epilepsy. M1 receptor knockout mice do not exhibit seizures following the administration of a dose of pilocarpine, which causes multiple tonic–clonic seizures in wild type mice (Hamilton et al., 1997). Similarly, AChE −/− mice were completely insensitive to pilocarpine-induced seizures. These results indicate that the level of M1 receptors is reduced in AChE −/− mice.

The M2 receptor plays a key role in the regulation of heart rate, temperature, and pain response (Gomeza et al., 1999a,b). M2 receptor knockout mice show strikingly reduced OXO-induced tremor, hypothermia, and analgesia, similar to the reduced response in AChE knockout mice. These results indicate that the level of functional M2 receptors is reduced in AChE −/− mice.

The M3 receptor is the major muscarinic receptor involved in glandular secretion. M3 receptor knockout mice, unlike M1 and M2 knockout mice, are resistant to agonist-induced salivation (Matsui et al., 2000). As shown here, AChE −/− mice had a dramatic decrease in OXO-induced salivation. We also observed a marked decrease in pilocarpine-induced glandular secretion in AChE −/− mice (data not shown), suggesting that the level of M3 receptors is reduced in AChE −/− mice. Overall, these data indicate a pronounced reduction in functional responses of multiple mAChR subtypes in AChE −/− mice.

4.2. Quantitation of the total number of muscarinic receptors

Receptor binding studies using [^{3}H]QNB indicated that the total number of muscarinic receptors in the AChE −/− mouse brain was reduced by 50%. On the other hand, the reactivity of the muscarinic receptors remained essentially unchanged—with the affinity for QNB binding being less than two-fold lower for wild type mice. This suggested that AChE −/− mice have adapted to excess acetylcholine by reducing the total number of muscarinic receptors rather than altering binding affinity. The overall decrease in the number of muscarinic receptors is a major factor responsible for loss of response to the muscarinic agonists, OXO and pilocarpine, in AChE −/− mice.

4.3. mRNA levels

The regulation of muscarinic gene expression by muscarinic receptor agonists differs according to cell type and muscarinic receptor subtype. Our results showed that the function of muscarinic receptors was downregulated without reducing the total receptor mRNA levels in AChE −/− mice. Our results are supported by the work of others in rats and cells. Studies in the rat brain in vivo demonstrated that acute and chronic treatments with AChE inhibitors induced a decrease of M4 receptors in the striatum without altering the mRNA level (Liste et al., 2002). Long-term muscarinic agonist treatment induced downregulation of M2, M3, and M4 receptors, but was not associated with a decrease of muscarinic receptor mRNA levels in cultured cell lines and primary cells (Haddad et al., 1995; Janossy et al., 2001; Lenz et al., 1994).

We did not detect any significant change in muscarinic receptor mRNA levels using whole brain homogenate. However, we cannot rule out the possibility that muscarinic receptor mRNA levels may have changed in selected areas. Yagle and Costa (1996) found that OP exposure can differentially regulate mRNA levels for muscarinic receptor subtypes in different brain areas. Rats treated with 2 mg/kg/

day disulfoton for 14 days had a 28% decrease in [^{3}H]QNB binding and 81% decrease in AChE activity in the cortex. There was a 23% decrease in M1 receptor mRNA levels in the hippocampus after the disulfoton treatment, while no change was observed in the cortex, corpus striatum, medulla, or cerebellum. The M2 subtype mRNA was decreased in both hippocampus (24%) and medulla (19%), but not in the cortex, striatum, or cerebellum. The M3 receptor mRNA levels were decreased in the cortex (10%), but no change was observed in the hippocampus, medulla, cerebellum, or lymphocytes.

4.4. Protein levels

Downregulation of G protein-coupled receptors, defined as a decrease in the total number of receptors in a cell, is observed following activation of G-coupled receptors (Bohm et al., 1997). Downregulation serves to decrease the responsiveness of a cell to a certain stimulus. We found that the protein levels for M1, M2, and M4 receptors in whole forebrain homogenates were reduced in AChE −/− mice. This indicates that the downregulation of functional mAChR in AChE −/− mice, as observed in behavioral assays, was accompanied by a net loss in the total number of mAChR.

Multiple processes are involved in the regulation of mAChR in response to agonist stimulation. The abundance and availability of G protein-coupled receptors at the cell surface are regulated by the neuronal environment and is the result of complex intraneuronal trafficking (Bernard et al., 1998, 1999; Edwardson and Szekeres, 1999; Koenig and Edwardson, 1996; Liste et al., 2002; Logsdon, 1999; Roseberry et al., 2001). Sequestration of receptors within the cell is an established mechanism for downregulation of functional muscarinic receptors. Studies by Bernard et al. found a marked depletion of cell surface M2 receptor, associated with an accumulation of M2 receptors in the endoplasmic reticulum and Golgi complex of AChE −/− mouse brain (Bernard et al., in press). Therefore, it is possible that enhanced mAChR downregulation results from muscarinic receptor sequestration in AChE −/− mice. Bernard et al. also demonstrated that blockade of cholinergic stimulation in AChE −/− mouse neurons provokes translocation of M2 receptors from the cytoplasm to the cell surface. These data strongly suggested that the absence of M2 receptors at the cell surface was due to chronic hyperstimulation by endogenous acetylcholine.

Phosphorylation is also involved in muscarinic receptor regulation. When muscarinic receptors are phosphorylated by G protein-coupled receptor kinases and other kinases (Tobin and Nahorski, 1993; Tsuga et al., 1998; Waugh et al., 1999), they become uncoupled from the G protein signaling system. It will be interesting to determine in future studies whether phosphorylation of muscarinic receptors is also involved in downregulation of muscarinic receptors in AChE −/− mice.

4.5. Functional implications

The finding that heterozygous mice, with 50% of the normal AChE activity, had intermediate sensitivity to low-dose OXO-induced hypothermia, tremor, and salivation, as well as intermediate sensitivity to pilocarpine-induced seizures and an intermediate LD_{50} value for atropine, suggested that the functional muscarinic receptors were reduced in AChE+/− mice. Duysen et al. (2001) have shown that AChE+/− mice are more sensitive to organophosphorus toxicants than wild type mice. However, by visual inspection, AChE+/− mice are indistinguishable from AChE+/+ mice. They do not have any obvious impairment. They have a normal lifespan and have normal reproductive abilities. This report shows that AChE+/− mice respond differently to muscarinic agonists. We expect that, in the future, people who have one deficient AChE allele will be identified. These people will be healthy, but will have an unexpected response to muscarinic agonists, muscarinic antagonists, and organophosphorus pesticides.

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