

Altered Striatal Function and Muscarinic Cholinergic Receptors in Acetylcholinesterase Knockout Mice

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ABSTRACT

Cholinesterase inhibitors are commonly used to improve cognition and treat psychosis and other behavioral symptoms in Alzheimer's disease, Parkinson's disease, and other neuropsychiatric conditions. However, mechanisms may exist that down-regulate the synaptic response to altered cholinergic transmission, thus limiting the efficacy of cholinomimetics in treating disease. Acetylcholinesterase knockout (AChE^{-/-}) mice were used to investigate the neuronal adaptations to diminished synaptic acetylcholine (ACh) metabolism. The striatum of AChE^{-/-} mice showed no changes in choline acetyltransferase activity or levels of the vesicular ACh transporter but showed striking 60% increases in the levels of the high-affinity choline transporter. This transporter takes choline from the synapse into the neuron for resynthesis of ACh. In addition, the striata of AChE^{-/-} mice showed dramatic reductions in

levels of the M₁, M₂, and M₄ muscarinic ACh receptors (mAChRs), but no alterations in dopamine receptors or the β 2 subunit of nicotinic receptors. M₁, M₂, and M₄ also showed decreased dendritic and cell surface distributions and enhanced intracellular localizations in striatal neurons of AChE^{-/-} mice. mAChR antagonist treatment reversed the shifts in mAChR distribution, indicating that internalized receptors in AChE^{-/-} mice can recover to basal distributions. Finally, AChE^{-/-} mice showed increased sensitivity to mAChR antagonist-induced increases in locomotor activity, demonstrating functional mAChR down-regulation. mAChR down-regulation in AChE^{-/-} mice has important implications for the long-term use of cholinesterase inhibitors and other cholinomimetics in treating disorders characterized by perturbed cholinergic function.

Acetylcholine plays a crucial role in memory, learning, and movement (Graybiel, 1990; Jerusalinsky et al., 1997; Pisani et al., 2001). Perturbed cholinergic transmission is involved in devastating disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and schizophrenia (Graybiel, 1990; Holt et al., 1999; Pisani et al., 2001). Cholinesterase inhibitors to enhance cholinergic transmission provide the primary treatment for Alzheimer's disease and may ameliorate hyperkinetic movement disorders associated with cholinergic hypofunction such as Huntington's chorea and tardive dyskinesia (Davis and Berger, 1978; Tammenmaa et al., 2002). Furthermore, the antipsychotic properties of these drugs have spurred new interest in the role of cho-

linomimetics in schizophrenia treatment (White and Cummings, 1996; Shannon et al., 2000) and the neuropsychiatric symptoms of Parkinson's disease (Mori, 2002). Although cholinomimetics show promise in treating a variety of neurological and psychiatric disorders, long-term treatment with cholinesterase inhibitors has been of limited efficacy (Farlow and Evans, 1998; Francis et al., 1999). It is possible that mechanisms that compensate for long-term loss of cholinesterase activity down-regulate the neuronal responsiveness to increased ACh and restrict the effectiveness of these agents. Potential targets for adaptive responses to altered cholinergic transmission include proteins involved in ACh synthesis and release and receptors involved in neurotransmission.

Recently developed AChE knockout (^{-/-}) mice provide a valuable tool for examining the effects of long-term complete and selective abolition of AChE activity (Xie et al., 2000; Duysen et al., 2002). These mice show normal development of cholinergic pathways (Mesulam et al., 2002), but they exhibit

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ABBREVIATIONS: ACh, acetylcholine; AChE, acetylcholinesterase; mAChR, muscarinic receptor; CHT, high affinity choline transporter; ChAT, choline acetyltransferase; VACHT, vesicular acetylcholine transporter; SPR, substance P receptor; CNS, central nervous system; GPCR, G protein-coupled receptor.

tremor, a classic symptom of basal ganglia dysfunction and hypercholinergic activity (Xie et al., 2000). In this study, we investigated the expression and function of key molecules involved in cholinergic transmission in the striatum. The striatum is densely innervated by cholinergic interneurons that are crucial for motor behavior (Pisani et al., 2001), and this structure is highly enriched in cholinergic markers. AChE^{-/-} mice showed dramatic and selective reduction in mAChR protein levels, a marked redistribution of mAChRs to intracellular compartments, up-regulation of the high-affinity choline transporter (CHT), and altered locomotor activity induced by mAChR antagonists. In contrast, there was no change in the activity of choline acetyltransferase (ChAT), the biosynthetic enzyme for ACh, or in the levels of the vesicular ACh transporter (VACHT) and the $\beta 2$ subunit of nicotinic receptors. These findings indicate that the cholinergic system exhibits a dramatic adaptive response to long-term loss of AChE activity; the most profound effects resulted from down-regulation of mAChR in AChE^{-/-} mice. These results have important therapeutic implications for the design of pharmacotherapeutic agents targeted at long-term modulation of AChE or mAChR activity to treat CNS disorders involving perturbed cholinergic function.

Materials and Methods

AChE^{-/-} Mice. The AChE^{-/-} mice were generated in the 129Sv strain and characterized as described previously (Xie et al., 2000). The mice survive to adulthood when maintained on a liquid diet (Duysen et al., 2002).

ChAT Activity. ChAT activity in striatal homogenates was determined as described previously (Levey et al., 1982) except that Ready Organic scintillant (Beckman Coulter Inc., Fullerton CA) was used. Background was subtracted from the values obtained. The assays were performed in triplicate and the data represent an average from three AChE^{+/+} and three AChE^{-/-} mice.

Immunoblotting and Quantitation. The striatum was dissected and the samples were homogenized in buffer containing 10 mM Tris-Cl, pH 7.4, 1 mM EDTA, pH 8.0, and protease inhibitor cocktail (Roche Diagnostics Corporation, Indianapolis, IN). The samples were solubilized in Laemmli sample buffer, subjected to SDS-polyacrylamide gel electrophoresis and transferred to membrane. After blocking in Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE), blots were incubated in primary antibodies to the following antigens: $\beta 2$ subunit of the nicotinic ACh receptor (1:200; Covance, Inc., Princeton, NJ), D1 dopamine receptor (1:500; Levey et al., 1993), D2 dopamine receptor (1:100; Levey et al., 1993), M₁ (1 μ g/ml), M₂ (1:500), M₄ (1 μ g/ml; Levey et al., 1991), VACHT (1 μ g/ml; Gilmor et al., 1996), CHT (1 μ g/ml; S. Ferguson, A. Levey and R. Blakely, unpublished observations). To control for loading, primary antibody solutions also contained antibodies to either total extracellular signal-regulated kinase 1/2 (1:500; Cell Signaling Technologies, Beverly, MA) or Na⁺/K⁺ ATPase (1:40,000; Upstate Biotechnologies, Waltham, MA). Blots were rinsed and incubated in AlexaFluor 680 donkey anti-mouse or anti-rat (1:10,000; Molecular Probes, Eugene, OR) and IRDye 800 goat anti-rabbit (1:10,000; Rockland, Gilbertsville, PA) secondary antibodies in blocking buffer. Blots were scanned, and quantitated with an Odyssey Infrared Imaging System (Li-Cor Biosciences). To quantitate immunoreactivity of mAChRs, a box was drawn around the bands corresponding to M₁, M₂, and M₄, which are approximately 65 kDa. Glycosylation of the receptors alters the molecular mass of the receptors, resulting in a few mAChR-specific bands within the 65-kDa range. The Odyssey program calculated the integrated intensity of the bands and subtracted background from the values. Background, or 0% immunoreactivity,

is calculated using pixels around the perimeter of the area being quantified. The mAChR immunoreactivity for each mouse (three individual AChE^{+/+} mice and three AChE^{-/-} mice) was normalized to the loading control within each lane. The average mAChR immunoreactivity (\pm S.E.M.) in the AChE^{-/-} mice was expressed as a percentage of the average immunoreactivity (\pm S.E.M.) in the AChE^{+/+} mice. Similar methods were performed for quantitation of other markers. Immunoblotting was performed a minimum of three times and data were analyzed with SPSS software (SPSS Inc., Chicago, IL) using analysis of variance with Fisher's least significant difference post hoc test or independent *t* test.

Immunofluorescence and Quantitation of Confocal Images.

Immunofluorescence, confocal imaging, and quantitation were performed as described previously (Zhang et al., 2002a). Coronal sections were incubated in antibodies to the following antigens diluted in buffer containing 50 μ g/ml biotin and 1% normal horse serum: D1 (1:200), D2 (1 μ g/ml), M₁ (1 μ g/ml), M₂ (1:100), M₄ (1 μ g/ml), substance P (neurokinin 1) receptor (1:500; Chemicon International, Temecula, CA). Some sections were also treated with Hoechst 33258 (1:10,000). Unless otherwise indicated in the figure legends, all immunostaining was performed in a minimum of three AChE^{+/+} mice and three AChE^{-/-} mice.

Drug Treatments. Mice received three intraperitoneal injections of saline or 5 mg/kg atropine dissolved in saline every 30 min; the total time of treatment was 90 min. The mice were then perfused according to the methods described above.

Locomotor Activity. Motor behavior was tested using a motion detector consisting of a dark box in a sound-proof room containing the home cage and a red light-emitting diode. Horizontal activity was measured as the number of photobeam interruptions. Mice were acclimated to the test cage for 2 h before the experiment was begun. Each animal received s.c. injections of 0.05 mg/kg or 0.5 mg/kg scopolamine (Sigma, St. Louis, MO) dissolved in 0.1 M potassium phosphate, pH 7.0, or injections of 0.1 M potassium phosphate alone (vehicle). Locomotor activity was measured for 90 min in consecutive 2-min intervals.

Results

Activity and Expression of Enzymes and Transporters Involved in ACh Synthesis and Release. Long-term diminished ACh metabolism in AChE^{-/-} mice may lead to changes in neurotransmitter synthesis and release. To evaluate potential presynaptic changes, we first measured the expression and activity of the three key enzymes and transporters essential for ACh synthesis and release, ChAT, VACHT, and CHT, in striatal homogenates from AChE^{+/+} and AChE^{-/-} mice. Activity of ChAT, which synthesizes ACh, was measured, and in AChE^{+/+} mice, approximately 0.29 fmol/mg of protein of ACh were formed in 25 μ l of homogenate in 40 min; in AChE^{-/-} mice, approximately 0.31 fmol/mg of protein of ACh was formed. Thus, an equivalent amount of ACh was formed in the striatal homogenates from AChE^{+/+} mice and AChE^{-/-} mice (Fig. 1a), indicating no change in ChAT activity in response to deficient synaptic ACh metabolism. VACHT regulates the uptake of ACh into synaptic vesicles from the cytosol; thus, altered VACHT expression could change the amount of ACh released into the synapse. VACHT (Fig. 1b) showed quantitatively similar immunoreactivity in the striatum of AChE^{+/+} mice and AChE^{-/-} mice. Overall, these data suggest that the CNS does not respond to diminished ACh metabolism by altering either ChAT activity or expression of VACHT. Choline uptake via the CHT is the rate limiting step of ACh synthesis (Kuhar and Murrin, 1978; Joje, 1979) and changes in CHT may

mediate adaptive response to altered synaptic metabolism of ACh. ChT expression was significantly increased by ~60% in striatal homogenates from AChE^{-/-} mice relative to AChE^{+/+} control mice (Fig. 1c).

Cholinergic Expression in the Brains of AChE^{-/-} Mice. Changes in cholinergic receptor expression could modulate neuronal responsiveness to increased stimulation in response to chronically reduced synaptic ACh metabolism. Because the striatum contains three distinct mAChR proteins (Levey et al., 1991), M₁, M₂, and M₄, these subtypes were measured individually. Levels of all three mAChRs were substantially reduced in homogenates from AChE^{-/-} striatum compared with AChE^{+/+} controls (Fig. 2, a-c). Compared with wild-type mice, quantitation by immunoblot of receptor levels in AChE^{-/-} mice showed a statistically significant reduction of M₁, M₂, and M₄ immunoreactivity in the striatum by ~40, ~56, and ~64%, respectively. Therefore, the M₁, M₂, and M₄ mAChRs showed consistent and dramatic reductions in the striatum of AChE^{-/-} mice. Nicotinic cholinergic receptors expressed in the striatum also provide a potential target for adaptive responses to reduced ACh metabolism. The $\beta 2$ subunit of the nicotinic receptor, which is expressed in striatal dopamine terminals (Zoli et al., 2002), showed similar levels in AChE^{-/-} mice relative to AChE^{+/+} mice (Fig. 2f). Hence, modulatory G protein-coupled mAChR, but not inotropic nicotinic receptors, are altered in AChE^{-/-} mice.

Expression of Receptors Involved in Dopaminergic Transmission in the Striatum of AChE^{-/-} Mice. A bal-

ance between the cholinergic and dopaminergic system in the striatum is important for motor behavior (Graybiel, 1990), and M₁ and M₄ play important roles in striatal dopaminergic transmission (Gomez et al., 1999b; Gerber et al., 2001; Zhang et al., 2002b). M₁ and M₄ in medium spiny neurons are coexpressed with the D1 and D2 dopamine receptors, which are also members of the G protein-coupled receptor family (Graybiel, 1990; Ince et al., 1997; Yan et al., 2001). Given the marked changes in expression of mAChRs, we examined the effect of diminished ACh metabolism in AChE^{-/-} mice on expression of dopamine receptors. Levels of D1 and D2 dopamine receptor immunoreactivities in the striatum were not significantly altered in AChE^{-/-} mice relative to AChE^{+/+} control mice (Fig. 2, d and e). Overall, these data demonstrate that diminished ACh metabolism in the striatum selectively reduced mAChR protein levels without affecting dopamine receptors.

Localization of Postsynaptic mAChRs to Dendrites and the Cell Surface in AChE^{-/-} Mice. Agonist-induced mAChR endocytosis promotes down-regulation (Tsuga et al., 1998; Shockley et al., 1999); thus, increased internalization may contribute to the reduced mAChR levels in AChE^{-/-} mice. In addition, decreased availability of mAChRs at the cell surface and dendrites may provide another mechanism by which postsynaptic neurons respond to long-term diminished synaptic metabolism of ACh. The subcellular distributions of the postsynaptically expressed M₁ and M₄ mAChRs were analyzed in medium spiny neurons of AChE^{+/+} and AChE^{-/-} mice (Fig. 3, a and b). Previous immunohistochemical and electron microscopic studies demonstrate localization of M₁ and M₄ to cell bodies and dendrites of medium spiny neurons of the striatum (Hersch et al., 1994). In the striatum of AChE^{+/+} mice, M₁ and M₄ showed a dendritic neuropil distribution (Fig. 3, +/+). In AChE^{-/-} mice, both M₁ (Fig. 3a) and M₄ (Fig. 3b) showed markedly reduced staining intensity in the neuropil. In addition, the appearance of prominent staining around the nuclei of some neurons in AChE^{-/-} mice indicates increased intracellular localization of the receptors (Fig. 3, -/-).

The substance P receptor (SPR) and the M₂ mAChR are both expressed in cholinergic interneurons of the striatum (Kaneko et al., 1993). Double labeling for M₂ and SPR was used to evaluate the redistribution of mAChRs in neurons of AChE^{-/-} mice. In striatal interneurons of AChE^{+/+} mice, M₂ localized predominantly to the perikaryal cell surface (Fig. 3c) with occasional regions of enhanced immunoreactivity in subdomains at the cell surface and scattered intracellular puncta. M₂ also localized to small puncta throughout the striatum, consistent with localization to presynaptic terminals (Hersch et al., 1994). The SPR showed a continuous distribution along the plasma membrane and colocalized with M₂ within domains along the cell surface (Fig. 3, arrows). In AChE^{-/-} mice, the distribution of the SPR was not altered, but M₂ no longer localized to the cell surface and showed an enhanced intracellular distribution (Fig. 3c, bottom). These data confirm the dramatic redistribution of mAChRs from the cell surface to intracellular compartments in AChE^{-/-} mice. Furthermore, the shifts in subcellular distribution are selective for mAChRs and do not reflect generalized alterations or impairment in G protein-coupled receptor trafficking.

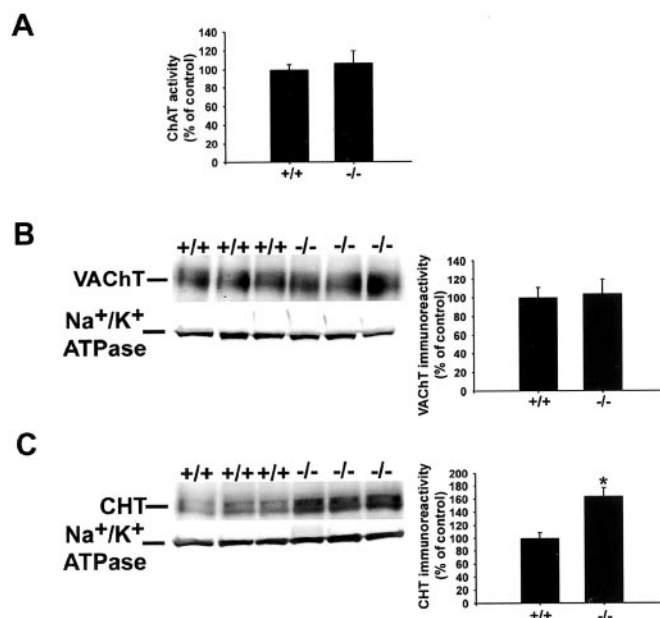


Fig. 1. Protein levels of the high-affinity ChT are increased in the striatum of AChE^{-/-} mice. **A**, ChAT activity was measured in striatal homogenates from 3 individual AChE^{+/+} and 3 AChE^{-/-} mice. The amount of ACh formed in striatal homogenates from AChE^{+/+} mice was equivalent to AChE^{-/-} mice (P = nonsignificant, NS). **B**, VACHT (**B**) and CHT (**C**) immunoreactivities were analyzed in striatal homogenates from 3 individual AChE^{+/+} and 3 AChE^{-/-} mice. Blots were also probed for Na⁺/K⁺ ATPase to demonstrate equivalent loading. For quantitation, VACHT and CHT values were divided by Na⁺/K⁺ ATPase values to normalize for loading. **B**, VACHT immunoreactivity was equivalent between AChE^{+/+} and AChE^{-/-} mice (P = not significant). **C**, CHT immunoreactivity in AChE^{-/-} mice showed a statistically significant increase in immunoreactivity relative to AChE^{+/+} mice (*, P < 0.05).

Altered M_4 Subcellular Localization Results from Enhanced Activity-Dependent Internalization in D1-Dopamine Receptor Expressing Neurons. mRNA studies demonstrate that M_4 is expressed in both D1-expressing striatonigral medium spiny neurons and D2-expressing striatopallidal medium spiny neurons (Yan et al., 2001). D2 is enriched in the striatal matrix in which AChE is also enriched, whereas D1 is more concentrated in AChE-poor striosomes of the striatum (Graybiel, 1990). In AChE $^{+/+}$ mice, M_4 mAChR protein and D2 dopamine receptor protein do not colocalize in the striatum (Fig. 4a). However, M_4 and D1 colocalize extensively in the dendritic neuropil (Fig. 4b), consistent with quantitative electron microscopic studies, demonstrating that approximately 95% of M_4 protein colocalizes with D1 protein-expressing medium spiny neurons, whereas it colocalizes with only 1% of D2 protein-expressing neurons (Ince et al., 1997). Therefore, the redistribution of M_4 in AChE $^{-/-}$ mice occurs selectively in D1-expressing striatonigral medium spiny neurons.

The M_4 redistribution in AChE $^{-/-}$ mice relative to the AChE $^{+/+}$ control mice could result from ACh-induced internalization of the receptors or impaired trafficking of the receptors from biosynthetic compartments to the cell surface. To distinguish among these possibilities, the mice were treated with the mAChR antagonist atropine to prevent ACh-induced mAChR activation and internalization. AChE $^{+/+}$ mice and AChE $^{-/-}$ mice received injections of atropine or saline (total time of treatment was 90 min). In saline-treated AChE $^{-/-}$ mice, M_4 showed reduced localization to dendritic neuropil and enhanced intracellular localization within cell bodies (Fig. 4b). D1 distribution did not seem to be altered in AChE $^{-/-}$ mice. M_4 and D1 showed minimal colocalization in AChE $^{-/-}$ mice as a result of the

altered subcellular distribution of M_4 . After a 90-min treatment with atropine, M_4 in AChE $^{-/-}$ mice showed a neuropil distribution similar to that of saline-treated AChE $^{+/+}$ mice, and M_4 and D1 colocalized extensively.

Quantification of M_4 and D1 colocalization in the striatum showed that M_4 overlap with D1 was significantly reduced by ~52% in saline-treated AChE $^{-/-}$ mice compared with saline-treated AChE $^{+/+}$ mice (Fig. 4c). Treatment with atropine significantly increased M_4 /D1 colocalization in the AChE $^{-/-}$ mice to levels equivalent to those of AChE $^{+/+}$ mice. Atropine-induced recovery of M_4 to basal distributions may result from increased protein expression of M_4 or redistribution of M_4 from intracellular compartments to the cell surface and dendritic neuropil. Immunoblots of M_4 were thus performed after 90-min atropine treatments to evaluate possible recovery of protein levels (Fig. 4d). M_4 immunoreactivity in atropine-treated AChE $^{-/-}$ mice did not increase relative to saline-treated AChE $^{-/-}$ mice. Therefore, inhibiting acetylcholine-induced M_4 activation for 90 min did not increase M_4 protein levels but allowed M_4 to traffic from intracellular compartments back to the dendritic neuropil. Similarly, M_2 also returned to the cell surface of cholinergic interneurons after atropine treatment without an increase in protein levels (data not shown). Overall, these data show that although mAChR localization is dramatically altered in AChE $^{-/-}$ mice, the receptors can rapidly return to a localization similar to control mice, and the redistribution of mAChRs to intracellular compartments in AChE $^{-/-}$ mice results from acetylcholine-induced internalization.

Effects of mAChR Blockade on Locomotor Activity in AChE $^{-/-}$ Mice. To determine whether the altered expression and distribution of striatal mAChRs are reflected in behavioral changes, we examined locomotor activity in the

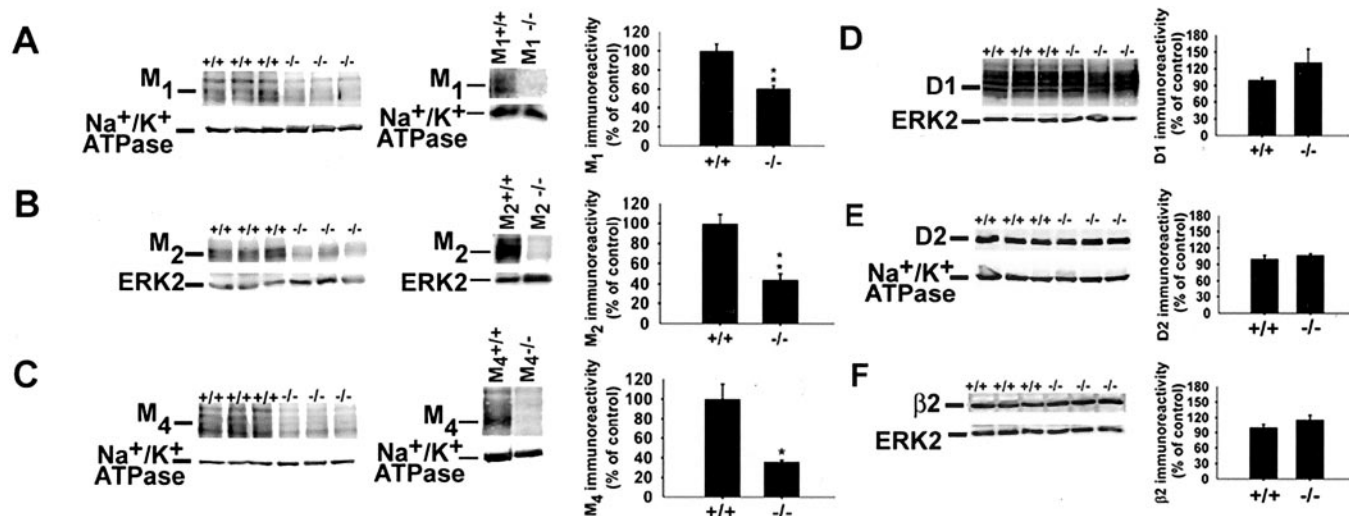


Fig. 2. In the striata of AChE $^{-/-}$ mice, M_1 , M_2 , and M_4 mAChRs show dramatically reduced immunoreactivity, but protein levels of dopamine receptors or the $\beta 2$ subunit of nicotinic ACh receptors are unaltered. Immunoreactivities of M_1 (A), M_2 (B), M_4 (C), D1 dopamine receptor (D), D2 dopamine receptor (E), and the $\beta 2$ subunit of the nicotinic ACh receptor (F) were measured in striatal homogenates from three individual AChE $^{+/+}$ and three AChE $^{-/-}$ mice. Blots were also probed for Na $^{+}$ /K $^{+}$ ATPase or total extracellular signal-regulated kinase 2 (ERK2) to demonstrate equivalent loading. For quantitation of immunoblots, receptor immunoreactivities were normalized to loading controls. A, M_1 showed significantly decreased immunoreactivity in AChE $^{-/-}$ mice ($61 \pm 3\%$) compared with AChE $^{+/+}$ mice ($100 \pm 7\%$) (**, $P < 0.01$). B, M_2 showed significantly decreased immunoreactivity in AChE $^{-/-}$ mice ($44 \pm 6\%$) compared with AChE $^{+/+}$ mice ($100 \pm 9\%$) (**, $P < 0.01$). C, M_4 also showed significantly reduced immunoreactivity in AChE $^{-/-}$ mice ($36 \pm 2\%$) compared with AChE $^{+/+}$ mice ($100 \pm 15\%$) (*, $P < 0.05$). In addition, forebrain homogenates from M_1 , M_2 , and M_4 $^{+/+}$ and M_1 , M_2 , and M_4 $^{-/-}$ mice (Gomez et al., 1999a,b; Gerber et al., 2001) were probed with their respective antibodies. Each mAChR showed immunoreactivity in the $^{+/+}$ mice but not in the corresponding subtype-specific $^{-/-}$ mice, demonstrating antibody specificity. D1 (D) and D2 (E) dopamine receptors showed equivalent immunoreactivities in the striatum of AChE $^{-/-}$ mice relative to AChE $^{+/+}$ mice. The $\beta 2$ subunit of nicotinic ACh receptors (F) also did not show altered immunoreactivity in the striatum of AChE $^{-/-}$ mice compared with AChE $^{+/+}$ mice.

AChE^{-/-} mice. Administration of mAChR antagonists, such as the high-affinity antagonist scopolamine, increases locomotor activity (Sipos et al., 1999), and M₁^{-/-} mice and M₄^{-/-} mice show enhanced locomotor activity (Gomez et al., 1999b; Gerber et al., 2001; Miyakawa et al., 2001). Therefore, the changes M₁ and M₄ in AChE^{-/-} mice may induce similar behavioral changes or cause enhanced sensitivity to the effects of mAChR antagonists on locomotor activity. At baseline, AChE^{+/+} mice and AChE^{-/-} mice did not show a statistically significant difference in locomotor activity (Fig. 5). After administration of a low dose (0.05 mg/kg) of scopolamine, AChE^{-/-} mice showed a dramatic increase in locomotor activity compared with injection of vehicle. The AChE^{+/+} mice did not show increased locomotor activity at this dose, and the difference in locomotor activity between the AChE^{+/+} mice and AChE^{-/-} mice was statistically significant. Administration of 0.5 mg/kg scopolamine increased locomotor activity in both AChE^{+/+} mice and AChE^{-/-} mice, and the two genotypes did not show a statistically significant difference in motor activity after administration of the higher dose of scopolamine. Therefore, AChE^{-/-} mice showed markedly enhanced sensitivity to mAChR antagonist-induced increases in motor behavior.

Discussion

This study shows that long-term lack of AChE activity leads to pre- and postsynaptic changes in molecules involved in cholinergic transmission. In the presynaptic terminal, expression of the CHT is increased in AChE^{-/-} mice relative to AChE^{+/+} mice, with no alterations in ChAT activity or VACHT expression. In postsynaptic, cholinergic neurons in striatum, there is a robust decrease in protein levels of the M₁, M₂, and M₄ mAChRs. These alterations are selective for cholinergic metabotropic receptors because AChE^{-/-} mice show no alterations in levels of D1 and D2 dopamine receptors or the β 2 subunit of nicotinic ACh receptors. In addition, M₁, M₂, and M₄ show dramatic shifts in cell surface and dendritic localizations in AChE^{-/-} mice that are atropine-reversible. The down-regulation and altered distribution of mAChRs results in altered motor function in the AChE^{-/-} mice, with enhanced sensitivity to mAChR antagonist-induced increases in locomotor activity. This is the first study to demonstrate that the CNS responds to complete and selective loss of AChE activity by (1) selective regulation of CHT, which mediates the rate-limiting step in ACh synthesis, and (2) selectively decreasing expression of all mAChR

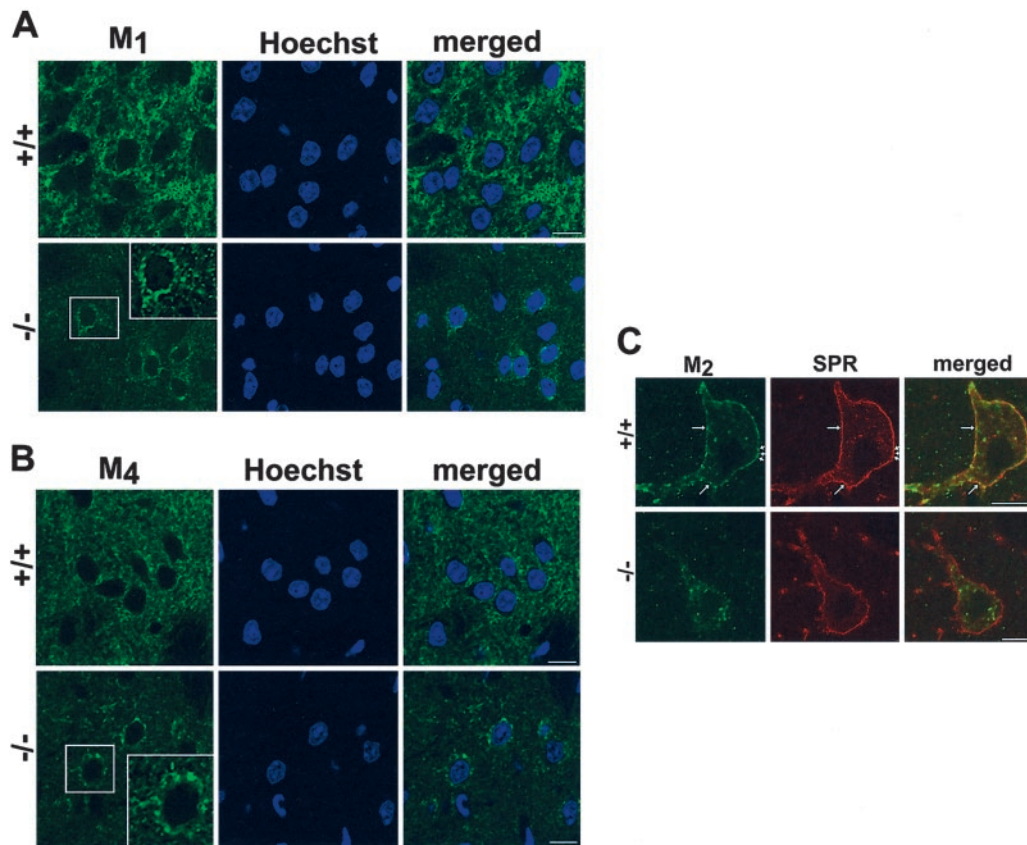


Fig. 3. mAChRs show enhanced intracellular localization within striatal cell bodies of AChE^{-/-} mice. mAChR immunoreactivity in striatal neurons or was assessed in 50-μm coronal sections from the striatum of AChE^{+/+} and AChE^{-/-} mice. M₁, M₂, and M₄ are visualized in green; blue hoechst staining of nuclei allows visualization of the localization of medium spiny neuron cell bodies; and SPR staining (red) allows visualization of the cholinergic interneuron cell surface. A, M₁ in the striatum of AChE^{+/+} mice localized to the dendritic neuropil. In AChE^{-/-} mice, M₁ showed reduced neuropil distribution and enhanced intracellular localization within medium spiny neuron cell bodies. A higher magnification inset in the AChE^{-/-} mice allows visualization of intracellular M₁ within the medium spiny neuron cell body. Scale bar, 10 μm. B, M₄ showed distribution throughout the dendritic neuropil in the striatum of AChE^{+/+} mice. In AChE^{-/-} mice, M₄ showed reduced neuropil distribution and an enhanced intracellular localization. A higher magnification inset in the AChE^{-/-} mice allows visualization of intracellular M₄ within the medium spiny neuron cell body. Scale bar, 10 μm. C, in AChE^{+/+} mice, M₂ localized primarily to the cell surface of cholinergic interneurons, where it colocalized with the SPR within domains (yellow in the merged image, arrows) at the cell surface. In AChE^{-/-} mice, M₂ localized intracellularly and M₂ and the SPR no longer showed colocalization. Scale bar, 10 μm.

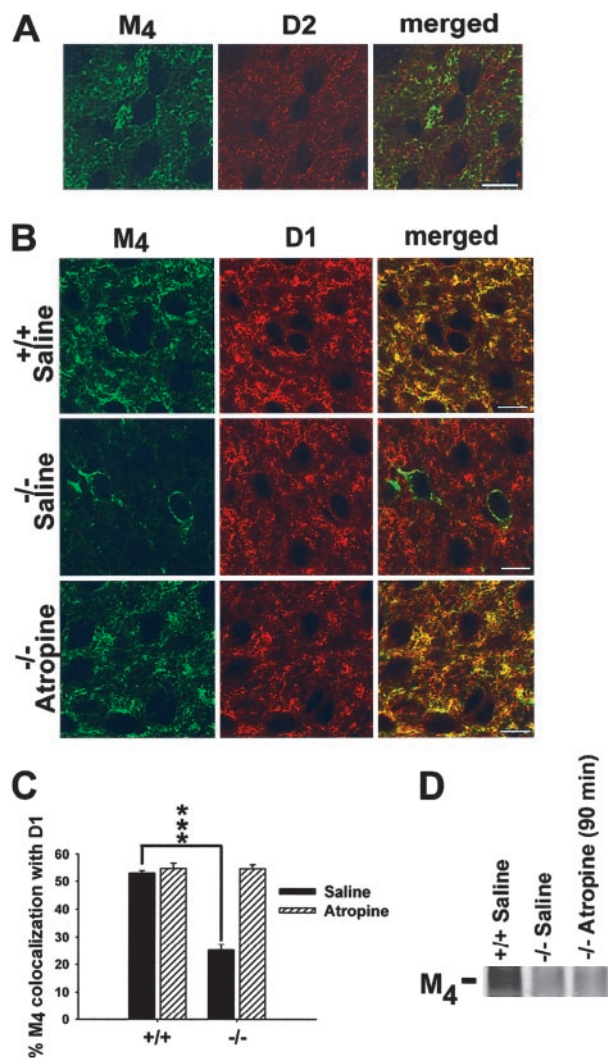


Fig. 4. Inhibiting cholinergic activity in AChE $^{-/-}$ mice allows M₄ recovery to distributions similar to AChE $^{+/+}$ mice. **A**, double-labeling immunofluorescence in AChE $^{+/+}$ mice demonstrated that both M₄ (green) and the D2 dopamine receptor (red) showed neuropil distributions in the striatum. M₄ and the D2 dopamine receptor do not colocalize in the striatum (merged image). **B**, mice received three i.p. injections of either saline or atropine every 30 min. In saline-treated AChE $^{+/+}$ mice, M₄ (green) and D1 (red) localized to the dendritic neuropil of the striatum, where they colocalized (yellow in the merged image). In AChE $^{-/-}$ mice, M₄ showed reduced neuropil staining and an enhanced intracellular localization. The distribution of D1 seemed unaltered in AChE $^{-/-}$ mice; accordingly, M₄ and D1 no longer showed colocalization. In atropine-treated AChE $^{-/-}$ mice, M₄ showed a neuropil distribution similar to that of saline-treated AChE $^{+/+}$ mice, and M₄ and D1 colocalized extensively. Immunofluorescence was performed on sections from 2 individual saline-treated AChE $^{+/+}$ mice and AChE $^{-/-}$ mice, two atropine-treated AChE $^{+/+}$ mice, and three AChE $^{-/-}$ mice [$n = 24$ (eight fields from two individual mice)]. **C**, quantitation of confocal images demonstrated significantly reduced M₄ colocalization with D1 in saline-treated AChE $^{-/-}$ mice [$n = 16$ fields analyzed (eight fields from two individual mice)] relative to saline-treated AChE $^{+/+}$ control mice ($n = 16$) (***, $P < 0.001$; statistically significant difference between saline-treated AChE $^{+/+}$ and AChE $^{-/-}$ mice). Ninety minutes of atropine treatment significantly increased M₄/D1 colocalization such that the extent of overlap in AChE $^{-/-}$ mice [$n = 24$ (eight fields from three mice)] was similar to that of AChE $^{+/+}$ mice ($n = 16$). **D**, immunoblots were performed for M₄ in forebrain homogenates from saline-treated AChE $^{+/+}$ mice, saline-treated AChE $^{-/-}$ mice, and AChE $^{-/-}$ mice treated for 90 min with atropine. M₄ showed reduced immunoreactivity in saline-treated AChE $^{-/-}$ mice compared with saline-treated AChE $^{+/+}$ mice. M₄ immunoreactivity in atropine-treated AChE $^{-/-}$ mice did not increase compared with saline-treated AChE $^{-/-}$ mice.

subtypes expressed in the striatum without affecting nicotinic ionotropic acetylcholine receptors or GPCRs activated by dopamine. These findings have important implications for understanding the regulation of mAChRs in the CNS and the application of AChE inhibitors and mAChR agonists as therapeutic agents.

High-Affinity Choline Transporter Protein Levels Are Increased in Response to Diminished Metabolism of ACh. High-affinity choline uptake is the rate-limiting step in the synthesis of ACh. Similar to AChE $^{-/-}$ mice, mice in which the dopamine transporter gene has been deleted (dopamine transporter $^{-/-}$ mice) show hyperdopaminergic activity (Giros et al., 1996). These mice show reduced expression of tyrosine hydroxylase, the rate-limiting enzyme involved in dopamine synthesis, and the vesicular monoamine transporter, which mediates uptake of dopamine into synaptic vesicles (Jaber et al., 1999). In contrast to the findings in DAT $^{-/-}$ mice, ChAT activity and VACHT expression were not affected in AChE $^{-/-}$ mice. However, AChE $^{-/-}$ mice did show a dramatic increase in CHT expression. Regulation of signaling cascades through presynaptic mAChR activation may be involved in controlling CHT expression, possibly through modulation of cAMP activity (Breer and Knipper, 1990). In addition to synthesis of ACh, presynaptic choline is used for synthesis of phosphatidylcholine, a component of the plasma membrane. Therefore, CHT may be

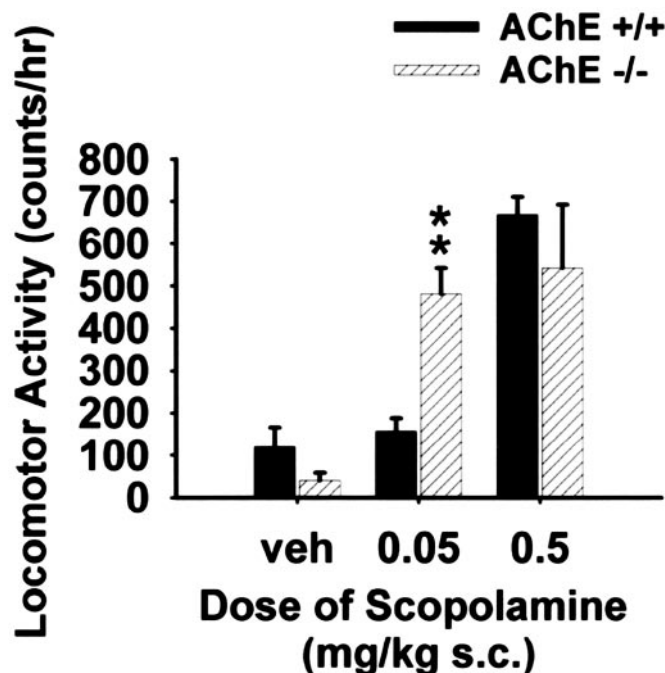


Fig. 5. AChE $^{-/-}$ mice show enhanced sensitivity to the effects of mAChR antagonists on locomotor activity. Vehicle (0.05 mg/kg) or scopolamine (0.5 mg/kg) was injected s.c. into female mice. Locomotor activity was expressed as the total number of times the animal crossed a photobeam per hour. Vehicle-treated AChE $^{+/+}$ mice ($n = 7$) and AChE $^{-/-}$ mice ($n = 6$) did not show statistically significant differences in total locomotor activity. The low dose of scopolamine increased locomotor activity in AChE $^{-/-}$ ($n = 5$) mice but not AChE $^{+/+}$ mice ($n = 5$) (*, $P < 0.01$; significant difference between AChE $^{+/+}$ mice and AChE $^{-/-}$ mice treated with 0.05 mg/kg scopolamine). Administration of the high dose of scopolamine increased locomotor behavior in AChE $^{+/+}$ mice ($n = 10$) and AChE $^{-/-}$ mice ($n = 4$) ($P < 0.001$). There was not a statistically significant difference in locomotor activity between AChE $^{+/+}$ mice and AChE $^{-/-}$ mice after the high dose of scopolamine.

increased as a response to reduced metabolism of ACh into choline and acetic acid in AChE^{-/-} mice.

mAChRs Show Selective Reductions in Protein Levels and Shifts in Subcellular Distribution in AChE^{-/-} mice. The most dramatic response found in AChE^{-/-} mice was decreased immunoreactivity of striatal M₁, M₂, and M₄. Levels of the D1 and D2 dopamine receptors were not altered in AChE^{-/-} mice, demonstrating the selectivity of the effect for cholinergic receptors. Furthermore, levels of the β 2 subunit of the nicotinic receptor was not altered in AChE^{-/-} mice, suggesting that metabotropic mAChRs, but not nicotinic ion channels, are particularly sensitive to persistently diminished ACh metabolism. Northern blot analyses demonstrate that mRNA levels of M₁, M₂, and M₄ are not altered in AChE^{-/-} mice (Li et al., 2003). Therefore, reduced mAChR immunoreactivity most probably results from receptor degradation and not from decreased mAChR synthesis, consistent with electron microscopy studies showing that after long-term stimulation, a proportion of mAChRs localize to intracellular organelles associated with degradation (Liste et al., 2002).

M₁, M₂, and M₄ also show decreased localization to the cell surface and dendrites and an enhanced intracellular distribution in AChE^{-/-} mice. These data support previous findings that the M₂ mAChR shows decreased cell surface localization in AChE^{-/-} mice (Bernard et al., 2003; Decossas et al., 2003) and extend these findings to all the mAChR subtypes predominately expressed in the CNS. Treatment of AChE^{-/-} mice with atropine for 90 min causes mAChR subcellular localization to recover to distributions similar to those of AChE^{+/+} mice, confirming that the shift in mAChR distribution results from ACh-induced internalization, not from impaired trafficking from biosynthetic pathways to the cell surface. The recovery of mAChRs to basal distributions in atropine-treated AChE^{-/-} mice results from a return of intracellular receptors back to the cell surface, not from increased receptor synthesis, demonstrating that internalized mAChRs in neurons can rapidly recycle to the cell surface. Overall, these data indicate that mAChR internalization after long-term stimulation and consequently decreased mAChR localization to dendrites and the cell surface provides a compensatory mechanism by which the CNS responds to persistently enhanced cholinergic stimulation.

mAChR Function Is Impaired in AChE^{-/-} Mice. Decreased expression and receptor redistribution corresponds to altered mAChR function in AChE^{-/-} mice, as demonstrated by enhanced sensitivity to scopolamine-induced increases in locomotor behavior. The increased sensitivity to antagonist probably results from mAChR down-regulation in the striatum. M₁ and M₄ play important roles in the control of locomotor activity, as demonstrated by increased locomotor activity in M₁^{-/-} mice and M₄^{-/-} mice (Gomez et al., 1999b; Gerber et al., 2001; Miyakawa et al., 2001). It has been suggested that M₁ and M₄ stimulation decreases motor behavior by altering activity of the striatopallidal and striatonigral pathways (Gomez et al., 1999b; Miyakawa et al., 2001). Reduction or inhibition of M₁ and M₄ would consequently increase locomotor activity. Low doses of scopolamine increased motor behavior in AChE^{-/-} mice but not in AChE^{+/+} mice. Because mAChR levels are reduced in AChE^{-/-} mice, lower concentrations of antagonist are required to produce increased locomotor activity. Thus,

AChE^{-/-} mice showed enhanced sensitivity to mAChR antagonist-induced increases in motor activity, indicative of functional down-regulation of striatal mAChRs.

Therapeutic Implications. The profound alterations in mAChR expression and subcellular localization after long-term loss of AChE activity in AChE^{-/-} mice have important therapeutic implications for cholinergic drug therapy in humans. Long-term treatment with cholinesterase inhibitors is the primary therapy for Alzheimer's disease (Farlow and Evans, 1998; Francis et al., 1999) and shows promise for other disorders, including dementia associated with Parkinson's disease, Huntington's chorea, and tardive dyskinesia (Davis and Berger, 1978; Mori, 2002; Tammenmaa et al., 2002). Antipsychotic effects of AChE inhibitors have also fostered development of cholinomimetic treatments for schizophrenia (White and Cummings, 1996; Shannon et al., 2000). Because mAChR stimulation by enhanced synaptic activity of ACh most probably mediates the therapeutic response in these conditions, the marked down-regulation of mAChRs after long-term inhibition of AChE activity would limit efficacy of cholinomimetic drug treatment for CNS disorders.

The implications of the findings in this study may also extend beyond mAChRs in the CNS. For example, increased acetylcholine resulting from acetylcholinesterase inhibition will persistently activate mAChRs in the neuromuscular junction, smooth muscles of airway, the heart, exocrine glands, and gastrointestinal and urinary tracts. Therefore, expression and subcellular localization of mAChRs are most probably altered throughout the body. In addition, similar to mAChR activation by cholinomimetics, many clinical treatments persistently stimulate various GPCRs, such as prostaglandins for vascular disease, serotonergic receptors for depression, α -adrenergic receptors for hypertension, and hypothalamic hormones used to treat a wide variety of disorders, ranging from tumors to infertility to diabetes. In fact, dopamine receptors of Parkinson's disease patients show an increased intracellular distribution after prolonged treatment with levodopa (Muriel et al., 1999). Thus, similar to mAChRs, long-term administration of a variety of therapeutics may alter subcellular localization and function of multiple GPCR subtypes.

Although GPCRs may show dramatically altered expression and subcellular localization after long-term drug administration, it may be possible to improve the efficacy of therapies that stimulate GPCRs. For example, we show that the effects on mAChR subcellular localization are rapidly reversible in the AChE^{-/-} mice. Therefore, it is possible that other variables, such as the temporal pattern of cholinesterase inhibitor administration or the degree of inhibition, can influence the internalization, recycling, and down-regulation of mAChRs. In addition, the efficacy of cholinomimetic therapies may be enhanced by the development of selective mAChR ligands that activate the receptors without causing internalization or extensive down-regulation, as has been demonstrated for nonselective mAChR partial agonists (Nathanson et al., 1984) and μ -opioid receptor ligands (Keith et al., 1998). Hence, our findings in AChE-deficient mice identify novel mechanisms that regulate cholinergic neurotransmission, providing potential insights to the therapeutic limitations of long-term inhibition of AChE activity in humans. A better understanding of the factors regulating ex-

pression and localization of molecules involved in cholinergic transmission is important to improve the efficacy of long-term cholinergic drug treatments.

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