Neurobiology of Disease

Deletion of M_1 Muscarinic Acetylcholine Receptors Increases Amyloid Pathology *In Vitro* and *In Vivo*

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Alzheimer's disease (AD) is a progressive neurological disorder that causes dementia and poses a major public health crisis as the population ages. Aberrant processing of the amyloid precursor protein (APP) is strongly implicated as a proximal event in AD pathophysiology, but the neurochemical signals that regulate APP processing in the brain are not completely understood. Activation of muscarinic acetylcholine receptors (mAChRs) has been shown to affect APP processing and AD pathology, but less is known about the roles of specific mAChR subtypes. In this study, we used M_1 mAChR knock-out mice (M_1KO) to isolate the effects of the M_1 mAChR on APP processing in primary neurons and on the development of amyloid pathology in a transgenic mouse model of AD. We demonstrate that the loss of M_1 mAChRs increases amyloidogenic APP processing in neurons, as evidenced by decreased agonist-regulated shedding of the neuroprotective APP ectodomain APPsα and increased production of toxic Aβ peptides. Expression of M_1 mAChRs on the M_1KO background rescued this phenotype, indicating that M_1 mAChRs are sufficient to modulate nonamyloidogenic APP processing. In $APP_{Swe/Ind}$ transgenic mice, the loss of M_1 mAChRs resulted in increased levels of brain Aβ and greater accumulation of amyloid plaque pathology. Analysis of APP metabolites in $APP_{Swe/Ind}$ brain tissue indicates that the loss of M_1 mAChRs increases amyloidogenic APP processing. These results indicate that the M_1 mAChR is an important regulator of amyloidogenesis in the brain and provide strong support for targeting the M_1 mAChR as a therapeutic candidate in AD.

Introduction

Alzheimer's disease (AD) is the most prevalent type of dementia, affecting nearly half of individuals over the age of 85 and causing tremendous emotional distress and economic loss (Evans et al., 1989; Hebert et al., 2003; Alzheimer's Association, 2009). Multiple lines of evidence implicate the amyloid precursor protein (APP) and particularly its aberrant proteolytic processing in the pathophysiology of AD, but less is understood about the exact mechanisms that control APP processing and the production of its neurotoxic β-amyloid (Aβ) derivative *in vivo* (Selkoe et al., 1996; Thinakaran and Koo, 2008).

Because the accumulation of pathogenic Aβ peptides is implicated as a proximal event in AD, it is important to understand the regulatory mechanisms governing their production. Activation of muscarinic acetylcholine receptors (mAChRs) has been shown to stimulate nonamyloidogenic APP processing in cultured cells and brain slices (Nitsch et al., 1992; Farber et al., 1995), and treatment with cholinergic drugs has shown promise in a range of model systems, including trials in human patients (Farber et al., 1995; Beach et al., 2001b; Hock et al., 2003; Caccamo et al., 2006).

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The vast majority of previous studies have relied on agonists and antagonists that are not selective for the five known mAChR subtypes (M_1–M_5). Multiple "M_1-preferring" agonists have shown encouraging results, but they activate other mAChR subtypes in addition to the M_1 mAChR (Haring et al., 1994; DeLapp et al., 1998; Nitsch et al., 2000; Hock et al., 2003). Given the diversity in expression patterns of mAChR subtypes in various cell types throughout the brain, cholinergic regulation of APP processing has the potential to be highly mAChR subtype specific (Buckley et al., 1988; Levey et al., 1991, 1995). Thus, determining the mAChR subtypes responsible for regulating APP processing in the brain is critical for optimizing outcomes and limiting off-target effects.

The lack of subtype selective drugs has also hampered progress in the small number of studies performed *in vivo*. For example, Caccamo et al. (2006) demonstrated that AF267B, claimed to be selective for the M_1 mAChR subtype, reduces amyloid pathology in an AD mouse model. However, Jones et al. (2008) recently showed that this compound activates M_3 mAChRs as or more potently than M_1 mAChRs. Since these two receptors similarly activate signaling pathways and α-secretase processing of APP, the selective role of M_1 receptor-specific regulation of amyloidogenesis *in vivo* remains unknown.

In the present study, we designed experiments to examine the regulation of APP processing by the M_1 mAChR subtype. We demonstrate that the genetic deletion of M_1 receptors results in a loss of cholinergic regulation of APP processing in primary neurons. By crossing APP-transgenic mice with M_1 knock-out mice, we show that M_1 receptor deletion exacerbates amyloid pathol-

ogy *in vivo*. These results establish the M_1 mAChR as a critical regulator of amyloidogenesis *in vivo* and provide a logical foundation for the development of a new generation of M_1-selective drugs for the treatment of AD.

Materials and Methods

Primary neuron culture. Primary cortical neuron cultures were prepared from wild-type mice and M_1 knock-out mice at embryonic day E18. The generation and characterization of these mice has been described previously (Miyakawa et al., 2001). Time-pregnant dams were anesthetized with isoflurane and decapitated. Embryos were dissected and cortical hemispheres were isolated in dissection buffer (HBSS, 10 mM HEPES, 1% penicillin/streptomycin). Tissue was digested with 0.25% trypsin (Invitrogen) and 0.01% DNase in dissection buffer for 15 min at 37°C and rinsed twice with dissection buffer and twice with plating medium [buffered MEM (Invitrogen), 0.6% glucose (Invitrogen), 2 mM L-glutamine (Cellgro), 10% heat-inactivated horse serum (Invitrogen), 1% penicillin/streptomycin]. Tissue was mechanically dissociated by trituration through a fire-polished Pasteur pipette, and viable cells were determined by Trypan blue exclusion. Neurons were plated at a density of 80,000 cells/cm^2 on poly-L-lysine-coated 60 mm culture dishes. Cultures were maintained in Neurobasal medium (Invitrogen) containing B-27 supplement (Invitrogen), 2 mM L-glutamine, and 1% penicillin/streptomycin at 37°C, 5% CO_2. Lentivirus vectors encoding human APP695swe and human M_1 mAChR were added at the time of plating at a multiplicity of infection (MOI) ~1 and allowed to incubate for 72 h before removal. Cytosine arabinoside was added at a final concentration of 5 μM on day 3 *in vitro* to control proliferation of non-neuronal cells.

Neuron viability assay. Viability in lentivirus-transduced neurons was assessed using the CellTiter96 Cell Proliferation (MTS) Assay (Promega). E18 cortical neurons were plated onto poly-L-lysine-coated 96-well culture plates at a density of 50,000 cells/cm^2 and either infected with hAPP lentivirus (MOI = 1) or mock-infected, and allowed to incubate for 72 h. The MTS assay was performed according to the manufacturer's instructions, and plates were read on a SpectraMax Plus plate reader (Molecular Devices).

$APP_{Swedish/Indiana}$ × M1KO mice. Line J20 transgenic mice expressing human amyloid precursor protein incorporating the Swedish and Indiana mutations ($APP_{Swe/Ind}$) were generously provided by Dr. Lennart Mucke (The Gladstone Institute of Neurological Disease, San Francisco, CA) and have been previously described (Mucke et al., 2000). $APP_{Swe/Ind}$ heterozygous mice were bred to $M_1^{-/-}$ mice, and the resulting offspring were then crossed to $M_1^{+/-}$ mice to generate $M_1^{+/+}$ and $M_1^{-/-}$, $APP_{Swe/Ind}$ littermates for analysis.

Primary antibodies. Antibodies used in this study included: 6E10 (APP Aβ domain, Signet), C8 (APP C terminus, gift from Dr. Dennis Selkoe, Harvard Medical School, Boston, MA), Aβ42 [rabbit polyclonal, BioSource (Invitrogen)], β-actin (goat polyclonal, Santa Cruz Biotechnology), and EF1α (Millipore).

Tissue collection. Animals were killed by sodium pentobarbital overdose and perfused with normal saline. Brains were rapidly removed and sectioned along the sagittal plane. One hemibrain was immersion fixed in 4% buffered paraformaldehyde, and cerebral cortex and hippocampus were isolated from the other hemibrain, snap frozen in liquid nitrogen, and stored at −80°C until analysis. Individual tissue fractions were not subjected to more than one freeze/thaw cycle.

Sequential amyloid extraction. Cortical hemispheres were homogenized using a Dounce tissue grinder (Kontes) in PBS with protease inhibitor cocktail (Roche) and sonicated (~30 s at level 7 using a Branson Sonifier 250, Krackeler Scientific) in the presence of 2% SDS, then pelleted by centrifugation for 1 h at 100,000 × *g* at 8°C in an Optima TLX Ultracentrifuge (Beckman-Coulter). The supernatant was collected and the pellet resuspended in an equal volume of 70% formic acid (FA) and resonicated. FA-soluble fractions were neutralized using 1.0 M Tris, pH 11. SDS-soluble and neutralized FA-soluble fractions were diluted in ELISA sample diluent (50 mM Tris base, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate, 0.1 mg/ml phenylmethylsulfonyl fluoride, protease inhibitor cocktail, pH 7.4).

Tissue fractionation. Cortical hemispheres were homogenized in PBS with protease inhibitor cocktail using a Dounce tissue grinder at a concentration of 100 mg/ml. Soluble proteins were isolated from membrane fractions by differential centrifugation. Crude homogenates were centrifuged at 1000 × *g* to remove nuclei and debris (P1). The supernatant (S1) was centrifuged at 10,000 × *g* for 20 min to isolate larger organelles and membrane proteins (P2), and the resulting supernatant (S2) was subjected to centrifugation at 100,000 × *g* to enrich for soluble proteins (S3). The P2 fraction was rinsed with 500 mM NaCl to remove membrane associated proteins and further centrifuged at 10,000 × *g* for 20 min to pellet membrane proteins (P2′). The washed membrane proteins were then incubated with detergent buffer (50 mM Tris, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate) and solubilized proteins recovered by centrifugation at 15,000 × *g* for 5 min. All steps were performed at 4°C.

Western blotting. Cell lysates, conditioned media, and fractionated proteins from cortical homogenates were prepared in Laemmli sample buffer (Laemmli, 1970), separated by SDS-PAGE, and transferred to PVDF Immobilon-P membranes (Millipore). Membranes were blocked at room temperature and incubated with primary antibodies overnight at 4°C. Blots were rinsed and incubated with fluorophore-conjugated secondary antibodies (Invitrogen and Rockland) for 1 h at room temperature. Blots were imaged and band intensities were quantified using an Odyssey Image Station (LI-COR).

ELISA measurement of Aβ peptides. $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels in conditioned media and tissue homogenates were measured using hAmyloid ELISA (HS) kits (The Genetics Company) according to the manufacturer's instructions. Plates were read at 450 nm on a Spectra Max Plus plate reader (Molecular Devices).

Histochemical amyloid plaque analysis. Sagittal hemibrains were immersion fixed with 4% paraformaldehyde for 2 h at 4°C, cryoprotected in 30% sucrose, and sectioned at 50 μm on a freezing-sliding microtome. For thioflavin S plaque staining, sections were mounted on glass slides, treated with 1% thioflavin S solution for 10 min, and rinsed in 80% ethanol and water. For $A\beta_{42}$ immunohistochemistry, free floating sections were fixed with 2% glutaraldehyde, treated with sodium borohydride to quench unreacted glutaraldehyde, and incubated with 70% formic acid to retrieve antigens. Following treatment with hydrogen peroxide, sections were blocked with normal serum and incubated with an anti-$A\beta_{42}$ antibody overnight at 4°C. Sections were then incubated with a biotinylated secondary antibody and signal was visualized using the avidin-biotin-peroxidase complex method (Vector Laboratories) with diaminobenzidine. Mounted sections were dehydrated with sequential ethanol and Histoclear and images were captured using an Olympus BX51 microscope and Olympus software. Quantitation of extent of amyloid pathology was performed as previously described (Dodson et al., 2008). Briefly, thioflavin-stained plaques were manually counted in a blinded manner using MetaMorph image analysis software (Molecular Devices). Total amyloid burden was quantified by measuring $A\beta_{42}$-immunopositive surface area in a blinded manner using MetaMorph image analysis software. Plaque quantitation is shown as the mean plaque number or surface area per section as determined from 4 sagittal sections distributed evenly across ~1 mm of tissue.

Statistical analysis. All statistical comparisons were performed using Prism 4.0 software (GraphPad). Primary neuron culture APPs, CTF, and Aβ levels were analyzed using paired *t* tests and repeated-measures ANOVA followed by Dunnett's multiple-comparison test. Two-way ANOVA was performed to detect M_1 genotype effect on Aβ accumulation in $APP_{Swe/Ind}$ mice across age groups, followed by Mann–Whitney nonparametric *t* tests to compare Aβ levels within each age group and histochemical measures of amyloid plaque pathology. Levels of APP metabolites in cortical homogenates were compared using unpaired *t* tests.

Results

APP is a large type I transmembrane protein that can be proteolytically cleaved by two competing enzymatic processes: a nonamyloidogenic pathway initiated by α-secretase cleavage that results in the shedding of a soluble ectodomain termed "APPsα" and precludes the generation of Aβ, and an amyloidogenic path-

way initiated by β-secretase cleavage that gives rise to toxic Aβ peptides, which aggregate and ultimately deposit as amyloid plaques. To test whether the M_1 mAChR subtype is necessary for cholinergic regulation of nonamyloidogenic APP processing in cells relevant to AD, we performed a series of experiments in primary neurons. Because AD is an intrinsically human disease, we chose to study the processing of human sequence APP, which differs from the rodent sequence by three amino acid substitutions within the Aβ domain and likely accounts for the development of AD pathology in humans and other species that share this sequence. Cortical neurons from E18 embryonic wild-type and M_1KO mice were cultured *in vitro* and transduced with a lentivirus vector to achieve expression of human sequence APP (hAPP). Because of the high efficiency of retroviral gene delivery, a low copy number of transgene per target cell is sufficient to achieve a high percentage of transduced cells. As shown in Figure 1*A*, lentiviral transduction of mouse cortical neuron cultures resulted in efficient and consistent hAPP expression at levels approximately twofold to threefold over basal. To assess potential toxicity induced by lentivirus transduction of primary neurons, we performed an MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium] assay on neurons infected with hAPP lentivirus. Levels of formazan reaction product were not different between treatment groups (mock infection = 100% ± 13.8, hAPP = 104% ± 13.7, $p > 0.05$), indicating that lentivirus infection does not cause measurable toxicity in our system.

To measure mAChR-regulated APP processing, wild-type neurons were allowed to condition medium for 8 h in the presence or absence of the nonselective mAChR agonist carbachol (CCh, 100 μM). Western blot analysis of conditioned media samples and cell lysates showed a significant increase in levels of APPsα (66%) and the APP C-terminal fragment resulting from α-secretase cleavage (CTFα) (68%) following carbachol stimulation (Fig. 1*B*,*D*). Pretreatment with the muscarinic receptor antagonist atropine (1 μM) blocked the CCh-induced increase in APPsα (vehicle = 100 ± 13%, atropine plus CCh = 102 ± 29%). Levels of CTFβ were unchanged following CCh treatment ($p = 0.22$). Expression of full-length APP was not altered between treatment groups (vehicle = 100% ± 20.5, CCh = 110% ± 9.59; $p = 0.28$). This result is consistent with other reports from cultured cells and brain slices (Nitsch et al., 1992; Farber et al., 1995), and indicates that the cellular machinery required for mAChR-mediated signaling and APP processing is intact and functional in the primary neuron culture system.

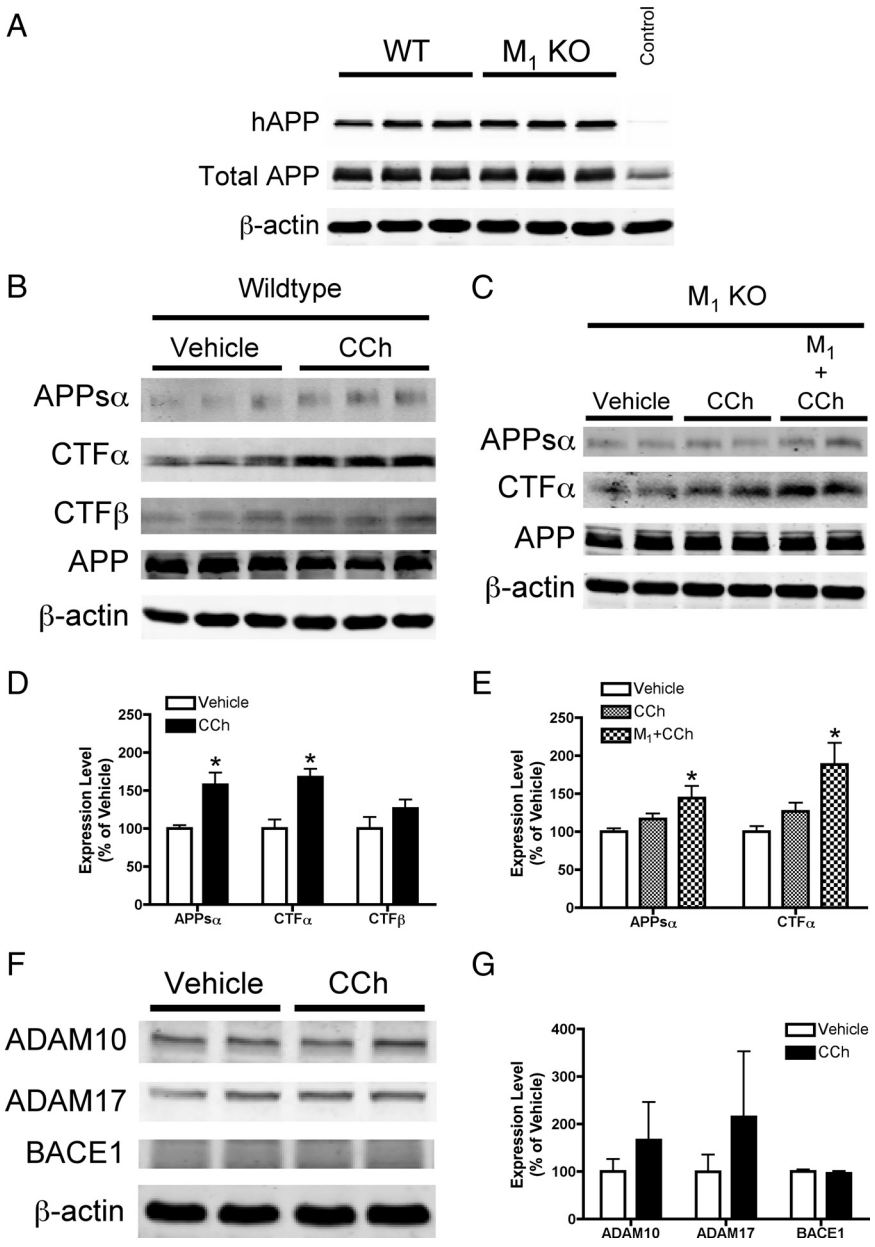

Figure 1. M_1 mAChR regulation of APP processing in primary neurons. ***A***, Western blot analysis of cell lysates from primary cortical neuron cultures derived from wild-type (WT) and M_1 mAChR knock-out (M_1KO) mice and transduced with a lentiviral vector encoding human sequence amyloid precursor protein (hAPP). A virus encoding green fluorescent protein was used as a control. The human-specific APP antibody 6E10 was used to detect expression of hAPP, and total APP (human plus endogenous murine) was detected using C8 (a C-terminal APP antibody). β-Actin is shown as a loading control. ***B***, ***D***, Representative Western blots and densitometric analysis of conditioned media and cell lysates from WT neuron cultures treated with the mAChR agonist CCh (100 μM). Quantitation of band intensity shows a 66% increase in APPsα shedding into conditioned media of WT neuron cultures treated with CCh ($p < 0.05$ vs vehicle), and a 68% increase in the level of CTFα ($p < 0.01$). Data are shown as mean ± SEM from three to five independent experiments. ***C***, ***E***, Western blots and densitometry from conditioned media and cell lysates from M_1KO neuron cultures. APPsα and CTFα levels were unchanged in cultures treated with CCh. In M_1KO neuron cultures transduced with an M_1 mAChR lentivirus, CCh treatment resulted in a 44% increase in APPsα shedding into conditioned media ($p < 0.05$), and an 88% increase in the levels of CTFα ($p < 0.05$). Data are shown as mean ± SEM of three independent experiments. ***F***, ***G***, Western blot and densitometric analysis demonstrates no change in levels of ADAM10 ($p = 0.16$), ADAM17 ($p = 0.16$), or BACE1 ($p = 0.34$) with CCh treatment in WT neuron cultures. Data are shown as the mean ± SD.

Having established regulated APP cleavage in wild-type neurons, we performed a similar experiment using neurons from M_1KO mice. As shown in Figure 1, *C* and *E*, deletion of the M_1 receptor results in a significant reduction in the amount of CCh-

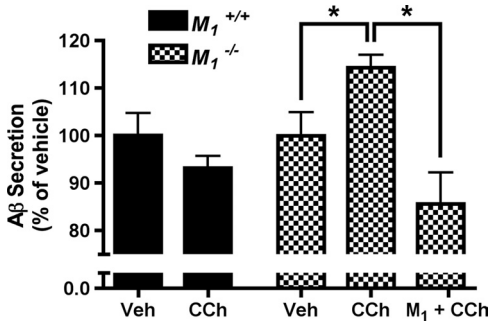

Figure 2. Aβ peptide levels in conditioned media from WT and M_1KO primary neuron cultures. In WT neurons, CCh treatment (100 μM) resulted in a trend toward decreased Aβ production, although this difference did not reach statistical significance. In M_1KO neurons, CCh treatment caused an increase in Aβ production ($p < 0.05$ vs vehicle). Following rescue of M_1KO neuron cultures by transduction with an M_1 lentivirus, CCh treatment resulted in a significant reduction in Aβ levels compared to CCh-treated M_1KO neurons ($p < 0.01$). Data are shown as mean ± SEM from three independent experiments.

stimulated APP processing, with no statistically significant difference in APPsα or CTFα between CCh- and vehicle-treated neurons. To test whether M_1 receptor activation is sufficient to regulate nonamyloidogenic APP processing, we exogenously expressed the M_1 mAChR subtype on the M_1 mAChR knock-out background. Total mAChR levels in M_1 mAChR KO cultures measured by [^{3}H]-NMS binding were reduced by 69% compared to WT levels, consistent with previous studies quantifying the relative abundance of mAChR subtypes in rodent brain (Levey et al., 1991). Total mAChR levels in M_1KO neurons transduced with M_1 lentivirus were increased by 156% compared to WT. In M_1KO neurons transduced with both human APP and M_1 mAChR viruses, carbachol stimulation resulted in significant increases in levels of APPsα (increased by 44%, $p < 0.05$) and CTFα (increased by 88%, $p < 0.05$) compared to vehicle-treated neurons (Fig. 1*C*,*E*). Expression of full-length APP was not altered across treatment groups (vehicle = 100% ± 15.8, CCh = 106% ± 7.74, M_1 + CCh = 112% ± 6.45; one-way ANOVA $p = 0.33$). We also measured levels of the putative α-secretase enzymes ADAM10 and ADAM17, as well as BACE1, the enzyme responsible for β-secretase cleavage of APP, in WT neuron cultures treated with CCh. Levels of all three enzymes were statistically similar in CCh-treated versus vehicle-treated neurons, although there was a greater degree of variability in ADAM levels (Fig. 1*F*,*G*).

We next examined the effects of manipulating M_1 mAChR signaling on the production of Aβ. In wild-type neuron cultures, CCh treatment resulted in a trend toward reduction of Aβ secretion, but this effect did not reach statistical significance (Fig. 2). In M_1KO neurons, CCh treatment resulted in a small but significant increase in secretion of total Aβ (increased by 14%, $p < 0.05$ vs vehicle). The fact that CCh stimulation caused a decrease in Aβ secretion in wild-type neurons but an increase in secretion in M_1KO neurons indicates that not only is M_1 mAChR activation necessary for regulated nonamyloidogenic APP processing, but that there are also other mAChR subtypes capable of promoting amyloidogenic APP processing. This result is consistent with a previous report using an M_2/M_4-preferring antagonist to modulate release of APPs in brain slices (Farber et al., 1995). In M_1KO neuron cultures rescued with M_1 lentivirus and stimulated with CCh, Aβ levels were reduced to 85% of baseline levels and were significantly lower than Aβ levels in CCh-treated M_1KO cultures ($p < 0.01$). Together, these data indicate that the M_1 mAChR is essential for carbachol-mediated nonamyloidogenic APP processing in cortical neurons.

Table 1. ELISA measurements of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ peptide levels in $M_1^{+/+}$ and $M_1^{-/-}$ $APP_{Swe/Ind}$ cortex at 3, 6, 12, and 16 months of age

	Age			
	3 months (mean ± SD)	6 months (mean ± SD)	12 months (mean ± SD)	16 months (mean ± SD)
$A\beta_{1\text{-}40}$				
$M_1^{+/+}$	318 ± 90	301 ± 49	4221 ± 2369	7050 ± 9668
$M_1^{-/-}$	287 ± 63	309 ± 161	4002 ± 1726	41,435 ± 19806
p value	0.7396	0.2284	1.0000	0.0190
$A\beta_{1\text{-}42}$				
$M_1^{+/+}$	72 ± 10	55 ± 20	97,038 ± 77,335	148,472 ± 175,542
$M_1^{-/-}$	69 ± 17	92 ± 108	100,188 ± 46,472	583,435 ± 321,325
p value	0.5362	0.8518	0.9048	0.0381

Values include both SDS- and formic acid-soluble Aβ fractions and are shown as the mean ± SD of the absolute concentrations (pg/ml). At 16 months, levels of both $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ are increased in $M_1^{-/-}$ mice compared to $M_1^{+/+}$ littermates ($A\beta_{1\text{-}40}$ increased by 488%, $p < 0.05$; $A\beta_{1\text{-}42}$ increased by 293%, $p < 0.05$).

Given the decreased APPsα shedding and increased Aβ production in primary neurons from M_1KO mice, we designed an *in vivo* experiment to determine the impact of loss of M_1 signaling on the development of amyloid pathology in the brain. Cohorts of $M_1^{+/+}$ and $M_1^{-/-}$ littermates carrying the $APP_{Swe/Ind}$ transgene were generated and aged to 3, 6, 12, and 16 months. Cerebral cortex homogenates were subjected to sequential extraction with SDS and formic acid (FA) to recover Aβ peptides, and levels of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ were determined using sandwich ELISA. $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels in $M_1^{+/+}$ and $M_1^{-/-}$ mice are shown in Table 1. We observed an age-dependent increase in both $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ in both $M_1^{+/+}$ and $M_1^{-/-}$ mice beginning between 6 and 12 months of age, consistent with previous reports of amyloid plaque accumulation in this line of transgenic mice (Mucke et al., 2000). The loss of M_1 mAChRs had a significant effect on the accumulation of Aβ (two-way ANOVA genotype effect $p < 0.0001$). By 16 months of age, $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels in $M_1^{-/-}$ mice were increased by 488 and 293%, respectively, as compared to $M_1^{+/+}$ littermates ($p < 0.05$ for both effects), indicating that the loss of M_1 receptors exacerbates the accumulation of Aβ in the brain. This effect was consistent for both SDS- and FA-soluble fractions of Aβ.

To determine whether the loss of M_1 mAChRs affects the development of amyloid plaque pathology, we performed histochemical evaluation of plaque burden on brain sections from $APP_{Swe/Ind}/M_1^{+/+}$ and $APP_{Swe/Ind}/M_1^{-/-}$ mice. Plaque counts of thioflavin S-positive amyloid deposits (Fig. 3*A*) and surface area measurements of $A\beta_{42}$-immunopositive brain sections (Fig. 3*B*,*C*) were used to quantify amyloid pathology. Consistent with our results from biochemical measurement of Aβ, deletion of M_1 had a significant effect on amyloid plaque pathology at 16 months of age. Plaque counts demonstrated a 227% increase in amyloid plaque number in $M_1^{-/-}$ mice compared to $M_1^{+/+}$ littermates ($p = 0.0159$) at this age (Fig. 3*A*). This effect was consistent for both cortex and hippocampus. Quantitation of amyloid plaque burden by measuring the surface area of $A\beta_{42}$-immunopositive tissue revealed a 129% increase in cortical plaque load in $M_1^{-/-}$ mice compared to $M_1^{+/+}$ littermates (Fig. 3*B*) ($p = 0.0381$). In cortex, the increased accumulation of cortical plaque pathology in $M_1^{-/-}$ mice was most striking in anterior regions (Fig. 3*C*). In the hippocampus, there was no statistically significant difference in total $A\beta_{42}$-immunopositive surface area between $M_1^{+/+}$ and $M_1^{-/-}$ mice.

To investigate the mechanism underlying the observed increased in amyloid pathology in $M_1^{-/-}$ mice, we performed Western blot analysis of APP fragments in cortical tissue homog-

enates from 16-month-old $APP_{Swe/Ind}$ mice. Full-length APP levels were unchanged ($M_1^{+/+}$ = 100.0 ± 13.7% vs $M_1^{-/-}$ = 99.7 ± 12.5%), as were levels of CTFα ($p = 0.65$). Levels of CTFβ were significantly increased in $M_1^{-/-}$ mice compared to $M_1^{+/+}$ mice (Fig. 4*A*) (increased to 157% of wild-type; $p < 0.05$). In contrast to our results from primary neuron experiments, we did not observe significant differences in levels of APPsα ($p = 0.2954$) or APPsβ ($p = 0.1830$). One likely explanation for this discrepancy is that APPs derivatives secreted into brain tissue are presumably cleared into CSF and thus are not retained in the tissue homogenate used for APP metabolite analysis in this study. It is also possible that there is a difference in steady-state brain levels of APP metabolites in aged animals as compared to dynamic changes induced by CCh-treatment in regulated APP processing experiments in primary neurons. Nevertheless, our results from both *in vitro* and *in vivo* experiments strongly indicate that the loss of M_1 mAChRs results in increased accumulation of amyloidogenic APP derivatives, leading to increased Aβ production and amyloid pathology.

As chronic mAChR stimulation has been reported to promote α-secretase cleavage of APP by increasing levels of the α-secretase candidate ADAM17 (Caccamo et al., 2006), we examined the effect of M_1 deletion on steady-state levels of both ADAM10 and ADAM17 in cortex from 16 month-old $M_1^{+/+}$ and $M_1^{-/-}$ mice. As shown in Figure 4, *C* and *D*, we found no difference in expression levels of ADAM10 ($p = 0.56$) and ADAM17 ($p = 0.89$) between $M_1^{+/+}$ and $M_1^{-/-}$ mice.

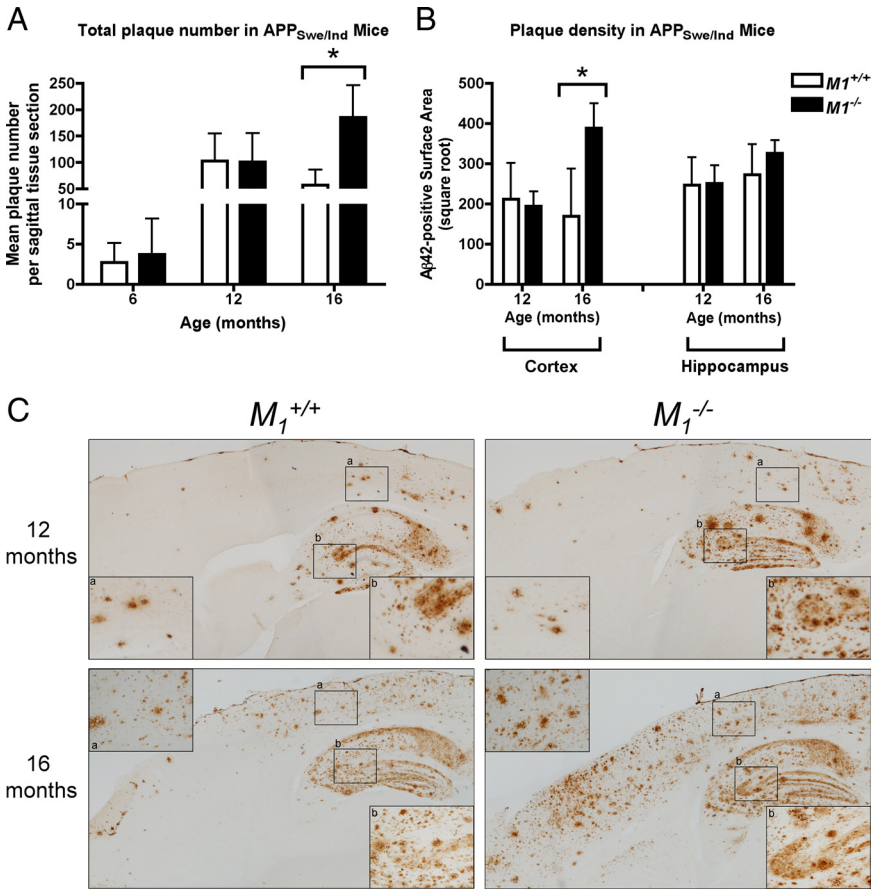

Figure 3. Amyloid pathology in $M_1^{+/+}$ and $M_1^{-/-}$ $APP_{Swe/Ind}$ mice. ***A***, Total number of thioflavin S-positive plaques is significantly increased at 16 months of age in $M_1^{-/-}$ mice (mean ± SD, $p < 0.01$). ***B***, Amyloid plaque density (mean surface area of Aβ_{42}-positive immunoreactivity (pixels) per tissue section) is significantly increased in the cortex of 16-month-old $M_1^{-/-}$ mice (mean ± SD, $p < 0.05$). ***C***, Light micrographs of Aβ_{42}-immunopositive plaques (brown deposits) in $M_1^{+/+}$ and $M_1^{-/-}$ $APP_{Swe/Ind}$ mice at 12 and 16 months of age. High-power magnification corresponding to boxed regions of cortex (***a***) and hippocampus (***b***) are shown as insets.

Discussion

In the present study, we investigated the role of the M_1 mAChR in regulating amyloidogenesis in primary neurons and in the development of amyloid pathology in a transgenic mouse model of AD. While previous studies have established that M_1 mAChR overexpression and semiselective agonists with preferential activation of M_1 and other mAChR subtypes enhance α-secretase processing of APP, our study is the first to use genetic models to definitively isolate the M_1 mAChR subtype, thus avoiding the ambiguity associated with nonselective agonists. Here we show that the M_1 mAChR is necessary and sufficient to regulate nonamyloidogenic APP processing in primary cortical neurons. Furthermore, we demonstrate that $APP_{Swe/Ind}$ transgenic mice lacking M_1 mAChRs develop increased amyloid pathology as measured by increased brain Aβ levels and amyloid plaque burden. APP metabolite analysis in brain tissue from aged $APP_{Swe/Ind}$ mice suggests that this exacerbation of pathology is due to increased amyloidogenic processing of APP. Together, these data validate the M_1 mAChR as a critical regulator of amyloidogenesis *in vivo*.

Our analysis of APP metabolites in CCh-stimulated primary neuron cultures has important implications regarding the mechanism by which M_1 mAChR signaling may influence neuronal physiology. We observed the largest changes in levels of APPsα and CTFα, the products of α-secretase cleavage. The APPsα ectodomain has been shown to be neuroprotective in some systems, and may play a role in memory enhancement, possibly by facilitating synapse formation (Mattson et al., 1993; Meziane et al., 1998; Bell et al., 2008). A recent study has also proposed a role for APPsα in the disruption of APP dimers on the cell surface, which the authors argue is important for regulating cell survival (Gralle et al., 2009). Regardless of the combination of mechanisms by which APPsα exerts a beneficial effect in the CNS, it is logical to conclude that signaling pathways that promote its secretion may be important for normal physiological brain function.

One potential explanation for our observed effects of M_1 activation on APP processing is through modulation of one or more of the secretase enzymes. As M_1 activation had the largest effect on α-secretase-mediated processing events, it is likely that the mechanism involves α-secretase, either through direct activation or by regulating traffic of ADAM enzymes and/or APP substrate. The cell biology of APP trafficking has been the focus of much study (for review, see (Thinakaran and Koo, 2008)), but further work will be required to appreciate exactly how M_1 activation may participate in this process. We did not observe an acute effect of M_1 activation on regulation of ADAM expression levels, nor did we find that deletion of M_1 altered ADAM levels *in*

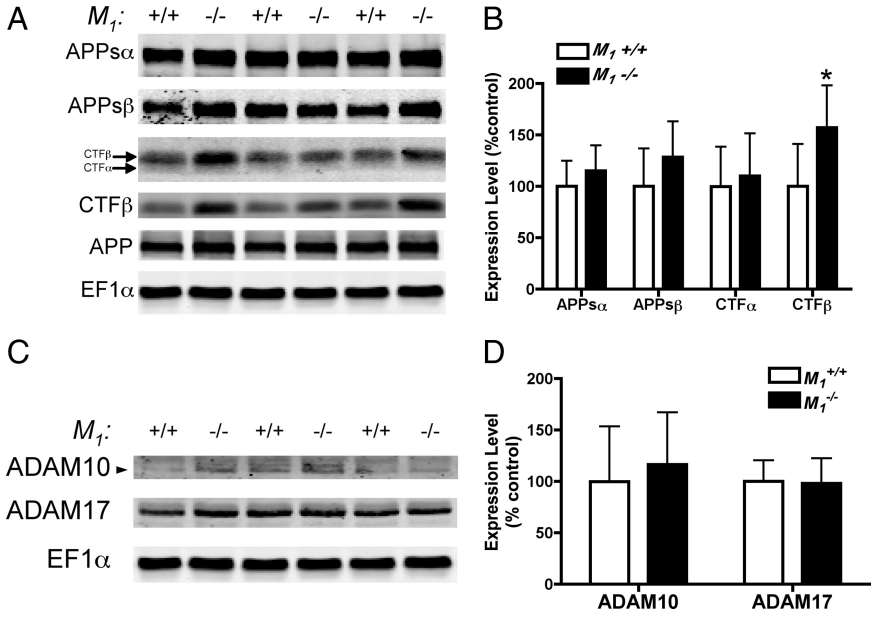

Figure 4. ***A***, ***B***, Immunoblot analysis of APP metabolites in $M_1^{+/+}$ and $M_1^{-/-}$ $APP_{Swe/Ind}$ cortex. Membrane and soluble proteins from cortical homogenates of $M_1^{+/+}$ ($n = 7$) and $M_1^{-/-}$ ($n = 6$) mice were fractionated by differential centrifugation and subjected to SDS-PAGE and Western blotting with antibodies to multiple APP metabolites. Representative immunoblots are shown probed with 6E10 to detect full-length APP, APPsα, and CTFβ, 192swe to detect the Swedish mutation form of APPsβ, and C8 to detect both CTFα and CTFβ. EF1α is shown as a loading control. For quantitation, APPs levels were normalized to EF1α, and CTF levels were normalized to full-length APP. Densitometric quantitation revealed a 57% increase in CTFβ in $M_1^{-/-}$ mice (mean ± SD, $p < 0.05$). ***C***, ***D***, Immunoblot analysis of ADAM10 and ADAM17 in $M_1^{+/+}$ and $M_1^{-/-}$ $APP_{Swe/Ind}$ cortex. Levels of both proteins were unchanged in $M_1^{+/+}$ compared to $M_1^{-/-}$ (data shown as mean ± SD).

vivo, but it is possible that chronic mAChR stimulation may induce ADAM upregulation, as suggested by Caccamo et al. (2006).

We also found that M_1 mAChR activation by CCh induced a trend toward decreased Aβ secretion in wild-type neurons, and that CCh actually increased Aβ secretion in M_1KO neurons. This finding suggests that other mAChR subtypes may stimulate amyloidogenic APP processing and agrees with a previous report showing that an M_2/M_4-preferring antagonist can potentiate CCh-stimulated APPs release from brain slices (Farber et al., 1995). These data indicate that mAChR signaling may be important for regulating multiple aspects of APP processing and amyloidogenesis in neurons, and therefore, that a loss of M_1 mAChR signaling may have multiple deleterious consequences in the context of AD pathogenesis. Previous work has also demonstrated that nicotinic acetylcholine receptors (nAChRs) can modulate APP processing, and carbachol is capable of activating nAChRs at high concentrations. However, nAChR stimulation has been associated with decreased, not increased, Aβ secretion, indicating that nAChR activation is not responsible for the increase in Aβ secretion we observed in CCh-stimulated M_1KO neurons (Kim et al., 1997; Lenzken et al., 2007; Nie et al., 2008).

Our data from $APP_{Swe/Ind}$ mice represent the first assessment of M_1 mAChR loss on the development of amyloid pathology *in vivo*. We demonstrate that M_1 mAChR deletion results in increased levels of pathogenic Aβ peptides in brain, as well as increased accumulation of amyloid plaque pathology. These findings are consistent with the important role that the M_1 mAChR plays in regulating APP processing as well as reports from several model systems, including human data, demonstrating that manipulation of mAChR signaling can modulate the development of amyloid pathology *in vivo* (Beach et al., 2000, 2001a,b; Nitsch et al., 2000; Perry et al., 2003; Caccamo et al., 2006). Our results from analysis of APP metabolites in aged $APP_{Swe/Ind}$ mice support the conclusions drawn from cellular models, implicating the M_1 mAChR as a pivotal regulator of nonamyloidogenic APP processing in the brain. What is less clear, however, is the mechanism by which loss of M_1 signaling results in increased brain Aβ levels and amyloid plaque pathology. Increased steady-state levels of Aβ in the brain could be the result of either increased Aβ production, or decreased Aβ clearance and/or degradation. Clearance of Aβ peptides, and the effects of this process on amyloid pathology, are areas of intense focus in AD research, and it is not known at this point how cholinergic signaling may participate in this process.

Further research, including follow-up studies in APP transgenic mice, will be required to more fully understand the implications of M_1 mAChR regulation of amyloidogenesis in the brain. In addition to the observed effects on amyloidogenesis, it will be important to investigate whether loss of M_1 mAChRs has an effect on learning and memory impairment in APP-transgenic mice. Accumulation of neurotoxic Aβ species impairs synaptic function (Walsh et al., 2005) and multiple lines of APP-transgenic mice show deficits in learning and memory tasks (Woodruff-Pak, 2008), so it is logical to hypothesize that the increase in amyloid pathology induced by deletion of M_1 would exacerbate cognitive deficits. Given the role that the M_1 mAChR plays in certain aspects of working memory and memory consolidation (Anagnostaras et al., 2003), the loss of M_1 mAChR signaling accompanied by increased accumulation of amyloid pathology may have an additive detrimental effect on cognition.

In conjunction with studies examining the effects of M_1 mAChR deletion on amyloid pathology and memory impairment, it will be important to evaluate the potential for M_1-selective agonists in reducing amyloid pathology and promoting cognitive processes. All of the mAChR-targeted therapies tried to date have shown only modest efficacy for AD symptoms, but it remains to be seen whether newer generations of M_1-selective agonists will be able to offer more meaningful therapeutic benefit. Our data from cultured cells indicates that M_1-selective agonists are effective at promoting nonamyloidogenic APP processing and are therefore excellent candidates for therapies aimed at reducing amyloid pathology *in vivo* (Jones et al., 2008).

The findings of the present study validate the long-standing hypothesis that the M_1 mAChR is an important regulator of APP processing in the brain. Our approach using M_1KO mice is the first of its kind to genetically isolate the M_1 mAChR subtype, circumventing the limitations imposed by the use of semiselective mAChR agonists in previous studies. We observed that M_1 mAChR loss decreased the shedding of the neuroprotective APPsα molecule in primary neurons, and increased the production of neurotoxic Aβ in primary neurons and *in vivo*, ultimately exacerbating amyloid pathology in a transgenic mouse model of AD. These data suggest that the M_1 mAChR may regulate multi-

ple aspects of neuronal physiology and AD pathology, emphasizing the importance of drug development to target molecules with disease-modifying potential, including M_1. The intimate relationship between the M_1 mAChR and higher cognitive functions including working memory and consolidation further underscores the potential benefit of M_1-based therapies for AD and other cognitive disorders.

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