Deletion of M1 muscarinic acetylcholine receptors increases amyloid pathology in vitro and in vivo

Albert A. Davis, Emory University
Jason Jon Fritz, Emory University
Jurgen Wess, National Institutes of Health
James J Lah, Emory University
Allan I Levey, Emory University

Journal Title: Journal of Neuroscience Nursing
Volume: Volume 30, Number 12
Publisher: Society for Neuroscience | 2010-03-24, Pages 4190-4196
Type of Work: Article | Post-print: After Peer Review
Publisher DOI: 10.1523/JNEUROSCI.6393-09.2010
Permanent URL: http://pid.emory.edu/ark:/25593/fh8vr

Final published version: http://www.jneurosci.org/content/30/12/4190

Copyright information:
© 2010 the authors

Accessed December 12, 2019 6:40 PM EST
Deletion of M₁ muscarinic acetylcholine receptors increases amyloid pathology in vitro and in vivo

Albert A. Davis¹, Jason J. Fritz¹, Jürgen Wess², James J. Lah¹, and Allan I. Levey¹
¹Center for Neurodegenerative Disease and Department of Neurology, Emory University School of Medicine, Atlanta, GA 30322, USA
²Molecular Signaling Section, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA

Abstract

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 (mACChRs) has been shown to affect APP processing and AD pathology, but less is known about the roles of specific mACChR subtypes. In this study, we used M₁ mACChR knockout mice (M₁KO) to isolate the effects of the M₁ mACChR 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 M₁ mACChRs 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 M₁ mACChRs on the M₁KO background rescued this phenotype, indicating that M₁ mACChRs are sufficient to modulate non-amyloidogenic APP processing. In APPSwe/Ind transgenic mice, the loss of M₁ mACChRs resulted in increased levels of brain Aβ₁-40 and greater accumulation of amyloid plaque pathology. Analysis of APP metabolites in APPSwe/Ind brain tissue indicates that the loss of M₁ mACChRs increases amyloidogenic APP processing. These results indicate that the M₁ mACChR is an important regulator of amyloidogenesis in the brain and provide strong support for targeting the M₁ mACChR as a therapeutic candidate in AD.

Keywords

muscarinic acetylcholine receptor; β-amyloid; amyloid precursor protein; Alzheimer’s disease; transgenic mouse; neurotransmitter

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; 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 \textit{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 non-amyloidogenic 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).

The vast majority of previous studies have relied on agonists and antagonists that are not selective for the five known mACHR subtypes (M1-M5). Multiple “M1-preferring” agonists have shown encouraging results, but they activate other mAChR subtypes in addition to the M1 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; Levey et al., 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 \textit{in vivo}. For example, Caccamo et al. demonstrated that AF267B, claimed to be selective for the M1 mAChR subtype, reduces amyloid pathology in an AD mouse model (Caccamo et al., 2006). However, Jones et al. recently showed that this compound activates M3 mAChRs as or more potently than M1 mAChRs (Jones et al., 2008). Since these two receptors similarly activate signaling pathways and α-secretase processing of APP, the selective role of M1 receptor-specific regulation of amyloidogenesis \textit{in vivo} remains unknown.

In the present study, we designed experiments to examine the regulation of APP processing by the M1 mAChR subtype. We demonstrate that the genetic deletion of M1 receptors results in a loss of cholinergic regulation of APP processing in primary neurons. By crossing APP-transgenic mice with M1 knockout mice, we show that M1 receptor deletion exacerbates amyloid pathology \textit{in vivo}. These results establish the M1 mAChR as a critical regulator of amyloidogenesis \textit{in vivo} and provide a logical foundation for the development of a new generation of M1-selective drugs for the treatment of AD.

\textbf{Materials and Methods}

\textbf{Primary Neuron Culture}

Primary cortical neuron cultures were prepared from wildtype mice and M1 knockout 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 (Hanks Balanced Salt Solution (HBSS), 10 mM HEPES, 1% penicillin/streptomycin). Tissue was digested with 0.25% trypsin (Gibco) and 0.01% deoxyribonuclease in dissection buffer for 15 minutes at 37°C and rinsed twice with dissection buffer and twice with plating medium (buffered MEM (Gibco), 0.6% glucose (Gibco), 2 mM L-glutamine (Cellgro), 10% heat-inactivated horse serum (Gibco), 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/cm² on poly-L-lysine coated 60mm culture dishes. Cultures were maintained in Neurobasal medium (Gibco) containing B-27 supplement (Gibco), 2 mM L-glutamine, and 1% penicillin/
streptomycin at 37°C, 5% CO₂. Lentivirus vectors encoding human APP695swe and human M₁ mAChR were added at the time of plating at a multiplicity of infection (MOI) ~1 and allowed to incubate for 72 hours 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, Madison, WI). E18 cortical neurons were plated onto poly-L-lysine coated 96-well culture plates at a density of 50,000 cells/cm² and either infected with hAPP lentivirus (MOI=1) or mock-infected, and allowed to incubate for 72 hours. The MTS assay was performed according to the manufacturer’s instructions, and plates were read on a SpectraMax Plus plate reader (Molecular Devices, Sunnyvale, CA).

**APP<sub>Swe</sub>/Ind × M₁KO mice**

Line J20 transgenic mice expressing human amyloid precursor protein incorporating the Swedish and Indiana mutations (APP<sub>Swe/Ind</sub>) 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<sub>Swe/Ind</sub> heterozygous mice were bred to M₁<sup>−/−</sup> mice, and the resulting offspring were then crossed to M₁<sup>+/−</sup> mice to generate M₁<sup>+/+</sup> and M₁<sup>−/−</sup>, APP<sub>Swe/Ind</sub> littermates for analysis.

**Primary Antibodies**

Antibodies used in this study included: 6E10 (APP Aβ domain, Signet, Dedham, MA), C8 (APP C-terminus, gift from Dr. Dennis Selkoe, Harvard Medical School, Boston, MA), AB42 (rabbit polyclonal, BioSource (Invitrogen), Carlsbad, CA), β-actin (goat polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA), and EF1α (Upstate/Millipore, Billerica, MA).

**Tissue Collection**

Animals were euthanized 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, Vineland, NJ) in phosphate-buffered saline with protease inhibitor cocktail (Roche, Indianapolis, IN) and sonicated (~30 seconds at level 7 using a Branson Sonifier 250, Krackeler Scientific, Inc., Albany, NY) in the presence of 2% sodium dodecyl sulfate (SDS), then pelleted by centrifugation for 1 hour at 100,000 × g at 8°C in an Optima TLX Ultracentrifuge (Beckman-Coulter, Fullerton, CA). The supernatant was collected and the pellet resuspended in an equal volume of 70% formic acid (FA) and re-sonicated. 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 phosphate-buffered saline 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 minutes to isolate larger organelles and membrae 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 minutes 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 carried out 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, Billerica, MA). 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 (Molecular Probes, Eugene, OR and Rockland, Gilbertsville, PA) for one hour at room temperature. Blots were imaged and band intensities were quantified using an Odyssey Image Station (LI-COR, Lincoln, NE).

ELISA measurement of Aβ peptides

Aβ1-40 and Aβ1-42 levels in conditioned media and tissue homogenates were measured using hAmyloid ELISA (HS) kits (The Genetics Company, Schlieren, Switzerland) according to the manufacturer's instructions. Plates were read at 450 nm on a Spectra Max Plus plate reader (Molecular Devices, Sunnyvale, CA).

Histochemical Amyloid Plaque Analysis

Sagittal hemibrains were immersion fixed with 4% paraformaldehyde for 2 hours 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 minutes, and rinsed in 80% ethanol and water. For Aβ42 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-Aβ42 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, Burlingame, CA) 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 fashion using Metamorph image analysis software (Molecular Devices). Total amyloid burden was quantified by measuring Aβ42 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 M1 genotype effect on Aβ accumulation in APPSweInd mice across age groups, followed by Mann-Whitney non-parametric t-tests to compare Aβ levels within each group.
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 non-amyloidogenic pathway initiated by α-secretase cleavage that results in the shedding of a soluble ectodomain termed “APPα” and precludes the generation of Aβ, and an amyloidogenic pathway initiated by β-secretase cleavage that gives rise to toxic Aβ peptides which aggregate and ultimately deposit as amyloid plaques. In order to test whether the M₁ mAChR subtype is necessary for cholinergic regulation of non-amyloidogenic 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 wildtype and M₁ KO mice were cultured in vitro and transduced with a lentivirus vector to achieve expression of human sequence APP (hAPP). Owing to 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 1A, lentiviral transduction of mouse cortical neuron cultures resulted in efficient and consistent hAPP expression at levels approximately twofold to threefold over basal. In order 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)-2H-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, wildtype neurons were allowed to condition medium for eight hours in the presence or absence of the non-selective mAChR agonist carbachol (CCh, 100 µM). Western blot analysis of conditioned media samples and cell lysates showed a significant increase in levels of APPα (66%) and CTFα (68%) following carbachol stimulation (Fig 1B,D). Pre-treatment with the muscarinic receptor antagonist atropine (1 µM) blocked the CCh-induced increase in APPα (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.

Having established regulated APP cleavage in wildtype neurons, we performed a similar experiment using neurons from M₁ KO mice. As shown in Figures 1C and 1E, deletion of the M₁ receptor results in a significant reduction in the amount of CCh-stimulated APP processing, with no statistically significant difference in APPα or CTFα between CCh- and vehicle-treated neurons. In order to test whether M₁ receptor activation is sufficient to regulate non-amyloidogenic APP processing, we exogenously expressed the M₁ mAChR subtype on the M₁ mAChR knockout background. Total mAChR levels in M₁ mAChR KO cultures measured by [³H]-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 M₁ KO neurons transduced with M₁ lentivirus were increased by 156% compared to WT. In M₁ KO neurons transduced with both human APP and M₁ mAChR viruses, carbachol stimulation resulted in significant increases in levels of APPα.
(increased by 44%, p<0.05) and CTFα (increased by 88%, p<0.05) compared to vehicle-treated neurons (Fig 1C,E). Expression of full-length APP was not altered across treatment groups (vehicle = 100% +/- 15.8, CCh = 106% +/- 7.74, M₁ + 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 vs. vehicle-treated neurons, although there was a greater degree of variability in ADAM levels (Fig 1F,1G).

We next examined the effects of manipulating M₁ mACHR signaling on the production of Aβ. In wildtype neuron cultures, CCh treatment resulted in a trend towards reduction of Aβ secretion, but this effect did not reach statistical significance (Fig 2). In M₁KO 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 wildtype neurons but an increase in secretion in M₁KO neurons indicates that not only is M₁ mACHR activation necessary for regulated non-amyloidogenic 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 M₅/M₄-preferring antagonist to modulate release of APPs in brain slices (Farber et al., 1995). In M₁KO neuron cultures rescued with M₁ lentivirus and stimulated with CCh, Aβ levels were reduced to 85% of baseline levels and were significantly lower than Aβ levels in CCh-treated M₁KO cultures (p<0.01). Taken together, these data indicate that the M₁ mACHR is essential for carbachol-mediated non-amyloidogenic APP processing in cortical neurons.

Given the decreased APPsα shedding and increased Aβ production in primary neurons from M₁KO mice, we designed an in vivo experiment to determine the impact of loss of M₁ signaling on the development of amyloid pathology in the brain. Cohorts of M₁+/+ and M₁−/− littermates carrying the APPSwe/Ind transgene were generated and aged to 3, 6, 12, and 16 months. Cerebral cortex homogenates were subjected to sequential extraction with sodium dodecyl sulfate (SDS) and formic acid (FA) to recover Aβ peptides, and levels of Aβ1-40 and Aβ1-42 were determined using sandwich ELISA. Aβ1-40 and Aβ1-42 levels in M₁+/+/ and M₁−/− mice are shown in Table 1. We observed an age-dependent increase in both Aβ1-40 and Aβ1-42 in both M₁+/+ and M₁−/− 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 M₁ mACHRs had a significant effect on the accumulation of Aβ (two-way ANOVA genotype effect p<0.0001). By 16 months of age, Aβ1-40 and Aβ1-42 levels in M₁−/− mice were increased by 488 and 293%, respectively, as compared to M₁+/+ littermates (p<0.05 for both effects), indicating that the loss of M₁ 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 M₁ mACHRs affects the development of amyloid plaque pathology, we performed histochemical evaluation of plaque burden on brain sections from APPSwe/Ind/M₁+/+ and APPSwe/Ind/M₁−/− mice. Plaque counts of thioflavin S–positive amyloid deposits (Fig 3A) and surface area measurements of Aβ42-immunopositive brain sections (Fig 3B, C) were used to quantify amyloid pathology. Consistent with our results from biochemical measurement of Aβ, deletion of M₁ had a significant effect on amyloid plaque pathology at 16 months of age. Plaque counts demonstrated a 227% increase in amyloid plaque number in M₁−/− mice compared to M₁+/+ littermates (p=0.0159) at this age (Fig 3A). This effect was consistent for both cortex and hippocampus. Quantitation of amyloid plaque burden by measuring the surface area of Aβ42-immunopositive tissue revealed a 129% increase in cortical plaque load in M₁−/− mice compared to M₁+/+ littermates (Fig 3B, p=0.0381). In cortex, the increased accumulation of cortical plaque pathology in M₁−/− mice was most striking in anterior
regions (Fig 3C). In the hippocampus, there was no statistically significant difference in total Aβ immunopositive surface area between M1+/− and M1−/− mice.

In order to investigate the mechanism underlying the observed increased in amyloid pathology in M1−/− mice, we performed western blot analysis of APP fragments in cortical tissue homogenates from 16 month old APPsw/Ind mice. Full-length APP levels were unchanged (M1+/− = 100.0% ±/− 13.7 vs. M1−/− = 99.7% ±/− 12.5), as were levels of CTFα (p=0.65). Levels of CTFβ were significantly increased in M1−/− mice compared to M1+/− mice (Fig 4A, increased to 157% of wildtype; p<0.05). In contrast to our results from primary neuron experiments, we did not observe significant differences in levels of APPα (p=0.2954) or APPβ (p=0.1830). One likely explanation for this discrepancy is that APPs derivatives secreted into brain tissue are presumably cleared into cerebrospinal fluid 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 M1 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 M1 deletion on steady state levels of both ADAM10 and ADAM17 in cortex from 16 month-old M1+/− and M1−/− mice. As shown in Figure 4C and 4D, we found no difference in expression levels of ADAM10 (p=0.56) and ADAM17 (p=0.89) between M1+/− and M1−/− mice.

Discussion

In the present study, we investigated the role of the M1 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 M1 mAChR overexpression and semi-selective agonists with preferential activation of M1 and other mAChR subtypes enhance α-secretase processing of APP, our study is the first to use genetic models to definitively isolate the M1 mAChR subtype, thus avoiding the ambiguity associated with non-selective agonists. Here we show that the M1 mAChR is necessary and sufficient to regulate non-amyloidogenic APP processing in primary cortical neurons. Furthermore, we demonstrate that APPsw/Ind transgenic mice lacking M1 mAChRs develop increased amyloid pathology as measured by increased brain Aβ levels and amyloid plaque burden. APP metabolite analysis in brain tissue from aged APPsw/Ind mice suggests that this exacerbation of pathology is due to increased amyloidogenic processing of APP. Taken together, these data validate the M1 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 M1 mAChR signaling may influence neuronal physiology. We observed the largest changes in levels of APPα and CTFα, the products of α-secretase cleavage. The APPα 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 APPα 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 APPα 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 M₁ activation on APP processing is through modulation of one or more of the secretase enzymes. As M₁ 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 (reviewed in (Thinakaran and Koo, 2008)), but further work will be required to appreciate exactly how M₁ activation may participate in this process. We did not observe an acute effect of M₁ activation on regulation of ADAM expression levels, nor did we find that deletion of M₁ altered ADAM levels in vivo, but it is possible that chronic mAChR stimulation may induce ADAM upregulation, as suggested by Caccamo et al. (Caccamo et al., 2006).

We also found that M₁ mAChR activation by CCh induced a trend towards decreased Aβ secretion in wildtype neurons, and that CCh actually increased Aβ secretion in M₁KO neurons. This finding suggests that other mAChR subtypes may stimulate amyloidogenic APP processing and agrees with a previous report showing that an M₂/M₄-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 M₁ 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 M₁KO neurons (Kim et al., 1997; Lenzken et al., 2007; Nie et al., 2008).

Our data from APP<sub>Swe/Ind</sub> mice represent the first assessment of M₁ mAChR loss on the development of amyloid pathology in vivo. We demonstrate that M₁ 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 M₁ 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; Nitsch et al., 2000; Beach et al., 2001a; Beach et al., 2001b; Perry et al., 2003; Caccamo et al., 2006). Our results from analysis of APP metabolites in aged APP<sub>Swe/Ind</sub> mice support the conclusions drawn from cellular models, implicating the M₁ mAChR as a pivotal regulator of non-amyloidogenic APP processing in the brain. What is less clear, however, is the mechanism by which loss of M₁ 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 M₁ mAChR regulation of amyloidogenesis in the brain. In addition to the observed effects on amyloidogenesis, it will be important to investigate whether loss of M₁ 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 M₁ would exacerbate cognitive deficits. Given that the M₁ mAChR plays in certain aspects of working memory and memory consolidation (Anagnostaras et al., 2003), the loss of M₁ mAChR signaling accompanied by increased accumulation of amyloid pathology may have an additive detrimental effect on cognition.

*J Neurosci. Author manuscript; available in PMC 2010 September 24.*
In conjunction with studies examining the effects of M₁ mAChR deletion on amyloid pathology and memory impairment, it will be important to evaluate the potential for M₁-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 M₁-selective agonists will be able to offer more meaningful therapeutic benefit. Our data from cultured cells indicates that M₁-selective agonists are effective at promoting non-amyloidogenic 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 M₁ mAChR is an important regulator of APP processing in the brain. Our approach using M₁KO mice is the first of its kind to genetically isolate the M₁ mAChR subtype, circumventing the limitations imposed by the use of semi-selective mAChR agonists in previous studies. We observed that M₁ mAChR loss decreased the shedding of the neuroprotective APPα 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 M₁ mAChR may regulate multiple aspects of neuronal physiology and AD pathology, emphasizing the importance of drug development to target molecules with disease-modifying potential, including M₁. The intimate relationship between the M₁ mAChR and higher cognitive functions including working memory and consolidation further underscores the potential benefit of M₁-based therapies for AD and other cognitive disorders.

Acknowledgments

This work was supported by the National Institutes of Health Grants NS030454 (A.I.L.) and F30 AG029731 (A.A.D.), and a predoctoral fellowship from the PhRMA Foundation (A.A.D.). The authors are grateful to Yinghong Cui, Guofu Fang, Xinping Huang, Howard Rees, and Zoe White for expert technical assistance.

References


the M1 agonist xanomeline on processing of human beta-amyloid precursor protein (FAD, Swedish mutant) transfected into Chinese hamster ovary-m1 cells. Biochem Biophys Res Commun 1998;244:156–160. [PubMed: 9514902]


**Figure 1.**

M₁ mAChR regulation of APP processing in primary neurons. **A,** Western blot analysis of cell lysates from primary cortical neuron cultures derived from wildtype (WT) and M₁ mAChR knockout (M₁KO) 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 carboxyl-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 APPs\(\alpha\) shedding into conditioned media of WT neuron cultures treated with CCh (p<0.05 vs. vehicle), and a 68% increase in the level of CTF\(\alpha\) (p<0.01). Data are shown as mean \(\pm\) SEM from three to five independent experiments.\(\text{C, E,}\) Western blots and densitometry from conditioned media and cell lysates from M\textsubscript{1}KO neuron cultures. APPs\(\alpha\) and CTF\(\alpha\) levels were unchanged in cultures treated with CCh. In M\textsubscript{1}KO neuron cultures transduced with an M\textsubscript{1} mAChR lentivirus, CCh treatment resulted in a 44% increase in APPs\(\alpha\) shedding into conditioned media (p<0.05), and an 88% increase in the levels of CTF\(\alpha\) (p<0.05). Data are shown as mean \(\pm\) SEM of three independent experiments.\(\text{F, G,}\) 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.
Figure 2.
Aβ peptide levels in conditioned media from WT and M₁KO primary neuron cultures. In WT neurons, CCh treatment (100 μM) resulted in a trend towards decreased Aβ production, although this difference did not reach statistical significance. In M₁KO neurons, CCh treatment caused an increase in Aβ production (p<0.05 vs. vehicle). Following rescue of M₁KO neuron cultures by transduction with an M₁ lentivirus, CCh treatment resulted in a significant reduction in Aβ levels compared to CCh-treated M₁KO neurons (p<0.01).
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 ($p<0.01$). 

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 ($p<0.05$). 

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.
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, APP$\alpha$, and CTF$\beta$, 192swe to detect the Swedish mutation form of APP$\beta$, and C8 to detect both CTF$\alpha$ and CTF$\beta$. EF1$\alpha$ is shown as a loading control. For quantitation, APPs levels were normalized to EF1$\alpha$, and CTF levels were normalized to full-length APP. Densitometric quantitation revealed a 57% increase in CTF$\beta$ in $M_1^{-/-}$ mice ($p<0.05$).

C, D, Immunoblot analysis of ADAM10 and ADAM17 in $M_1^{+/+}$ compared to $M_1^{-/-}$ APP$_{Swe/Ind}$ cortex. Levels of both proteins were unchanged in $M_1^{+/+}$ compared to $M_1^{-/-}$. 

*J Neurosci. Author manuscript; available in PMC 2010 September 24.*
Table 1

ELISA measurements of Aβ_{1-40} and Aβ_{1-42} peptide levels in M_{1}^{+/+} and M_{1}^{-/-} APP_{Swe/Ind} cortex at 3, 6, 12, and 16 months of age. 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 Aβ_{1-40} and Aβ_{1-42} are increased in M_{1}^{-/-} mice compared to M_{1}^{+/+} littermates (Aβ_{1-40} increased by 488%, p<0.05; Aβ_{1-42} increased by 293%, p<0.05).

<table>
<thead>
<tr>
<th>Age</th>
<th>3 months (mean ± SD)</th>
<th>6 months (mean ± SD)</th>
<th>12 months (mean ± SD)</th>
<th>16 months (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aβ_{1-40}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M_{1}^{+/+}</td>
<td>318 ± 90</td>
<td>301 ± 49</td>
<td>4221 ± 2369</td>
<td>7050 ± 9668</td>
</tr>
<tr>
<td>M_{1}^{-/-}</td>
<td>287 ± 63</td>
<td>309 ± 161</td>
<td>4002 ± 1726</td>
<td>41435 ± 19806</td>
</tr>
<tr>
<td>p-value</td>
<td>0.7396</td>
<td>0.2284</td>
<td>1.0000</td>
<td>0.0190</td>
</tr>
<tr>
<td>Aβ_{1-42}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M_{1}^{+/+}</td>
<td>72 ± 10</td>
<td>55 ± 20</td>
<td>97038 ± 77335</td>
<td>148472 ± 175542</td>
</tr>
<tr>
<td>M_{1}^{-/-}</td>
<td>69 ± 17</td>
<td>92 ± 108</td>
<td>100188 ± 46472</td>
<td>583435 ± 321325</td>
</tr>
<tr>
<td>p-value</td>
<td>0.5362</td>
<td>0.8518</td>
<td>0.9048</td>
<td>0.0381</td>
</tr>
</tbody>
</table>