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The ATP-binding Cassette Transporter-2 (ABCA2) Promotes Amyloidogenic Processing of Amyloid Precursor Protein by Glu11 Site Cleavage

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Abstract

The ATP binding cassette transporter-2 (ABCA2) has been genetically linked to Alzheimer's disease but the molecular mechanisms are unknown. In this study, the effects of expression of human ABCA2 on endogenous amyloid precursor protein (APP) expression, trafficking and processing were examined in mouse N2a neuronal cells. ABCA2 expression increased the steady-state APP mRNA levels through transcription. ABCA2 also induced increased synthesis of APP holoprotein and altered APP processing and metabolite generation. ABCA2 expression promoted β -secretase (BACE1) cleavage of APP not at the common Asp1 amino acid site (β -site) of A β in APP but at the Glu11 site (β' -site) to increase C89 carboxyl-terminal fragment levels (β' -CTF/C89). The levels of *N*-terminally truncated A β_{11-40} peptides were also increased by ABCA2 expression. The delivery of newly synthesized APP to the cell surface through the secretory pathway was not perturbed by ABCA2 expression; however, ABCA2 expression increased the amount of APP in early-endosomal compartments, which also contained the highest levels of β' -CTF/C89 and is likely the site of increased BACE1 processing of APP. This report identifies ABCA2 as a key regulator of endogenous APP expression and processing and suggests a possible biochemical mechanism linking ABCA2 expression, APP processing and Alzheimer's disease.

Keywords

ATP-binding cassette transporter; ABCA2; Alzheimer's; Amyloid precursor protein; APP; Glu11 site; *N*-terminally truncated A β

Introduction

The ATP binding cassette transporter-2 (ABCA2) is a member of the A-subfamily of transporters that have been linked to the maintenance of cellular lipid homeostasis (1). ABCA2 is most highly expressed in the brain (2, 3) and two transcript variants have been identified (4). By confocal immunocytochemistry and electron microscopy, ABCA2 has been localized within late-endosomal/lysosomal and trans-Golgi network compartments (2, 5). Overexpression of ABCA2 in CHO cells has identified a possible role in trafficking of lipoprotein-derived cholesterol from late-endosomes/lysosomes to the endoplasmic reticulum for esterification (6). Two groups have generated ABCA2 knockout mice. The Tew group found that loss of ABCA2 caused abnormal myelin compaction, a tremor and hyperactivity (7). The Inagaki group also observed the tremor but did not find the

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abnormalities in myelin structure. Interestingly, they reported an abnormal sphingolipid metabolism phenotype; an accumulation of ganglioside GM1 and decrease in sphingomyelin (8).

The clinical relevance of a role for ABCA2 and Alzheimer's disease is suggested by two large studies that strongly linked the identical single nucleotide polymorphism (NCBI db SNP rs908832) to early-onset (9) and late-onset/sporadic Alzheimer's disease (AD) (10).

The molecular mechanisms responsible for ABCA2 function in the etiology of Alzheimer's disease are unknown; however, the expression of ABCA2 has been associated with molecular determinants of AD. Using microarray analysis of gene expression patterns, ABCA2 overexpression in HEK293 cells was associated with alterations in the expression of genes functionally linked to AD, including the amyloid precursor protein (APP) (11).

This study proposed to investigate the hypothesis that in neuronal cells, ABCA2 modulates endogenous APP expression, trafficking and processing. It was determined that stable overexpression of human ABCA2 in mouse N2a neuroblastoma cells increased APP mRNA and protein levels and promoted beta secretase (BACE1) cleavage of APP, not at the canonical β -site at amino acid Asp1 of A β within APP, which generates the C99 carboxy-terminal fragment (CTF), but primarily at the β' -site at Glu11 of A β in APP, to increase the level of the C89 CTF. The increased production of intracellular and secreted N-terminally truncated A β ₁₁₋₄₀ peptides were also a consequence of ABCA2 expression. Surface biotinylation studies suggested that ABCA2 expression did not alter the amount of total full-length APP reaching the cell surface but increased the amount of APP re-internalized into early-endosomal compartments, which also contained the highest levels of β' -CTF/C89. The results of this study suggest a possible biochemical mechanism linking ABCA2 expression, APP processing and AD.

Materials and Methods

Materials

Dulbecco's Modified Eagle medium (DMEM) and fetal bovine serum (FBS) were obtained from Hyclone. OptiMem I and DMEM methionine- and cysteine-free growth media were obtained from Invitrogen. Glutamine and Penstrep were obtained from Fisher. Brefeldin A and monensin were from Sigma. Sulfo-NHS-biotin and avidin-agarose beads were from Pierce. The BACE1 inhibitor [OM00-3]DR₉/N1920 was from Bachem. Antibodies used in this study are summarized in Table 1. [³⁵S]methionine (specific activity > 1000 Ci/mmol) was obtained from Perkin-Elmer.

Cell lines and culture

The N2a mouse neuroblastoma cells were obtained from ATCC (CCL-131). Cells were grown in DMEM/OptiMem I (50:50) supplemented with 5% fetal bovine serum (FBS) 2 mM glutamine and 1% Penstrep at 37° C and 5% CO₂.

The N2a cell line stably expressing ABCA2 was generated by transfection of a LacZeo 2 expression construct (Invitrogen) and selection of resistant cells with Zeocin (0.7 mg/ml) to generate a FlpIn line. N2a FlpIn cells were transfected with a 7.4 kilobase ABCA2 cDNA construct that was subcloned into the pcDNA5 FRT/TO vector (Invitrogen), followed by selection of resistant cells expressing ABCA2 with Hygromycin (0.5 mg/ml). The construct provided high-levels of constitutive ABCA2 expression under the control of the cytomegalovirus promoter (CMV). Individual clones were isolated and analyzed for relative ABCA2 expression level and two (A2.1, A2.4) were utilized for most of the experiments.

Western Blot

Cells were lysed in radioimmunoprecipitation (RIPA) buffer (150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, RIPA) supplemented with HALT protease inhibitor cocktail (Roche). For ABCA2 or BACE1 protein levels 30 µg of protein were fractionated on 4–12% NuPAGE gels (Invitrogen). For APP and metabolite protein levels, 25 µg of protein were fractionated on 4–12% NuPAGE gels. Proteins were transferred to nitrocellulose membranes and probed with primary rabbit polyclonal antibodies: ABCA2 c-terminal (1:300), APP/0443 (1:4000), BACE1 (1:2000), TGN38 (1:1000), Calnexin (1:2000), primary monoclonal antibodies: Flotillin-1 (1:1000), glyceraldehydes phosphate dehydrogenase (GAPDH, 1:2000). Secondary antibodies were goat anti-mouse- or anti-rabbit-HRP (1:1000). Blots were developed using the Dura-Signal enhanced chemiluminescence reagent (Pierce) and imaged using the ImageStation IS2000R and Molecular Imaging software (Kodak).

Metabolic Radiolabeling/Immunoprecipitation

Metabolic radiolabeling of N2a cells and immunoprecipitation experiments were performed as previously described (12). Approximately 5×10^6 cells were plated in 100 mm dishes and grown for 48 hours. For continuous labeling, cells were starved in methionine- and cysteine-free DMEM and 5% FBS for 30 min. Medium was supplemented with 100 µCi/ml [35 S]methionine and cells were cultured for 4 hours. For pulse-chase labeling, cells were incubated in medium containing 100 µCi/ml [35 S]methionine for 1 hour, washed with phosphate buffered saline (PBS) and chased with methionine- and cysteine-free DMEM, 5% FBS and 1 mM methionine for 10, 20, 30, 60, or 120 min. Cells were lysed in RIPA buffer and equivalent masses of total protein were immunoprecipitated with 2 µg of 0443 antibody for intracellular APP and c-terminal fragment (CTF) metabolites. For detection of intracellular and secreted Aβ peptides, FCA3340 or Asp1 β-Amyloid antibodies were utilized. To detect secreted sAPPα and sAPPβ, specific antibodies to these metabolites were utilized. Antibodies were incubated with protein extracts for 2 hours, then 30 µl of Protein A/G Plus-agarose (Santa Cruz Biotechnology) were added, followed by overnight incubation at 4° C on a rocking platform. Proteins were fractionated on 10–20% Tricine gels (Invitrogen), dried, exposed to storage phosphor screens and developed using the Storm Phosphorimager and ImageQuant software. Values were normalized to total cell protein in the experimental samples. Data obtained was the result of three independent experiments.

Biotinylation of cell surface APP

On day 0, cells were plated at 3.5×10^6 cells per 100 mm plate in 10 ml of medium and cultured for 48 hours at 37° C 5% CO₂. Cells were washed twice with ice-cold PBS and treated with 1 mg/ml membrane-impermeant sulfo-NHS-biotin in PBS for 30 min at 4° C. After washing twice with cold PBS and once with 3.75 mg /ml glycine in PBS, the medium was collected and the cells were lysed in RIPA buffer. Protein concentrations were determined and 300 µg of protein extract were incubated with 30 µl avidin-agarose beads overnight at 4° C on a rocking platform. Beads were centrifuged at $500 \times g$ for 3 min and washed 4 times in RIPA buffer. Proteins were separated by on 4–12% NuPAGE gels, transferred to nitrocellulose and analyzed by Western blot with APP/0433 primary antibody and secondary antibody goat-anti-rabbit-HRP. Total APP in cell extracts was determined by Western blot on 25 µg of total cell protein as described above. Blots were developed using the Dura-Signal reagent (Pierce) and imaged using the ImageStation IS2000R and Molecular Imaging software (Kodak).

Sucrose density gradient ultracentrifugation (Trans Golgi Network/Early Endosome)

On day 0, 4×10^6 cells were plated in 25 ml of DMEM/OptiMem I, 5% FBS in 175 mm flasks and grown to 90% confluency at 37° C 5% CO₂. The pellet was resuspended in 1 ml of homogenization buffer containing 0.25 sucrose, 10 mM Tris-HCl (pH 7.4), 1 mM magnesium acetate and HALT protease inhibitor cocktail. The cells were allowed to swell on ice for 20 min followed by Dounce homogenization. A post-nuclear supernatant was recovered after centrifugation at $10,000 \times g$ for 3 min and protein concentrations were determined. The supernatant containing 1 mg total protein in 630 μ l was layered onto a step gradient comprised of 0.625 μ l of 2 M sucrose, 1.04 ml of 1.3 M sucrose, 1.04 ml of 1.16 M sucrose, 0.833 ml of 0.8 M sucrose, 0.833 ml of 0.5 M sucrose prepared in 10 mM Tris-HCl (pH 7.4), 1 mM magnesium acetate (total 5 ml). Centrifugation was performed at $100,000 \times g$ for 2.5 h, twelve 400 ml fractions were recovered from the top of the tube and 300 μ l were precipitated by the methanol chloroform method. Total precipitated proteins were fractionated on 4–12% NuPAGE gels, transferred to nitrocellulose and probed for BACE1 (anti-BACE1), APP (0443), trans-Golgi Network-38 (TGN38), or early-endosomal protein A1 (EEA1).

Sucrose density gradient ultracentrifugation (Lipid raft)

On day 0, 4×10^6 cells were plated in 25 ml of DMEM/OptiMem I, 5% FBS in 175 mm flasks and grown to 90% confluency at 37° C 5% CO₂. The pellet was resuspended in 1 ml of MBS lysis buffer (25 mM MES pH 6.5, 150 mM NaCl, 0.5% Lubrol WX) and HALT protease inhibitor cocktail (Pierce) and incubated on ice for 1 h with periodic vortexing. Extracts were passed through a 25-gauge needle 7 times and debris was removed by centrifugation at $10,000 \times g$ for 3 min at 4° C. Protein concentrations were determined using the DC protein assay (Bio-Rad). Approximately 1 mg of total protein in 500 μ l of MBS buffer was mixed with 500 μ l of 80% sucrose in MBS and briefly mixed by vortexing. Subsequently, 2 ml of 35% sucrose in MBS and 2 ml of 5% of sucrose in MBS were carefully added for discontinuous gradient formation and samples were centrifuged at $160,000 \times g$ for 18 h at 4° C in AH650 rotor. Ten 480 μ l fractions were recovered from the top of the tube and 300 μ l were precipitated by the methanol chloroform method. Total precipitated proteins were fractionated on 4–12% NuPAGE gels, transferred to nitrocellulose and probed for APP (0443), flotillin-1 (raft), and calnexin (non-raft).

Results

Generation of N2a cell line stably expressing human ABCA2

In order to determine the effects of ABCA2 function on APP expression and processing, several stable cell lines were established in mouse N2a neuroblastoma cells that overexpressed human ABCA2. The relative protein expression of ABCA2 in the A2.1 and the A2.4 lines were ~9 fold greater than the parental N2a cell line (Fig 1).

ABCA2 increases endogenous APP mRNA

To examine the hypothesis that ABCA2 overexpression increases endogenous APP mRNA in neuronal cells, real-time PCR was performed on reverse transcribed total RNA isolated from N2a and A2.1 cells. The steady state level of APP mRNA was increased ~ 1.7-fold in A2.1 cells relative to N2a cells (Fig. 2A). To determine whether ABCA2 expression increased the stability of APP mRNA, N2a and A2.1 cells were treated with actinomycin D to block transcription over intervals extending from 2–16 hours and the decay of APP mRNA level was measured by real-time PCR. ABCA2 expression in A2.1 cells did not significantly alter the turnover of APP mRNA relative to N2a cells over the time course examined (Fig. 2B). Alternatively, ABCA2 expression could induce transcription of the

endogenous APP gene. To explore this possibility, a human APP promoter/luciferase construct extending from -488 to +100 base pairs relative to the transcription start site was transfected into N2a and N2aA2 cells and the relative promoter activity measured. ABCA2 expression induced the human APP promoter/luciferase activity ~ 1.7-fold in A2.1 cells relative to the level in N2a cells (Fig. 2C). These results indicate that ABCA2 expression induces the elevation of endogenous APP mRNA in neuronal cells through increased transcription.

Effect of ABCA2 expression on APP holoprotein level and processing

To investigate whether ABCA2 expression influences the steady-state level of APP protein mass, Western blot was performed on whole cell extracts from N2a, A2.1 and A2.4 cells. APP holoprotein levels were increased ~ 1.65-fold in A2.1 and A2.4 cells relative to N2a cells (Fig. 3A). In A2.1 and A2.4 cells compared to parental N2a cells, there was an increase of ~ 2- fold in the production of carboxy-terminal fragments (CTFs). To determine whether β -secretase-1 (BACE1) was the enzymatic activity responsible for cleavage of the APP holoprotein cells to generate the increase in CTFs in A2.1, N2a and A2.1 cells were incubated with the specific BACE inhibitor [OM00-3]DR₉. Following separation of proteins by PAGE and Western blot with antibodies to APP and metabolites, it was determined that the BACE inhibitor dose-dependently reduced β -CTF/C89 fragment levels, by ~ 50 % at the highest inhibitor concentration, in A2.1 cells with negligible non-specific effects on α -CTF/C83 levels (Fig. 3B). These results suggested that BACE1 was the major enzymatic activity responsible for generating CTFs in cells overexpressing ABCA2.

ABCA2 increases steady-state APP holoprotein synthesis

To complement Western blot analysis of the effect of ABCA2 expression on steady-state APP protein levels and processing, N2a and A2.1 cells were metabolically radiolabeled with [³⁵S]methionine for 4 hours and APP holoprotein and metabolites were immunoprecipitated with specific antibodies from cell extracts and culture medium. Following separation of radiolabeled proteins by PAGE, the relative expression of APP and metabolites were measured by autoradiography. The steady-state level APP holoprotein synthesis was elevated by ~ 2.3 fold in A2.1 cells relative to N2a (Fig. 4A). Consistent with the result of Western blot analysis, the α -CTF/C83 and β -CTF/C99 c-terminal fragments were reduced in A2.1 cells relative to N2a cells. In contrast, the β '-CTF/C89 fragment, which is the product of β -secretase cleavage at the Glu11 site instead of the Asp1 site was persistently elevated in A2.1 cells. The 6 kDa γ -CTF/C50 fragment, also termed the amyloid intracellular domain (AICD) that is the product of γ -secretase cleavage of α -CTF, β -CTF or β '-CTF, was increased in A2.1 radiolabeled cells. Intracellular A β levels were not detectable in these experiments. Using an antibody (FCA3340) that recognizes A β ₄₀ and P3 fragments, a low level of A β ₁₋₄₀ was detectable in radiolabeled N2a cells as well as higher levels of P3 fragments, the product of α - and γ -secretase cleavage of APP. (Fig. 4B). In contrast, in A2.1 cells the FCA3340 antibody detected increased levels of A β ₁₋₄₀ and N-terminally truncated A β ₁₁₋₄₀ compared to N2a cells. Although the P3 fragment and the A β ₁₁₋₄₀ fragment run at similar mobility, ~ 3 kDa, it is likely the A β ₁₁₋₄₀ fragment that was detected in the A2.1 cells since the precursor β '-CTF/C89 fragment was persistently elevated in these cells. Using an antibody (FCA18) that recognizes only the first free aspartyl and the N-terminus of A β _{1-x} and does not recognize aspartyl-1-deleted A β _{x-40} peptides, the A β ₁₋₄₀ peptide was detectable but A β ₁₁₋₄₀ was not observed. In A2.1 cells, the FCA18 antibody also detected increased levels of A β ₁₋₄₀ relative to N2a cells and also failed to detect N-terminally truncated A β peptides. These results indicate that the elevated β '-CTF/C89 fragment produced by BACE1 cleavage at the Glu11 site observed in A2.1 cells is the substrate for subsequent γ -secretase cleavage and elevated production of N-terminally truncated A β ₁₁₋₄₀. Metabolic radiolabeling and immunoprecipitation determined levels of secreted sAPP into

culture medium with specific antibodies to sAPP α and sAPP β . Levels of both secreted sAPP α and sAPP β levels were increased by slightly more than 2-fold, respectively in A2.1 cells relative to N2a (Fig. 4C). These results indicate that elevated APP holoprotein expression induced by ABCA2 provides the substrate for subsequent α - and β -secretase cleavage to release secreted sAPP fragments extracellularly.

To examine the kinetics of the effects of ABCA2 expression on APP synthesis and processing, pulse-chase radiolabeling experiments were performed. Following pulse-labeling with [35 S]methionine, chase in medium containing unlabeled methionine for increasing period of time up to 2 hours demonstrated that the APP holoprotein declined in both N2a and A2.1 cells with similar kinetics, $t_{1/2}$ of ~ 30 min (Fig. 5A). The level of the intracellular α -CTF/C83 fragment did not significantly change over the course of chase time until 60 min in N2a cells. The intracellular β -CTF/C89 increased 1.8-fold as early as 10-min of chase time in A2.1 cells and continued to accumulate (~ 2.2 -fold) up to 30 min of chase time and then gradually declined to control levels at 120 min. (Fig. 5B). These results show that ABCA2 expression does not alter the kinetics of APP holoprotein synthesis or processing of its CTF metabolites compared to control cells.

ABCA2 expression decreases the level of BACE1 protein

Since previous reports have demonstrated that overexpression of BACE1 could promote cleavage at the Glu11 site in APP to generate the β -CTF/C89 fragment (13, 14), the level of BACE1 was determined in N2a, A2.1 and A2.4 cells. BACE1 expression was reduced by $\sim 45\%$ in ABCA2 expressing cells (Fig. 6). Therefore paradoxically, ABCA2 expression decreases total BACE1 protein levels but promotes increased β -CTF/C89 fragment production and therefore, these results indicate that the remaining BACE1 level in A2.1 cells is not limiting for APP processing and CTF production.

Effect of protein trafficking inhibitors on APP processing by ABCA2

In normal APP processing in the secretory pathway, APP is synthesized in the endoplasmic reticulum and trafficked through the Golgi apparatus to the cell surface. At the cell surface, α -secretase acts to generate secreted sAPP α and intracellular α -CTF/C83, which is the substrate for subsequent γ -secretase cleavage to generate the non-amyloidogenic P3 fragment (15). In amyloidogenic processing, β -secretase acts on APP after reinternalization from the cell surface to generate secreted sAPP β and β -CTF/C99, which is the substrate for subsequent γ -secretase cleavage to generate the amyloidogenic A β fragments. To investigate the effect of protein trafficking inhibitors of the secretory pathway on the differential processing of APP by ABCA2 expression, cells were treated with brefeldin A or monensin followed by Western blot. Brefeldin A induces the resorption of the proximal Golgi into the endoplasmic reticulum and inhibits movement and maturation of APP to the cell surface (16, 17). The ionophore monensin inhibits maturation and transport of newly synthesized proteins beyond the trans-Golgi network (TGN) (18). Treatment of cells with brefeldin A increased APP holoprotein level in N2a by ~ 5.5 fold, as has been reported by others (17) (Fig. 7A). In ABCA2 expressing cells brefeldin A increased APP holoprotein expression by ~ 8.5 fold, relative to untreated N2a cells. In contrast, brefeldin A reduced the level of α -CTF/C83 in N2a cells by 70% and reduced the level of β -CTF/C89 by 70–85% in ABCA2 expressing cells. These results suggest that trafficking of newly synthesized APP from the endoplasmic reticulum to the Golgi apparatus is required for subsequent BACE1 processing and CTF production.

The levels of the APP holoprotein in N2a, A2.1 and A2.4 cells was increased by ~ 2.3 - to 2.6-fold by monensin addition (Fig. 7B). Monensin treatment increased the level of the β -CTF in A2.1 and A2.4 cells by ~ 5 -fold and surprisingly, induced an increase in β -CTF/C89

by 1.5-fold in N2a cells. These results suggest that inhibition of trafficking beyond the trans-Golgi network permits increased activity of BACE1 on APP holoprotein to increase CTF production.

Effect of ABCA2 expression on trafficking of APP to the cell surface

In order to investigate the mechanism of ABCA2 modulation of APP cleavage by α - and β -secretases, the trafficking APP to the cell surface was followed. Biotinylation of cell surface proteins with cell-impermeant sulfo-NHS-biotin was performed at 4° C to inhibit endocytosis and biotinylated proteins were recovered from cell extracts by immunoprecipitation with streptavidin-agarose. Following separation by PAGE and probing with antibodies to APP, an increase in the level of APP surface expression was observed between N2a and A2.1 cells of ~ 1.7-fold (Fig. 8). Western blot was also performed on cell extracts for total APP in N2a and A2.1 cells. Since the relative amount of APP holoprotein reaching the cell surface was similar to the relative amount of total cellular APP, these data suggest that ABCA2 does not perturb APP trafficking through the secretory pathway to the cell surface.

The processing of APP by β -secretase is reported to occur in lipid raft compartments, which are specialized regions of lipid membranes enriched in cholesterol and sphingomyelin (19). To determine whether ABCA2 exerts its effects on APP processing by its compartmentation in lipid rafts, cell extracts were subjected to detergent solubilization in MES containing Lubrol and Triton X-100 followed by sucrose density gradient ultracentrifugation, PAGE of individual fractions and Western blot with antibodies to APP, flotillin-1 (raft) and calnexin (endoplasmic reticulum/ER, non-raft). The raft fractions showing the highest flotillin-1 immunoreactivity were 4–6 and were not significantly different N2a and A2.1 cells (Fig. 9A). The non-raft fractions showing the highest calnexin immunoreactivity were 7–10 and were not significantly different in N2a and A2.1 cells. In N2a cells, the α -CTF/C83 fragment was distributed in predominantly in heavier fractions that co-localized with ER markers from 5–10, whereas in A2.1 cells the β -CTF fragment was enriched in lighter fractions 5–8 and virtually absent in the heaviest ER fractions 9 and 10 (Fig. 9B). Therefore these results suggest that A2.1 increases the processing of APP holoprotein by β -secretase in lighter fractions that do not localize to the heaviest non-raft ER markers.

Identification of the sub-cellular sites of APP processing in ABCA2 expressing cells

Since monensin inhibition of APP trafficking through the secretory at the trans-Golgi network was associated with increased β' -site processing, experiments were undertaken to determine whether the trans-Golgi network was the sub-cellular site where ABCA2 exerts its effects on APP holoprotein processing. Cell extracts from N2a and A2.1 cells were subjected to sucrose gradient ultracentrifugation and Western blot with APP-, BACE1, and trans-Golgi network-specific (TGN38) antibodies. BACE1 was predominantly localized to fractions 4–7, with the immature form predominant in fraction 4 and the mature form in fraction 7. The trans-Golgi network marker TGN was most prominent in fractions 4–6 (Fig. 10A). In N2a cells, the APP holoprotein was present in heavier endoplasmic reticulum fractions with progressively less in lighter fractions (Fig 10B). The α -CTF/C83 fragment was predominantly located in fractions 5–7 with greatest in fraction 6. In A2.1 cells, however, APP holoprotein and the β' -CTF/C89 fragment were predominantly localized to the lightest endosomal fractions 1–4. The early endosomal marker EEA1 was also most prominent in these light fractions. These results indicate that ABCA2 increases total APP levels, which are subsequently processed by BACE1 at the Glu11 site, following internalization from the cell surface into early-endosomal compartments, to increase production of β' -CTF/C89 and subsequently N-terminally truncated A β _{11–40}.

Discussion

ABCA2 has been genetically linked to Alzheimer's disease but mechanisms have not been established. In this report, a possible biochemical role for ABCA2 has been investigated in the modulation of APP expression and amyloidogenic processing. The results of this study indicate that ABCA2 overexpression in N2a neuronal cells elevates endogenous APP expression and promotes amyloidogenic processing through BACE1 cleavage at the β' -site/Glu11 of A β in APP to generate the β' -CTF/89 fragment. Cleavage of the β' -CTF/89 fragment by γ -secretase generates *N*-terminally truncated A β peptides that are linked with AD pathology. Identification of regulators of endogenous APP expression and amyloidogenic processing is important since the majority of AD cases have wild-type APP and not genetic mutants of APP associated with early-onset familial AD (FAD). ABCA2 induction APP synthesis, β' -CTF/89 fragment generation and subsequent γ -secretase cleavage to produce *N*-terminally truncated A β peptides suggest a possible biochemical mechanistic role for ABCA2 function in the etiology of AD pathology.

The steady-state level of APP mRNA was increased by ABCA2 expression at the level of transcription. There was no significant difference in the turnover of APP mRNA in control and human ABCA2 expressing cells by culture in actinomycin D to inhibit *de novo* transcription. In addition, transfection of a human APP promoter construct, -488 to +100 base pairs, showed an increase in relative promoter activity in ABCA2 expressing cells. The mechanism for how ABCA2 induces APP transcription remains to be determined but the APP promoter utilized in this study contains binding sites for transcription factors known to activate transcription of APP, including NF κ B and AP-1 (20, 21). ABCA2 function may activate signaling pathways that induce expression or activation of these or other transcription factors to elevate APP mRNA. Future work will be undertaken by this laboratory to delineate the signaling pathways and identify regulatory elements and transcription factors required for ABCA2 activation of APP transcription.

A key finding of this study is that ABCA2 expression elevates APP protein synthesis and promotes amyloidogenic processing. ABCA2 expression increases BACE1 cleavage of the APP holoprotein, not at the common Asp1 site of A β in APP that generates β -CTF/C99 fragment and A $\beta_{1-40/42}$ peptides but at the Glu11 site to generate a *N*-terminal truncated β' -CTF/C89 fragment and *N*-terminal truncated secreted A β_{11-40} peptides. The levels of secreted APP metabolites, sAPP α and sAPP β were increased in ABCA2 overexpressing cells.

The relative efficiency of BACE1 cleavage of APP at Asp1 or Glu11 is not understood. Possible mechanisms include increased BACE1 expression or prolonged association of BACE1 with its APP substrate in subcellular compartments. In HEK293 cell lines stably overexpressing BACE1, A $\beta_{11-40/42}$ peptides were the major A β species produced and both full-length APP and the β -CTF/C99 fragment could be used as substrates by BACE1 for A $\beta_{11-40/42}$ generation (13). In addition, with increasing BACE1 expression level, the *N*-terminally truncated forms A $\beta_{11-40/42}$ predominated and the levels of the A $\beta_{1-40/42}$ forms declined. Overexpression of BACE1 in human NT2 and differentiated NT2N neurons, increased the production of both secreted and intracellular A $\beta_{1-40/42}$ and *N*-terminally truncated forms A $\beta_{11-40/42}$, which indicates that the *N*-terminally truncated forms are produced before deposition extracellularly in senile plaques (14). In humans, *N*-terminally truncated A β species have also been detected in AD and Down's syndrome brain sections and in vascular amyloid deposits (22, 23). Paradoxically, in this study, ABCA2 expression was not associated with increased but decreased BACE1 protein level. The mechanism responsible for ABCA2 regulation of BACE1 protein level remains to be determined. Since ABCA2 decreases total BACE1 protein levels, the mechanism for how ABCA2 increases β' -

site cleavage of APP may involve the trafficking and localization of APP and BACE1 in close proximity within subcellular compartments.

Culture of cells overexpressing ABCA2 in brefeldin A to inhibit movement through the Golgi apparatus inhibited β' -CTF/C89 generation. In contrast, monensin treatment, which inhibits protein maturation and trafficking to post-Golgi compartments, elevated β' -CTF/C89 generation in both N2a and ABCA2 expressing cells. These results suggest that inhibition of trafficking of APP beyond the trans-Golgi network may be sufficient for increased BACE1 cleavage at the Glu11 site to generate the β' -CTF/C89 fragment.

To understand the mechanisms for how ABCA2 modulates APP processing, the trafficking of APP to the cell surface was followed in N2a and ABCA2 expressing cells. ABCA2 expression did not alter the relative amount of total APP reaching the cell surface. This finding suggested that the effect of ABCA2 on promoting BACE1 cleavage of APP occurred after transit to the cell surface.

Cleavage of APP by BACE1 occurs in cholesterol and sphingolipid rich lipid raft compartments (19). Detergent solubilization and density gradient ultracentrifugation to identify lipid raft compartments revealed that ABCA2 expression did not alter the formation of lipid rafts but that the β' -CTF/C89 metabolite was enriched in lighter fractions including lipid rafts but absent in the heavier endoplasmic reticulum non-raft fractions.

After synthesis in the endoplasmic reticulum, mature BACE1 has been localized to the trans-Golgi network and early endosomal compartments (24, 25). Sucrose density gradient ultracentrifugation and Western blot was employed and demonstrated a relative increase in BACE1 protein level in fractions identified as the trans-Golgi network in both control and ABCA2 overexpressing cells. Interestingly, in ABCA2 expressing cells, the β' -CTF/C89 was located predominantly in early-endosomal fractions that localized with the marker protein EEA1. These results suggest that ABCA2 increases both total APP expression and cell surface levels, which upon reinternalization into early-endosomal compartments are processed by BACE1.

In summary, this report identifies ABCA2 as a key regulator of endogenous APP expression and represents the first description of a protein other than BACE1 whose expression promotes β' -site processing of APP to generate β' -CTF/C89 and *N*-terminally truncated $A\beta_{11-40}$ in neuronal cells. The pathologic effects of *N*-terminally truncated $A\beta$ peptides may be due to the fact that they are less soluble and enhance aggregation of β -amyloid into neurotoxic, β -sheet fibrils (26). Since the presence *N*-terminally truncated $A\beta_{11-40/42}$ has been detected in senile plaques in AD patients and their effects on neurodegeneration have been reported in several *in vivo* murine models of AD and in human AD brain, the importance of ABCA2 in modulation of the production of these $A\beta$ species warrants further study. Future work will determine whether ABCA2 elevates APP holoprotein expression and increases production of *N*-terminally truncated $A\beta_{11-40/42}$ *in vivo* in primary neurons and mouse brain. These studies may increase our understanding of APP processing and establish a mechanistic link between ABCA2 and AD pathogenesis.

Abbreviations

ABC	ATP-binding cassette transporter
$A\beta$	β -amyloid
APP	amyloid precursor protein

BACE1	beta secretase cleaving enzyme 1
CTF	carboxy-terminal fragment
β-site	beta secretase cleavage site
ER	endoplasmic reticulum
TGN	trans-Golgi network
RIPA	radioimmunoprecipitation buffer
CMV	cytomegalovirus

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Figure 1. ABCA2 protein expression in N2a and N2aA2 cells

A representative Western blot is shown of ABCA2 protein expression in parental N2a cells and two clones expressing human ABCA2 protein (A2.1, A2.4). The anti-ABCA2 rabbit polyclonal antibody detects both endogenous mouse ABCA2 as well as the human protein.

Figure 2. ABCA2 increases endogenous mRNA APP expression by transcription

(A) Total RNA was isolated from N2a and A2.1 cells, reverse-transcribed and real-time PCR was performed with APP gene-specific primers as described in Methods. The data are expressed as fold-increase in APP expression in A2.1 relative to N2a cells. (B) *ABCA2 does not affect APP mRNA stability*. N2a and N2aA2 cells were cultured in 5 μ g/ml Actinomycin D to block de novo transcription for increasing periods of time before isolation of total RNA followed by reverse transcription and real-time PCR with APP gene-specific primers. (C) *ABCA2 induces transcription from the human APP promoter*. N2a and A2.1 cells were transfected with a human APP promoter/luciferase construct extending from -488 to +100

relative to the transcription start site. After 48 hours, cell extracts were prepared and luciferase activity measured as described in Methods.

Figure 3. ABCA2 expression increases the steady-state level of APP holoprotein and β' -CTF/C89
 (A) Protein extracts from N2a, A2.1 and A2.4 cells were fractionated on 4–12% NuPAGE gels transferred to nitrocellulose and probed with the APP/0443 antibody. (B) Specific inhibition of BACE1 activity. N2a and A2.1 cells were incubated with 4, 8.5 or 14 μ M of the specific BACE inhibitor [OM00-3]DR₉ for 24 hours. PAGE and Western blot were performed as above. Bands were quantified by densitometry as described in Methods. The data are expressed as the fold-change in expression relative to control N2a cells ($P < 0.01$, Student's *t* test).

Figure 4. ABCA2 increases APP holoprotein protein synthesis and enhances β'-site cleavage
N2a and A2.1 cells were metabolically radiolabeled with 100 μCi/ml [³⁵S]methionine for 4 hours followed by immunoprecipitation of APP and *c*-terminal fragment (CTF) metabolites with APP-specific antibodies (0443). Following PAGE and autoradiography signals corresponding to (A) APP holoprotein and C99, C89 and C83 CTFs were quantified as described in Methods. (B) The levels of secreted Aβ species with Aβ-specific antibodies, anti-Aβ40 (FCA3340) or anti-β amyloid/Asp1 (FCA18). (C) Secretion of sAPP species into culture medium with sAPPα and sAPPβ-specific antibodies.

Fig. 5. Kinetics of ABCA2 effects on APP synthesis and processing

N2a and A2.1 cells were starved in methionine- and cysteine-free DMEM for 30 min, metabolically radiolabeled with 250 μ Ci/ml [35 S]methionine for 1 hr. Cells were chased in methionine- and cysteine-free DMEM supplemented with unlabeled methionine for up to 2 hours before preparation of cell lysates, immunoprecipitation with the 0443 antibody as described in Methods.

Fig. 6. Effect of ABCA2 expression on BACE1 protein level

Protein extracts from N2a, A2.1 and A2.4 cells were fractionated on 4–12% NuPAGE gels transferred to nitrocellulose and probed with the anti-BACE1 antibody as described in Methods. Following PAGE and autoradiography, bands were quantified by densitometry as described in Methods. The data are expressed as the fold-change in expression relative to control N2a cells ($P < 0.01$, Student's *t* test).

Fig. 7. Effect of protein trafficking inhibitors on APP processing

A. Cells were treated with 10 μ g/ml Brefeldin A for 24 hours before protein extraction and Western blot for APP and CTF with the 0443 antibody. B. Cells were treated with 10 μ g/ml monensin for 24 hours before protein extraction and Western blot as described above. The data are expressed as fold-change relative to untreated cells.

Fig. 8. ABCA2 decreases APP level at cell surface

(A) Cells were treated with 1 mg/ml of membrane impermeant sulfo-NHS-biotin in PBS for 30 min at 4° C as described in Methods. Biotinylated proteins were collected with avidin-agarose beads. Following PAGE and transfer to nitrocellulose filters, blots were probed with the 0443 antibody and APP holoprotein levels measured by densitometry. (B) Total APP was measured in cell extracts following Western blot with 0443. The data are expressed as the fold-change in APP

A. **B.**

Fig. 9. Processing of CTFs in lipid raft and non-raft compartments in N2a and A2.1 cells
 Cell extracts were prepared by solubilization N2a and A2.1 cells in Triton X-100 and Lubrol in MBS followed by sucrose density gradient ultracentrifugation as described in Methods. Equal volumes of protein were precipitated and analyzed by Western blot with specific antibodies. A. Distribution of raft marker flotillin-1 and non-raft marker calnexin in N2a and A2.1 cells. B. Distribution of ABCA2, APP holoprotein and CTFs in N2a and A2.1 cells.

A. **B.**

Fig. 10. Sucrose density gradient fractionation to identify sites of BACE processing of APP
Cells were grown as described in Methods and fractionated on a sucrose step gradient followed by protein precipitation and Western blot with specific antibodies. A. Distribution of BACE1 and the trans-Golgi-network marker protein TGN38 is shown. B. Distribution of APP holoprotein and CTFs in N2a and A2.1 cells is shown as well as the early-endosomal marker protein EEA1. The experiment was performed four times and representative Western blots are shown.

Table 1

Antibodies used in this study

Antibody	Type	Epitope	Source
1. ABCA2 c-term	rabbit polyclonal	human aa 2417–2436	Invitrogen
2. APP (0433)	rabbit polyclonal	human aa 751–770	Calbiochem
3. BACE1	rabbit polyclonal	human aa 485–501	Calbiochem
4. Flotillin 1	mouse monoclonal	mouse aa 312–428	BD transduction
5. Calnexin	rabbit polyclonal	canine <i>N</i> -term	Stressgen
6. TGN38	goat polyclonal	human Cyt. Domain	Santa Cruz
7. EEA1	goat polyclonal	human <i>N</i> -term	Santa Cruz
8. GAPDH	mouse monoclonal	rabbit GAPDH	Santa Cruz
9. β -tubulin	rabbit polyclonal	human aa 416–430	Lab Vision
10. sAPP α	rabbit polyclonal	sAPP α peptide	Covance
11. sAPP β	rabbit polyclonal	sAPP β peptide	Covance
12. anti- β Amyloid (FCA3340)	rabbit polyclonal	human aa 704–711	Calbiochem
13. anti- β amyloid (Asp1) (FCA18)	rabbit polyclonal	human <i>N</i> -term	Calbiochem
14. goat-anti-mouse IgG-HRP			Pierce
15. goat-anti-rabbit IgG-HRP			Pierce
16. donkey-anti-goat IgG-HRP			Santa Cruz