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Kim, Woojin Scott, Rahmanto, Aldwin Suryo, Kamili, Alvin, Rye, Kerry-Anne, Guillemin, Gilles J, Gelissen, Ingrid C, Jessup, Wendy, Hill, Andrew F and Garner, Brett (2005) Role of ABCG1 and ABCA1 in regulation of neuronal cholesterol efflux to apolipoprotein E discs and suppression of amyloid- $\beta$  peptide generation. *Journal of Biological Chemistry*, 282 (5). pp. 2851-2861. ISSN 0021-9258

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# Role of ABCG1 and ABCA1 in Regulation of Neuronal Cholesterol Efflux to Apolipoprotein E Discs and Suppression of Amyloid- $\beta$ Peptide Generation\*

Received for publication, August 16, 2006, and in revised form, November 20, 2006. Published, JBC Papers in Press, November 22, 2006, DOI 10.1074/jbc.M607831200

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Maintenance of an adequate supply of cholesterol is important for neuronal function, whereas excess cholesterol promotes amyloid precursor protein (APP) cleavage generating toxic amyloid- $\beta$  (A $\beta$ ) peptides. To gain insights into the pathways that regulate neuronal cholesterol level, we investigated the potential for reconstituted apolipoprotein E (apoE) discs, resembling nascent lipoprotein complexes in the central nervous system, to stimulate neuronal [<sup>3</sup>H]cholesterol efflux. ApoE discs potently accelerated cholesterol efflux from primary human neurons and cell lines. The process was saturable (17.5  $\mu$ g of apoE/ml) and was not influenced by *APOE* genotype. High performance liquid chromatography analysis of cholesterol and cholesterol metabolites effluxed from neurons indicated that <25% of the released cholesterol was modified to polar products (e.g. 24-hydroxycholesterol) that diffuse from neuronal membranes. Thus, most cholesterol (~75%) appeared to be effluxed from neurons in a native state via a transporter pathway. ATP-binding cassette transporters ABCA1, ABCA2, and ABCG1 were detected in neurons and neuroblastoma cell lines and expression of these cDNAs revealed that ABCA1 and ABCG1 stimulated cholesterol efflux to apoE discs. In addition, ABCA1 and ABCG1 expression in Chinese hamster ovary cells that stably express human APP significantly reduced A $\beta$  generation, whereas ABCA2 did not modulate either cholesterol efflux or A $\beta$  generation. These data indicate that ABCA1 and ABCG1 play a significant role in the regulation of neuronal cholesterol efflux to apoE discs and in suppression of APP processing to generate A $\beta$  peptides.

Regulation of cholesterol balance is crucial for normal neuronal development, plasticity, and synaptic transmission (1, 2).

\* This work was supported in part by Australian National Health and Medical Research Council Grants 350810 (to B. G.) and 222722 (to K.-A. R. and W. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> Supported by a Vice Chancellor Fellowship from the University of New South Wales.

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The cholesterol content of neuronal membranes also modulates enzymatic processing of the amyloid precursor protein (APP),<sup>3</sup> which may be sequentially cleaved by  $\beta$ - and  $\gamma$ -secretase to generate amyloid- $\beta$  (A $\beta$ ) peptides of 39 to 42 amino acids (3–5). A $\beta$  peptides are neurotoxic and proinflammatory, impair memory, and represent a major constituent of cerebral amyloid plaques associated with Alzheimer disease (6–9). APP processing via the  $\alpha$ -secretase pathway is non-amyloidogenic as the cleavage occurs in the middle of the A $\beta$  sequence and thereby precludes A $\beta$  generation. Increases in cellular cholesterol regulate APP processing by inhibiting  $\alpha$ -secretase activity and stimulating  $\beta$ - and  $\gamma$ -secretase activities (10–13).

The molecular components of the  $\beta$ - and  $\gamma$ -secretases reside in cholesterol and sphingolipid-enriched lipid raft microdomains, and elevated membrane cholesterol concentration increases the extent to which APP is located in rafts (12, 14). Thus total neuronal cholesterol levels and membrane cholesterol distribution are important determinants not only of normal neuronal function but also of pathogenic APP processing. A precise understanding of the factors controlling neuronal cholesterol homeostasis is therefore clearly required to define the pathways contributing to neurodegeneration and to design therapeutic approaches for Alzheimer disease.

Neurons, like all cells, synthesize cholesterol via the mevalonate pathway and endocytose lipoproteins via the low-density lipoprotein receptor and members of the low-density lipoprotein receptor-related protein family (2). Apolipoprotein E (apoE) as a constituent of lipoprotein particles is an important ligand for these receptors in the central nervous system (15, 16). Both the synthetic and endocytic pathways are subject to feedback down-regulation, however, because the passive diffusion of cholesterol from cell membranes is extremely inefficient, when cholesterol levels are elevated beyond the needs of the cell, an export mechanism is required to remove the excess cholesterol. Neurons selectively express cholesterol 24-hydrox-

<sup>3</sup> The abbreviations used are: APP, amyloid precursor protein; A $\beta$ , amyloid  $\beta$ ; apoE, apolipoprotein E; HPLC, high performance liquid chromatography; ABC, ATP-binding cassette; CHO, Chinese hamster ovary; HEK, human embryonic kidney; 24-OH-Ch, 24-S-hydroxycholesterol; POPC, 1-palmitoyl-2-oleylphosphatidylcholine; PBS, phosphate-buffered saline; BSA, bovine serum albumin; LXR, liver X receptor; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PL, phospholipid.

## Neuronal Cholesterol Efflux and A $\beta$ Production

ylase and it has been shown that the cholesterol oxidation product 24-*S*-hydroxy-cholesterol (24-OH-Ch) can be released from cells and diffuse through the blood-brain barrier into the circulation (17, 18). As cholesterol cannot be enzymatically degraded, this pathway is thought to contribute to the removal of excess neuronal cholesterol and to balance cholesterol synthesis in the central nervous system (2, 19, 20). The exact contribution that 24-OH-Ch makes to total cholesterol flux from neurons is, however, not known. Previous research indicates that micromolar concentrations of 24-OH-Ch are neurotoxic and proinflammatory (21, 22) and that deletion of 24-OH-Ch oxidase activity in *Cyp46a1* knock-out mice resulted in a partial (~40%) reduction in brain cholesterol synthesis without neurological abnormalities (23). These observations suggest that in addition to the 24-OH-Chol pathway, another neuronal cholesterol efflux pathway may exist.

Several members of the ATP-binding cassette subfamily A transporters (ABCA1, A2, A3, A7, and A8) that are potentially involved in trans-membrane lipid transport are expressed in isolated human neurons and neuronal cell lines (24, 25). ABCG1 and ABCG4 are also strongly expressed in the brain and there is evidence that ABCG1 is expressed in mouse neuronal tissue (26, 27). ABCA/G transporters are well known to transport cholesterol across the plasma membrane to apolipoprotein acceptors in peripheral tissues and this constitutes the initial step in the reverse cholesterol transport pathway (28). Studies in macrophages have revealed that apoA-I initially interacts with ABCA1 to generate a partially lipidated discoidal complex that subsequently interacts with ABCG1 to acquire additional cholesterol, which may be esterified by the action of lecithin:cholesterol acyltransferase to generate core lipids and thus a spherical lipoprotein particle (29, 30). Whether a similar process involving neuronal ABC transporters occurs is not known.

It is clear that apoE is a major central nervous system cholesterol transport protein (31). ApoE isolated from cerebral spinal fluid is present in the form of both spherical and discoidal lipoprotein complexes (15, 32–38). Previous reports have suggested that apoE discs could participate in cellular cholesterol efflux (31, 33) and there is evidence consistent with a role for ABCA1 and ABCG1 in the regulation of cholesterol efflux from astrocytes and microglia, respectively (39, 40). Based on this earlier work and our identification of specific ABC transporters in human neurons (24) we hypothesized that apoE discs may stimulate cholesterol efflux from neurons via ABCA/G transporters. In the present work we have therefore investigated the potential for apoE discs to stimulate cholesterol efflux from neurons, the contribution that 24-OH-Ch makes to total cholesterol efflux, and the role that specific ABCA/G transporters expressed in neurons may play in this pathway. Because neuronal cholesterol balance regulates APP processing, we also examined the impact of ABCA/G transporters on A $\beta$  peptide generation.

### EXPERIMENTAL PROCEDURES

**Materials**—Cell culture media and additives were obtained from Invitrogen (Melbourne, Australia) unless stated otherwise. Recombinant human A $\beta$ -(1–40) and A $\beta$ -(1–42) were

purchased from rPeptide (Athens, GA), stored lyophilized at –20 °C until use and a 1 mM stock solution prepared in dimethyl sulfoxide directly before addition to cell culture medium. Human ABCA1, ABCA2, and apoE cDNAs were generously provided by Professor Mason Freeman (Harvard Medical School), Professor Kenneth Tew (Medical University of South Carolina), and Professor Karl Weisgraber (Gladstone Institutes, University of California, San Francisco).

**Cell Culture**—Human fetal brain tissues were obtained from 14–18-week-old aborted fetuses collected after therapeutic termination following informed consent (ethical approval from the University of New South Wales Human Research Ethics Committee, HREC03187). Neurons, astrocytes, oligodendrocytes, and microglia were isolated from the brain tissues and cultured as previously described (24). The cell lines SK-N-SH, NTERA-2 (NT2), HEK293, and BV2 were obtained from the ATCC (Manassas, VA). Human foreskin fibroblasts (AG01518) were obtained from the Coriell Institute (Camden, NJ). All cell lines were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, and 100  $\mu$ g/ml streptomycin at 37 °C in humidified air containing 5% CO<sub>2</sub>. HEK293 cells were grown on poly-D-lysine-coated plates to ensure maximal adhesion. The CHO cell lines stably expressing human ABCG1 (CHO-ABCG1) or the human 695-amino acid APP (CHO-APP) were generated as described previously (30, 41). The recombinant plasmids were maintained using Zeocin (200  $\mu$ g/ml) and puromycin (7.5  $\mu$ g/ml), respectively. Transfected cells were cultured in RPMI 1640 medium containing 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, and 100  $\mu$ g/ml streptomycin.

**Transfection**—Transient transfection was performed using Lipofectamine 2000 and Opti-MEM I (Invitrogen) following the manufacturer's protocol. Briefly, cells were seeded at ~90% confluence in 12-well plates using antibiotic-free medium. cDNA-Lipofectamine complex was added to the cells and after 24 h of incubation samples were collected for gene expression analysis. In the case of cholesterol efflux assays the cells were cultured for up to an additional 24 h.

**Cholesterol Efflux Assay**—Cellular cholesterol efflux was measured as described previously (42). In brief, cells were labeled with 2  $\mu$ Ci/ml [<sup>3</sup>H]cholesterol (Amersham Biosciences) for 24 h, rinsed with phosphate-buffered saline (PBS), and incubated for 2 h in medium containing 0.1% (w/v) bovine serum albumin (BSA) to allow equilibration of [<sup>3</sup>H]cholesterol in intracellular pools. The cells were rinsed once more in PBS and then incubated in serum-free medium containing 0.1% BSA with or without cholesterol acceptors for up to 24 h (*i.e.* 0.1% BSA is always present). Media samples were collected at specific time points and cleared of any cellular debris by centrifugation at 1000  $\times$  *g* for 5 min. The cells were lysed with 0.1 M NaOH and radioactivity in the media samples and cell lysates were measured by scintillation counting. Cholesterol effluxed to the medium was calculated as a percentage of total radioactivity in the cell lysates and medium. Experiments were routinely performed in triplicate and repeated three times. The cholesterol acceptors used were apoA-I, apoE3, reconstituted apoE2, apoE3, and apoE4 discs (see below) and BV2 microglial cell-conditioned media. Unless stated otherwise all experi-

ments using apoE discs contained the apoE3 isoform. Human apoA-I was purified from human high density lipoprotein by ultracentrifugation and anion exchange chromatography as previously described (42). Recombinant human apoE2, apoE3, and apoE4 were prepared from *Escherichia coli* as previously described (43, 44).

ApoE discs containing recombinant apoE, 1-palmitoyl-2-oleylphosphatidyl choline (POPC), and cholesterol were prepared using the cholate dialysis method and characterized as described previously (45). ApoE discs had an average diameter of 17.0 nm as judged by gel filtration chromatography (45). The disc size and therefore apoE conformation resembles astrocyte-secreted apoE discs that are reported to have a mean diameter of 15.4 nm (33). The POPC/cholesterol/apolipoprotein molar ratios of the apoE discs ranged from 114.7:12.8:1.0 to 94.4:8.4:1.0. This stoichiometry indicates that our reconstituted apoE discs were phospholipid-enriched as compared with astrocyte-secreted apoE discs that have been reported to contain phospholipid/cholesterol ratios in the order of 2:1 to 1:2 (33, 34, 38). Attempts to prepare reconstituted apoE discs with equimolar POPC/cholesterol ratios were not successful resulting in a loss of discoidal structure.<sup>4</sup> Nonetheless, the reconstituted apoE discs do resemble astrocyte-secreted discs and are more physiologically relevant than lipid-free apoE, which does not appear to be present in cerebral spinal fluid.

All cholesterol acceptors were used at a concentration of 15  $\mu$ g of protein/ml unless stated otherwise. Conditioned medium from microglial cells was prepared by culturing murine BV2 cells in 75-cm<sup>2</sup> flasks with serum-free medium for 24 h. The conditioned medium was collected and centrifuged at 1000  $\times$  g for 10 min and stored at 4 °C for up to 1 week until use. The concentration of apoE in BV2-conditioned medium was estimated by Western blot analysis using a rabbit anti-human apoE polyclonal antibody that cross-reacts with murine apoE (44).

**Western Blotting**—Cells expressing ABCA1, ABCA2, ABCG1 (or stably expressing Myc-tagged ABCG1) or APP 695 were cultured in 6-well plates, rinsed with cold PBS, and lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and protease and phosphatase inhibitors). Bicinchoninic acid protein assays were performed on lysates and equal amounts of protein were separated on SDS-PAGE gels (12% for ABCG1, 6% for ABCA1, ABCA2, and APP) and transferred onto 0.45- $\mu$ m nitrocellulose membranes at 100 volts for 30 min. Membranes were blocked overnight at 4 °C in PBS containing 5% nonfat dry milk and probed with the relevant antibodies to reveal the major bands at the appropriate molecular mass: ABCA1 250 kDa (Novus, rabbit polyclonal 1/1000), ABCA2 270 kDa (from Professor Kenneth Tew, rabbit polyclonal 1/1000), ABCG1 ~60 kDa (ABCG1 Santa Cruz, rabbit polyclonal 1/200, ABCG1-Myc Invitrogen, murine monoclonal 1/5000), APP 90-kDa (Sigma, 6E10 monoclonal 1/2000) at 22 °C for 2 h. The membranes were washed three times in PBS containing 0.1% Tween 20 and then incubated with horseradish peroxidase-conjugated goat anti-rabbit (Dako, 1/2000) or rabbit anti-

mouse (Dako, 1/1000) secondary antibody for 2 h. Signals were detected using enhanced chemiluminescence (ECL, Amersham Biosciences) and x-ray films. The signal intensity was quantified using NIH Image J software. Western blotting of secreted A $\beta$  peptides was carried out as previously described (41). Briefly, A $\beta$  in the culture medium was separated on 10–20% Tris/Tricine gels or 12% SDS-PAGE gels and transferred onto 0.2- $\mu$ m nitrocellulose membranes at 65 volts for 15 min. Membranes were boiled in Milli-Q H<sub>2</sub>O for 10 min, probed with anti-APP 6E10 monoclonal antibody followed by rabbit anti-mouse horseradish peroxidase-conjugated secondary antibody and ECL detection applied as described above.

**HPLC**—Total lipids were extracted from cell culture medium containing [<sup>3</sup>H]cholesterol (and potential derivatives) by mixing 150  $\mu$ l of culture medium, previously centrifuged at 1000  $\times$  g for 5 min to remove cellular debris, with 600  $\mu$ l of methanol. To this was added 150  $\mu$ l of chloroform and the mixture vortexed. To this was added 450  $\mu$ l of Milli-Q H<sub>2</sub>O and the mixture vortexed and centrifuged at 12,000  $\times$  g for 5 min. The upper aqueous phase was collected into a clean tube and 450  $\mu$ l of methanol added to the infranatant. This mixture was mixed and centrifuged as above and the supernatant removed from the protein pellet and added to the tube containing the original aqueous upper phase. This extract (containing all polar and non-polar compounds but excluding proteins) was dried under vacuum and redissolved in 100  $\mu$ l of methanol. Fifty  $\mu$ l of each sample was analyzed by reversed phase HPLC using a Supelco-sil 250  $\times$  4.6-mm C18 column (Supelco, Bellefonte, PA) and methanol mobile phase according to an established method (46). Fractions of 250  $\mu$ l were collected from the column and [<sup>3</sup>H] levels of each fraction assessed by scintillation counting. The same HPLC method was used to analyze oxysterol standards with UV210 nm detection.

**Quantitative Real-time PCR**—RNA was isolated from cells using TRIzol reagent (Invitrogen) following the manufacturer's protocol. All procedures were carried out using RNase-free reagents. Four  $\mu$ g of RNA was reverse transcribed into cDNA as previously described (24). cDNA was used as a template in the quantitative real-time PCR assay, which was carried out using a Mastercycler ep realplex S (Eppendorf) and the fluorescent dye SYBR Green (Eppendorf), following the manufacturer's protocol. Briefly, each reaction (20  $\mu$ l) contained 1 $\times$  RealMasterMix, 1 $\times$  SYBR Green, 5 pmol of primers, and 1  $\mu$ l of template. Amplification was carried out with 40 cycles of 94 °C for 15 s and 60 °C for 1 min. All gene expression was normalized to  $\beta$ -actin, which served as an internal control for the quality of RNA isolated from each cell sample. Experiments were performed in triplicate and at least three samples were analyzed for each cell type. Conventional PCR amplification was also carried out with 30 cycles of denaturation (94 °C, 30 s), annealing (60 °C, 30 s), and extension (72 °C, 30 s), and the PCR products were visualized after electrophoresis in 1% agarose gels. Details of all primers used are provided in Table 1.

**A $\beta$  Peptide Clearance**—Cells were seeded in 12-well plates using Dulbecco's modified Eagle's medium, 10% fetal calf serum as above. Once the cells were confluent the medium was replaced with serum-free medium containing 1  $\mu$ M of the recombinant A $\beta$  peptides (A $\beta$ -(1–40) or A $\beta$ -(1–42)) with

<sup>4</sup> K.-A. Rye, unpublished observation.

## Neuronal Cholesterol Efflux and A $\beta$ Production

**TABLE 1**  
Primer sequences and PCR product sizes

Gene	Primer <sup>a</sup> (5'-3')	Product size
		bp
ABCA1	F: AACTCTACATCTCCCTTCCCG R: CTCCTGTCGCATGTCACCTC	123
ABCA2	F: AGTGCTCAGCCTTCGTACAG R: AGGCGGTACAGGATTTTGG	188
ABCA3	F: ACGTCTGGAAGCTTCTCCT R: TGTGAGAAGGGATGTAGGCAA	187
ABCG1	F: TTTGAGGGATTTGGGTCTGAAC R: CCCCTTTAATCGTTTTGTCTGCT	229
APP	F: AGAGTACCAACTTGCATGAC R: TCCTCTGCTACTTCTACTAC	211
$\beta$ -Actin	F: TCATGAAGTGTGACGTGGACATCCGT R: CCTAGAAGCATTTGCGGTGCACGATG	285

<sup>a</sup> F, forward; R, reverse.

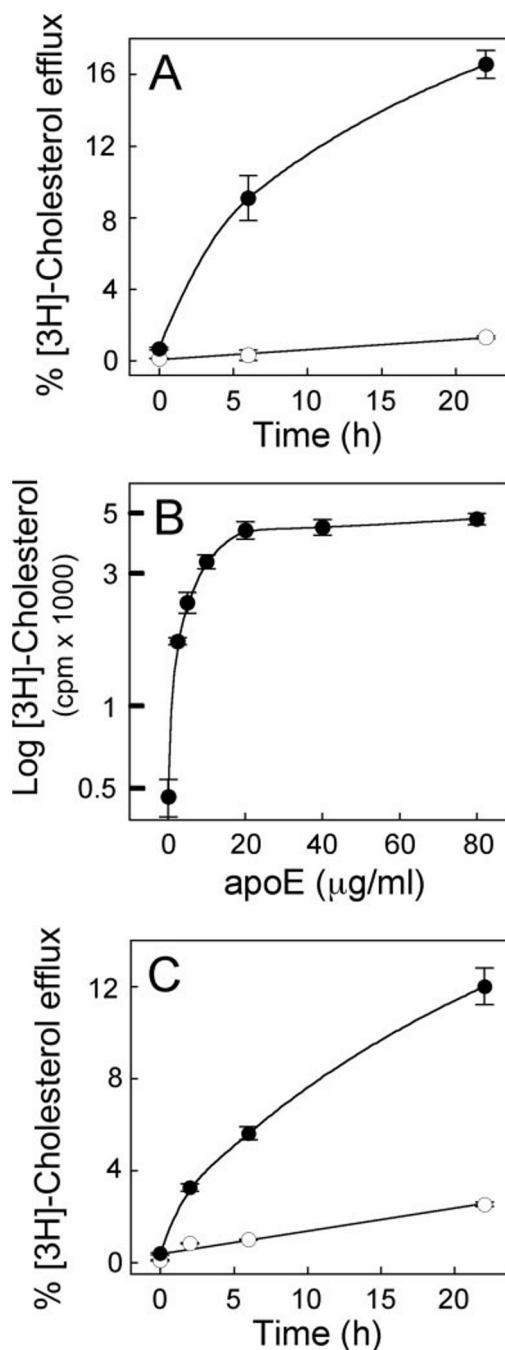
either 15  $\mu$ g/ml apoE disc or 0.1% (w/v) BSA. After 24 h medium samples were collected and cells removed by centrifugation. The samples were analyzed for A $\beta$  by Western blotting.

**Statistical Analysis**—Experiments were routinely performed in triplicate and repeated 3 times. Data are presented as mean  $\pm$  S.E. shown by error bars. Differences were considered significant where  $p < 0.05$  as determined by the two-tailed Student's  $t$  test for unpaired data.

### RESULTS

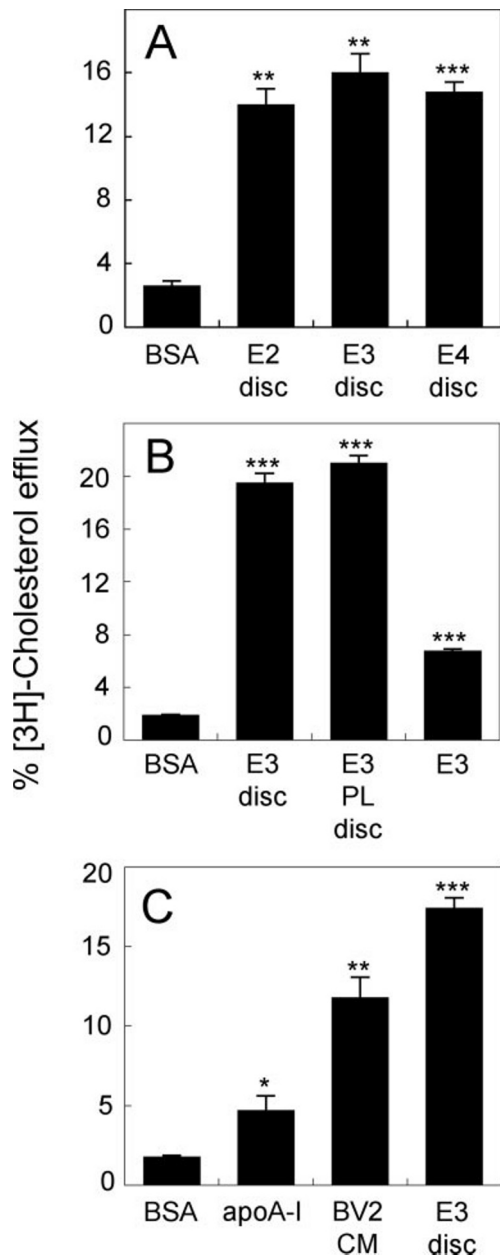
**Cholesterol Efflux from Neurons to ApoE Discs**—Previous work identified apoE discs in the human brain and raised the possibility that these lipid-poor complexes may promote neuronal cholesterol efflux (15, 31, 33, 34). To test this, we synthesized apoE3 discs (E3 is the most common *APOE* genotype) containing cholesterol and phospholipid (PL), thereby resembling lipidated apoE discs secreted from astroglial cells (see "Experimental Procedures"), and incubated these discs with [ $^3$ H]cholesterol-labeled human SK-N-SH neurons. ApoE discs potently stimulated neuronal cholesterol efflux (Fig. 1A). This process was saturated at an apoE protein concentration of 17.5  $\mu$ g/ml (Fig. 1B) and essentially identical results were obtained when cholesterol efflux from primary human neurons was assessed (Fig. 1C).

**Impact of ApoE Disc Composition on Neuronal Cholesterol Efflux**—Because apoE genotype is a strong predictor of Alzheimer disease risk (47), and neuronal cholesterol accumulation is associated with neurodegeneration (48), we assessed the efflux capacity of apoE discs containing each of the three common human apoE isoforms (E2, E3, and E4). ApoE discs comprising each of the three isoforms were equally potent in their ability to stimulate cholesterol efflux (Fig. 2A). As the apoE discs contain cholesterol, it was possible that the efflux of [ $^3$ H]cholesterol to apoE discs was at least partly accelerated in response to delivery of exogenous cholesterol to the cells (subsequent to up-regulation of cholesterol responsive genes). Further experiments were therefore conducted using apoE discs that contained PL only or, for the purpose of comparison, non-physiological lipid-free apoE. The absence of cholesterol in the apoE discs had no impact on neuronal [ $^3$ H]cholesterol efflux activity, whereas lipid-free (non-discoidal) apoE was a relatively poor cholesterol acceptor (Fig. 2B). Additional experiments revealed that apoA-I was also a relatively poor acceptor of neuronal cholesterol and



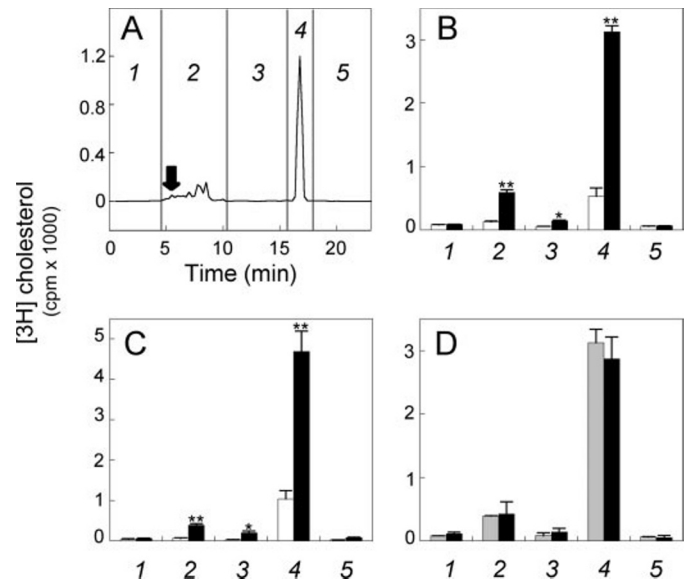
**FIGURE 1. Cholesterol efflux from human neurons to apoE discs.** A, SK-N-SH neurons were labeled with [ $^3$ H]cholesterol for 24 h followed by incubation in serum-free medium containing 0.1% BSA with (●) or without (○) apoE discs. Samples of the media were collected at 6 and 22 h, and the percent cholesterol efflux was calculated by dividing [ $^3$ H]cholesterol released to the medium by the total [ $^3$ H]cholesterol in the cells and medium. B, to determine the saturation concentration of apoE discs, SK-N-SH cells were labeled with [ $^3$ H]cholesterol and incubated with increasing concentrations of apoE (0, 2.5, 5.0, 10, 20, 40, and 80  $\mu$ g/ml) and cholesterol efflux determined at 6 h. C, primary human neurons were labeled with [ $^3$ H]cholesterol as above and incubated with (●) or without (○) apoE discs, and the percent cholesterol efflux was calculated at the time points indicated. Experiments were performed in triplicate and values are mean  $\pm$  S.E. represented by the error bars.

that murine BV2 microglial cell culture-conditioned medium, which contains apoE (49), stimulated cholesterol efflux to a similar degree as the reconstituted apoE discs (Fig. 2C). These



**FIGURE 2. Impact of apoE disc composition on neuronal cholesterol efflux.** *A*, the effect of different apoE isoforms (*E2*, *E3*, and *E4*) on cholesterol efflux was tested. SK-N-SH neurons were labeled with [ $^3$ H]cholesterol for 24 h followed by incubation in serum-free medium containing 0.1% BSA plus apoE discs of the isoform indicated. Samples of the media were collected at 24 h and the percent cholesterol efflux was calculated. *B*, to further characterize the effect of apoE disc composition on cholesterol efflux, SK-N-SH neurons were labeled with [ $^3$ H]cholesterol and exposed to apoE3 discs that contain phospholipid (PL) only (*E3 PL disc*). This was compared with lipid-free apoE3 (*E3*) and apoE3 discs that contain PL and cholesterol (*E3 disc*). *C*, the ability of apoA-I (*apoA-I*) or murine BV2 microglial cell-conditioned medium (*BV2 CM*) to stimulate cholesterol efflux from SK-N-SH neurons was also assessed. Acceptors were used at a protein concentration of 15  $\mu$ g/ml except for the BV2-conditioned medium that was used at a concentration of apoE approximating 15  $\mu$ g/ml (as assessed by Western blotting, see "Experimental Procedures"). Experiments were performed in triplicate and values are mean  $\pm$  S.E. represented by the error bars. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

data indicate that apoE discs (a structure that is physiologically relevant to the human central nervous system) can stimulate cholesterol efflux from neurons and that this process is not significantly dependent on *APOE* genotype.



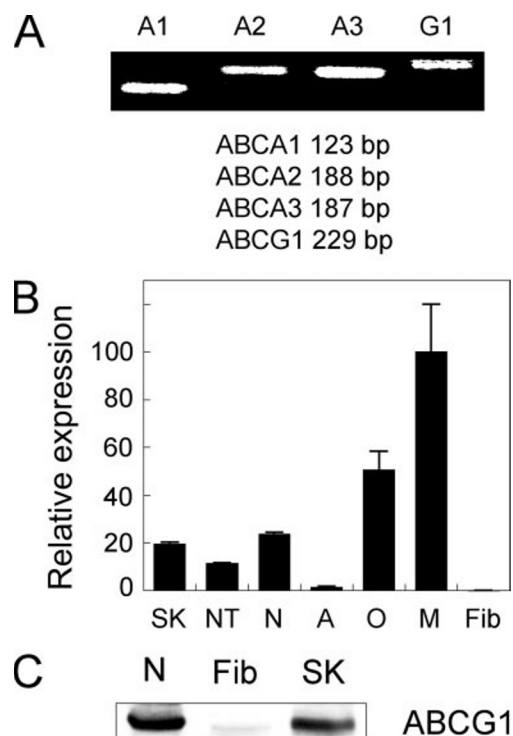
**FIGURE 3. Quantitation of non-modified [ $^3$ H]cholesterol effluxed from neurons to apoE discs.** *A*, SK-N-SH neurons were labeled with [ $^3$ H]cholesterol for 24 h followed by incubation in serum-free medium containing 0.1% BSA only or BSA plus apoE discs. Samples of the media were collected at 24 h and analyzed by reversed phase HPLC. Fractions eluting from the column were collected at 15-s intervals and the total [ $^3$ H] in each fraction analyzed separately by scintillation counting. The chromatogram was divided into five fractions (1–5). A series of small peaks was observed in fraction 2 indicating small amounts of different cholesterol metabolites. A peak eluting at 5.1 min (arrowed) co-eluted with 24-OH-Ch. The major peak was observed at 17 min and this represents the intact cholesterol, which contributes to at least 75% of the cholesterol effluxed from neurons. *B*, the five pooled fractions derived from SK-N-SH cells treated with BSA (open bar) or with apoE discs (filled bar) were analyzed. *C*, these five fractions from primary human neurons treated with BSA (open bar) or apoE discs (filled bar) were also analyzed. *D*, the five fractions from SK-N-SH cells treated with apoE discs containing PL only (hatched bar) or apoE discs containing both PL and cholesterol (filled bar) were also analyzed. Data are mean values derived from triplicates with S.E. represented by the error bars. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

**Quantitation of [ $^3$ H]Cholesterol and [ $^3$ H]Cholesterol Derivatives Effluxed from Neurons to ApoE Discs**—Previous work suggests that neuronal cholesterol 24-hydroxylase may contribute to cholesterol efflux in the central nervous system (2, 18, 19). It was of interest therefore to determine what proportion of the [ $^3$ H]cholesterol effluxed from neurons under our experimental conditions was in the form of cholesterol *versus* 24-OH-Ch or perhaps other polar [ $^3$ H]cholesterol derivatives. To assess the contribution of metabolic products of [ $^3$ H]cholesterol to total [ $^3$ H] detected in the medium, cell culture supernatants were collected and total lipid extracts analyzed by reversed phase HPLC. Fractions eluting from the column were collected at 15-s (250  $\mu$ l) intervals and the total [ $^3$ H] in each fraction analyzed separately by scintillation counting.

This analysis indicated that at least 75% of the cholesterol removed from neurons in the presence of apoE discs could be recovered as intact cholesterol (Fig. 3A). Greater than 97% of the [ $^3$ H] label was recovered from the HPLC column in these experiments and spiking experiments indicated that both cholesterol and 24-OH-Ch were stable to the extraction and analytical methods.<sup>5</sup> The fraction of [ $^3$ H] that did not elute in the cholesterol fraction was recovered as a series of partially

<sup>5</sup> W. S. Kim and B. Garner, unpublished observation.

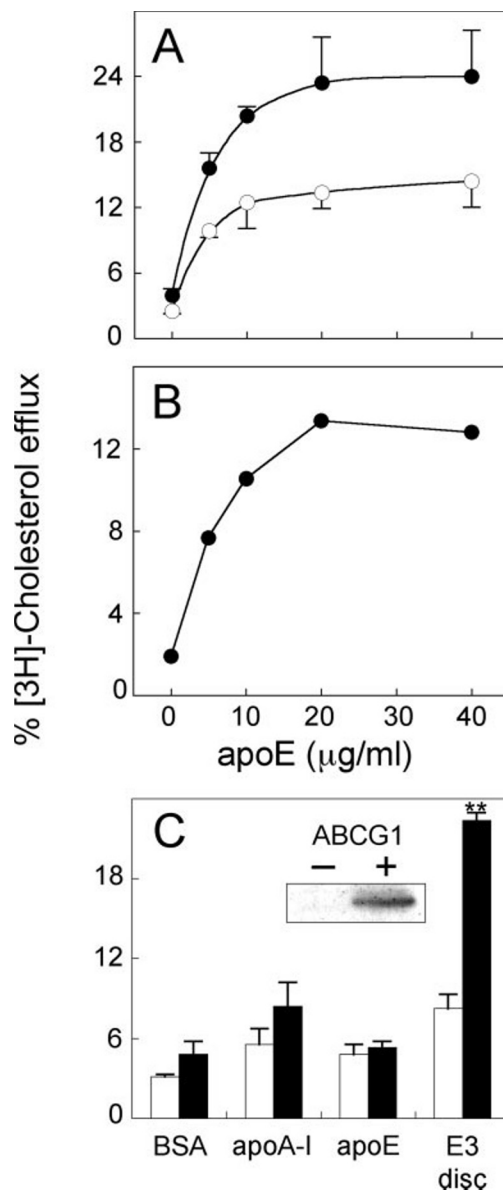
## Neuronal Cholesterol Efflux and A $\beta$ Production



**FIGURE 4. Analysis of ABCA/G transporter gene expression in human neurons.** *A*, RNA was isolated from primary human neurons and converted to cDNA that was then used as template to PCR amplify ABCA1, ABCA2, ABCA3, and ABCG1. The PCR products were visualized on a 1% agarose gel that shows a single product of appropriate size for each gene. *B*, quantitative real-time PCR was used to measure the expression levels of ABCG1 in neuronal cell lines SK-N-SH (SK) and NTERA-2 (NT), primary human neurons (N), astrocytes (A), oligodendrocytes (O), microglia (M), and fibroblasts (Fib). The expression levels were normalized to  $\beta$ -actin and are presented as percent values relative to the cell type that produced the highest expression. Data are means of three different cell preparations for each cell type and the error bars show S.E. *C*, the expression of ABCG1 in primary human neurons (N) and neuronal cell line SK-N-SH (SK) was verified by Western blotting. The expression in fibroblasts (Fib) was very low as expected. All lanes were loaded with 40  $\mu$ g of protein.

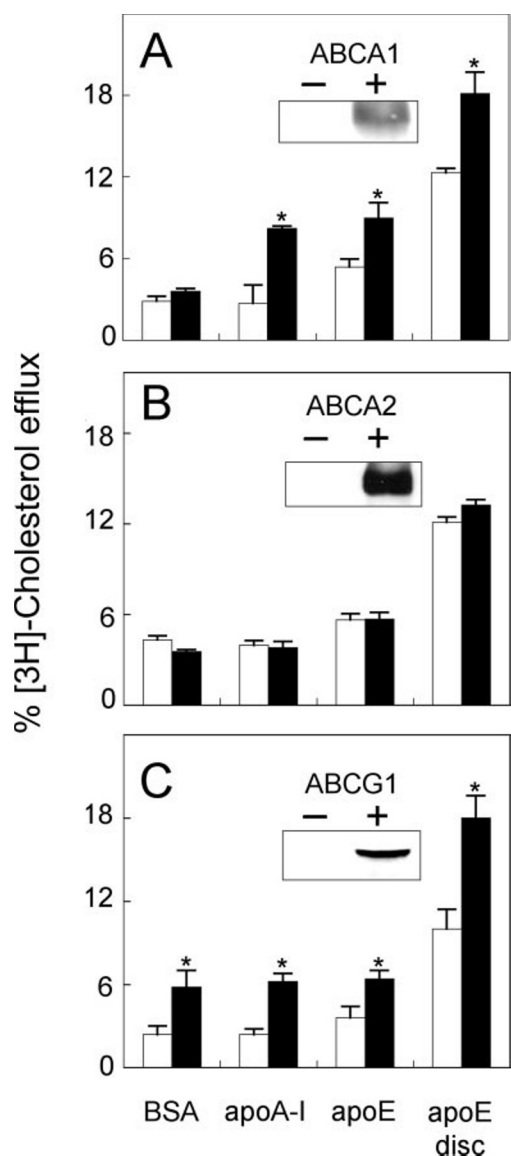
resolved peaks with retention times between 5 and 10 min. A small but reproducibly detectable peak eluting at 5.1 min co-eluted with 24-OH-Ch (Fig. 3A). Although this suggests that only a minor proportion of cholesterol was converted to 24-OH-Ch in neurons, it is possible that 24-OH-Ch was metabolized further to form other products that eluted within the 5–10-min fraction. There is evidence that 24-OH-Ch may be metabolized by neurons and glia, however, the products resulting from such metabolism are not definitively established (50). Several commercial oxysterol standards including 27- and 25-hydroxycholesterol eluted in this early region of the chromatogram; however, identifications of these more polar cholesterol products in the cell culture supernatants were not made (due to the small quantities present).

In additional experiments, the polar fractions containing [ $^3$ H] were pooled (as indicated Fig. 3A) and the total radioactivity in this fraction compared with that in the [ $^3$ H]cholesterol fraction. Whereas the levels of [ $^3$ H]cholesterol detected in the efflux medium that contained apoE discs were consistently higher than in the presence of BSA alone (as predicted), we were surprised to find that the levels of the polar [ $^3$ H]cholesterol products were also significantly higher in the presence of



**FIGURE 5. Role of ABCG1 in cholesterol efflux to apoE discs.** CHO cells that stably express human ABCG1 (CHO-ABCG1) were used to test whether ABCG1 was able to promote cholesterol efflux to apoE discs. *A*, CHO-ABCG1 ( $\bullet$ ) and CHO ( $\circ$ ) cells were labeled with [ $^3$ H]cholesterol for 24 h followed by incubation in serum-free medium containing 0.1% BSA or BSA plus apoE discs (0, 5.0, 10, 20, and 40  $\mu$ g/ml) and cholesterol efflux determined after 24 h. *B*, to determine the saturation concentration for cholesterol efflux by apoE discs, the cholesterol efflux achieved from CHO cells was subtracted from the CHO-ABCG1 cholesterol efflux values. *C*, the ability of ABCG1 to promote cholesterol efflux to lipid-free apoA-I or apoE was also examined. CHO (open bar) and CHO-ABCG1 (filled bar) cells that were labeled with [ $^3$ H]cholesterol were incubated with apoA-I (15  $\mu$ g/ml) and apoE (15  $\mu$ g/ml) and were compared with apoE discs (15  $\mu$ g/ml). The inset shows Western blotting of ABCG1 in CHO-ABCG1 (+) and CHO (-) cells. Data are mean values derived from triplicates with S.E. represented by the error bars. \*\*,  $p < 0.01$ .

apoE discs (Fig. 3B). This was observed irrespective of whether SK-N-SH neurons or primary human neurons were analyzed (Fig. 3B, see also C). This could indicate that at least a proportion of the polar cholesterol products are unable to diffuse from the plasma membrane in the absence of an acceptor (which is in contrast to 24-OH-Ch (51)) or that apoE discs stimulate a signaling pathway that results in [ $^3$ H]cholesterol modification.



**FIGURE 6. Ability of ABCA1, ABCA2, and ABCG1 to stimulate cholesterol efflux to different acceptors.** HEK293 cells were transfected (filled bars) with human ABCA1 (A), ABCA2 (B), or ABCG1 (C) or empty vector (open bars), labeled with [ $^3$ H]cholesterol, and then incubated with apoA-I (15  $\mu$ g/ml), apoE (15  $\mu$ g/ml), or apoE discs (15  $\mu$ g/ml) for 24 h. The insets show Western blotting of HEK293 cells transfected with each of the ABC transporters (+) or empty vector (-). Data are mean values derived from triplicates with S.E. represented by the error bars. \*,  $p < 0.05$ .

Similar to our observations regarding levels of total cholesterol efflux (Fig. 2B), apoE discs that contained PL only produced essentially identical quantitative profiles of [ $^3$ H]cholesterol and polar [ $^3$ H]cholesterol products as determined by HPLC (Fig. 3D). Thus, exogenously added cholesterol did not appear to be responsible for the increase in polar [ $^3$ H]cholesterol derivatives observed in the presence of apoE discs. The primary purpose of the HPLC analysis was to determine the amount of cholesterol removed from neurons that remains in a native state. It was clear that the majority (>75%) of cholesterol effluxed from neurons in the presence of apoE discs was not modified. This raised the possibility that a transporter mechanism may play a role in promoting cholesterol efflux to apoE discs.

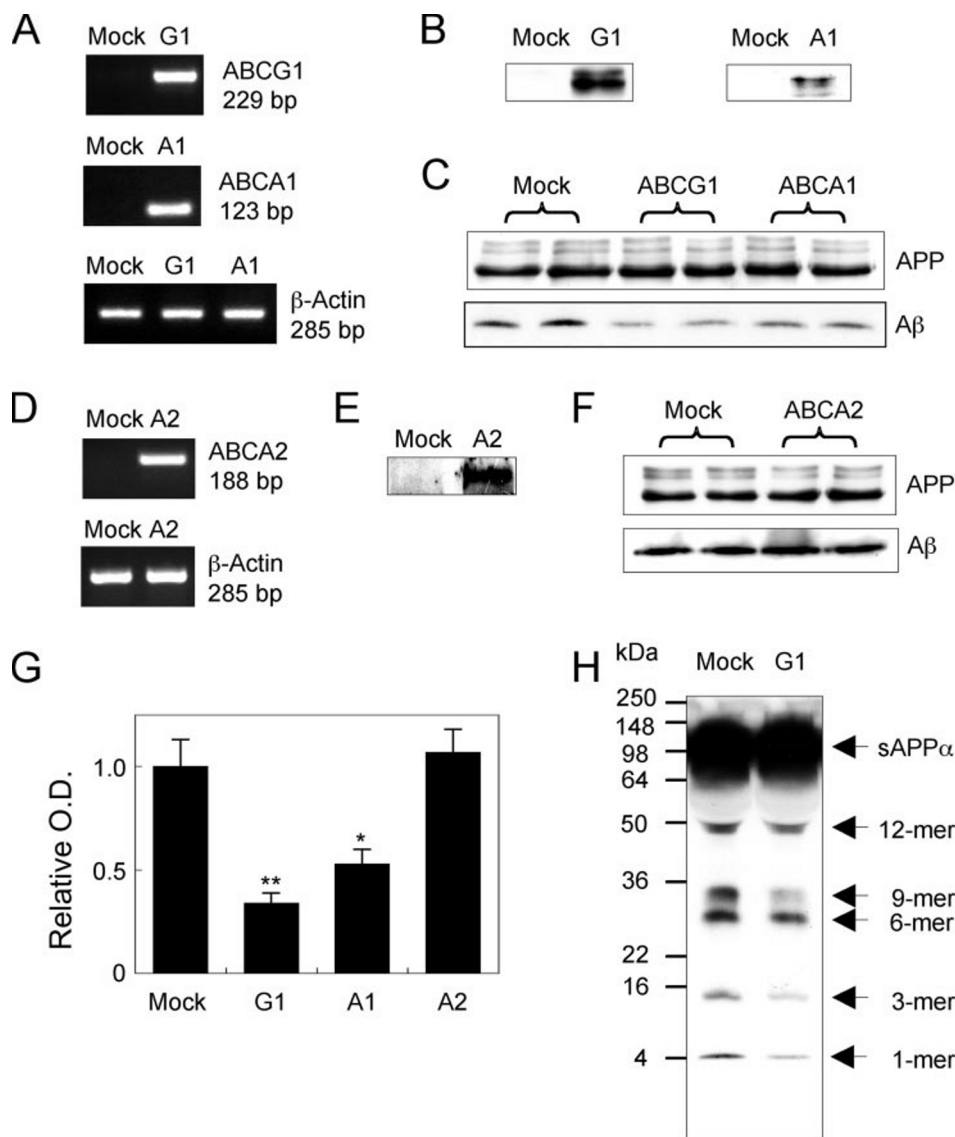
**Analysis of ABCA/G Transporter Gene Expression in Neurons**—Recent studies show that several ABC class A transporters are expressed in primary human neurons and neuronal cell lines (24). Of these ABCA1, A2, and A3 are the most highly expressed (24). Additional work indicates that ABCG1 transfers membrane cholesterol to lipoprotein complexes (30). Based on previous work, the expression of ABCG1 in primary human neurons is plausible (27, 52, 53); however, direct data demonstrating this is lacking. We addressed this issue and found that ABCG1 was clearly detected in primary human neurons (Fig. 4A). Real time PCR analysis of isolated human neurons, astrocytes, oligodendrocytes, and microglia revealed that ABCG1 was expressed in all of the cell types examined, although expression in astrocytes was relatively low (Fig. 4B). Analysis of the SK-N-SH and NTERA-2 neuroblastoma cell lines indicated ABCG1 was expressed at approximately the same level as primary neurons (Fig. 4B), whereas ABCG1 expression was very low in fibroblasts. Expression of ABCG1 protein in primary human neurons and SK-N-SH neuroblastoma cells was also confirmed by Western blotting (Fig. 4C). The finding that ABCG1 is expressed in neurons prompted us to further examine whether this particular transporter could accelerate cholesterol efflux to apoE discs.

**Role of ABCG1 in Cholesterol Efflux to ApoE Discs**—To examine a potential role for ABCG1 to promote cholesterol efflux to apoE discs we initially used CHO cells that stably express human ABCG1 (30). Cholesterol efflux to apoE discs was significantly accelerated when CHO-ABCG1 cells were compared with CHO cells (Fig. 5A). ABCG1-dependent cholesterol efflux to apoE discs was saturated at an apoE protein concentration of 18  $\mu$ g/ml (Fig. 5B). Consistent with previous observations using this CHO-ABCG1 cell line (30), efflux of cholesterol to lipid-free apoA-I or apoE was not significantly accelerated by ABCG1, although there was a trend toward increased efflux in the presence of BSA and apoA-I (Fig. 5C). In contrast, cholesterol efflux to apoE discs was consistently increased 2–3-fold from the cells expressing ABCG1 (Fig. 5C).

**Role of ABCA1 and ABCA2 in Cholesterol Efflux to ApoE Discs**—To determine whether cholesterol efflux to apoE discs could also be accelerated by specific ABCA transporters expressed in human neurons (ABCA1, ABCA2), we used a HEK293 transient transfection approach. In these experiments ABCA1 transfection more than doubled the rate of cholesterol efflux to apoA-I (Fig. 6A), consistent with previous work (54). Cholesterol efflux to lipid-free apoE was also significantly increased by ABCA1 transfection, also confirming previous observations (55). When cholesterol efflux to apoE discs was evaluated, we observed a significant increase in the order of 25–30% (Fig. 6A). In contrast, transfection of HEK293 cells with human ABCA2 cDNA did not result in increased cholesterol efflux to any of the acceptors used in these experiments (Fig. 6B). We also transiently transfected HEK293 cells with human ABCG1 cDNA and these experiments confirmed that cholesterol efflux was significantly accelerated by apoE discs (Fig. 6C). In these latter experiments, basal cholesterol efflux to BSA was also stimulated in the ABCG1 overexpressing cells and was not increased fur-



## Neuronal Cholesterol Efflux and A $\beta$ Production



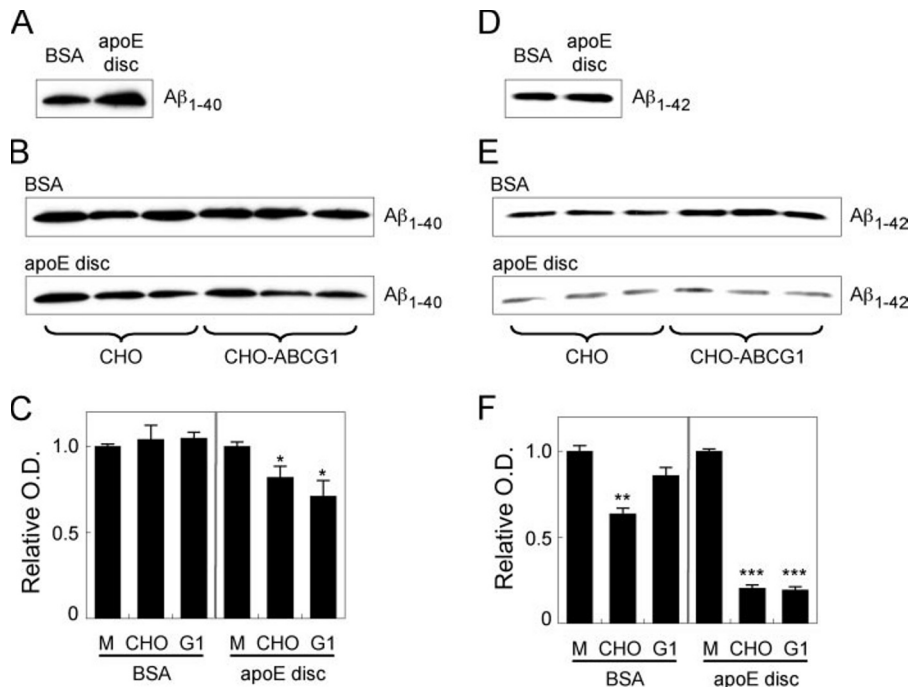
**FIGURE 7. Impact of ABCA/G transporter expression on A $\beta$  peptide generation.** To examine the potential impact of ABCA/G transporters on A $\beta$  peptide generation, CHO cells that stably express human APP (CHO-APP) were transiently transfected with human ABCG1, ABCA1, ABCA2, and empty vector (*Mock*). Transfection efficiency was confirmed by PCR amplification of cDNA (*A* and *D*) and Western blotting (*B* and *E*). PCR amplification of the internal control  $\beta$ -actin shows similar loading. Twenty four h after transfection samples were collected from the culture media and equal volumes of samples (40  $\mu$ l) were separated on 12% SDS-PAGE gels, transferred onto 0.2- $\mu$ m nitrocellulose membranes, and probed with anti-APP antibody 6E10 for A $\beta$  peptides quantitation (*C* and *F*, bottom panels). The total cell lysate for each sample (10  $\mu$ g of protein) was also analyzed by Western blotting for APP (*C* and *F*, top panels). The signal intensity of the Western blots was quantified using Image J software and reveals significant reductions in A $\beta$  levels in ABCG1- and ABCA1-transfected cells but not in ABCA2-transfected cells (*G*). A typical example of a blot depicting all secreted peptides is also shown for both the mock and ABCG1-transfected cells (*H*). All data are representative of three experiments performed in duplicate or triplicate. Data in *G* are means of triplicate samples with the S.E. represented by the error bars. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

ther by apoA-I or lipid-free apoE (Fig. 6C); consistent with published data from several groups (30, 56–58).

These data show that ABCA1 and ABCG1 promote cholesterol efflux to apoE discs. The expression of these transporters in human neurons together with our observations that most cholesterol effluxed from neurons is not modified to more polar compounds, such as 24-OH-Ch, is consistent with the proposal that the ABCA1/G1-apoE disc-cholesterol efflux pathway contributes to neuronal cholesterol homeostasis.

**Regulation of A $\beta$  Peptide Generation by ABCG1**—Previous work indicates that ABCG1 alters the distribution of cholesterol in membranes making it more accessible for efflux to lipidated apolipoproteins and to oxidation by cholesterol oxidase (59). Based on these studies and the knowledge that redistribution of cholesterol from liquid-ordered rafts inhibits amyloidogenic processing of APP (14), we were prompted to investigate the regulation of A $\beta$  peptide generation by ABCG1. To assess this we used CHO-APP cells stably expressing human APP cDNA (41). These cells were transiently transfected with ABCG1, ABCA1, or vector alone and the impact on A $\beta$  peptide generation assessed by Western blot analysis of cell culture media. ABCG1 and ABCA1 mRNA were both expressed to a similar degree after transfection (Fig. 7A) and expression at the protein level was confirmed by Western blot (Fig. 7B). Expression of either ABCG1 or ABCA1 significantly reduced the concentration A $\beta$  peptide generation although having no impact on cellular APP levels (Fig. 7C). In contrast to the inhibition of A $\beta$  peptide secretion induced by ABCG1 or ABCA1, transient expression of ABCA2 (Fig. 7, D and E) had no impact on A $\beta$  peptide secretion (Fig. 7F). The signal intensity of the monomeric A $\beta$  peptide detected by Western blot indicated that ABCG1 and ABCA1 inhibited A $\beta$  generation by 64 and 55%, respectively (Fig. 7G). The reduction of A $\beta$  peptide detected at 4 kDa was also paralleled by reductions in several higher molecular weight immunodetectable complexes (A $\beta$  peptide oligomers), whereas levels of soluble amyloid precursor protein- $\alpha$  (which accounts for ~95% of secreted proteolytic products of APP in the CHO-APP cell line) were not detectably altered (Fig. 7H).

To gain insights into the mechanism by which ABCG1 reduces A $\beta$  levels in the cell culture media, several approaches were taken. First, to determine whether the observed reduction in A $\beta$  levels could be due to enhanced uptake of A $\beta$  by the ABCG1 expressing cells, we incubated CHO and CHO-ABCG1 cell lines with exogenously added A $\beta$ -(1–40) or A $\beta$ -(1–42) either in the absence or presence of apoE discs. A $\beta$ -(1–40) accounts for ~90% of the total A $\beta$  peptide secreted from the CHO-APP cell line and the data in



**FIGURE 8. Clearance of A $\beta$  peptides by CHO and CHO-ABCG1 cells.** CHO and CHO-ABCG1 cells were grown to confluence in 12-well plates and incubated in the presence of 1  $\mu$ M A $\beta$ -(1–40) (A–C) or A $\beta$ -(1–42) (D–F) in media containing 0.1% (w/v) BSA or 0.1% (w/v) BSA plus apoE discs (15  $\mu$ g of apoE/ml). After 24 h, A $\beta$  levels in cell-free medium (A and D) were compared with levels detected in medium taken from cells (B and E) by Western blotting as described in the legend to Fig. 7 and the signal intensity of the Western blots was quantified (C and F). Data are representative of two experiments performed in triplicate. Data in C and F are means of triplicates with the S.E. represented by the error bars. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

Fig. 8 indicate that after 24 h in the presence of BSA there was no detectable clearance of A $\beta$ -(1–40) from the medium by either CHO or CHO-ABCG1 cells (Fig. 8, A–C). In the presence of apoE discs, however, A $\beta$ -(1–40) levels were significantly reduced by both CHO and CHO-ABCG1 cells (by 18 and 25%, respectively). Under identical experimental conditions, A $\beta$ -(1–42) was significantly cleared (36% reduction) from the medium by CHO cells in the presence of BSA and a nonsignificant trend was also observed for clearance (15% reduction) from the medium by CHO-ABCG1 cells (Fig. 8, D–F). In the presence of apoE discs, dramatic reductions in A $\beta$ -(1–42) levels were observed after 24 h incubation with either CHO or CHO-ABCG1 cells (both by 80%). These data show that apoE discs promote A $\beta$  peptide clearance and that ABCG1 does not accelerate this process. These results also suggest that ABCG1 predominantly regulates A $\beta$  peptide production (rather than clearance) under our experimental conditions.

In a final series of experiments using CHO-APP-transfected cells we investigated the potential for apoE discs to further reduce A $\beta$  production (in addition to the reduction achieved by ABCG1 transfection). The rationale was that stimulation of cholesterol efflux could potentially promote further reductions in membrane cholesterol levels that would inhibit A $\beta$  generation. The presence of apoE discs resulted in a nonsignificant trend toward reduced A $\beta$  production in mock transfected cells (Fig. 9). In the ABCG1-transfected cells, A $\beta$  production was significantly reduced compared with the mock transfected cells (consistent with our earlier experiments); however, the presence of apoE discs did not result in a further decrease in A $\beta$  generation

(Fig. 9). We interpret this as indicating that under our experimental conditions the stimulation of cholesterol efflux is not a strict requirement for ABCG1-mediated suppression of A $\beta$  generation.

## DISCUSSION

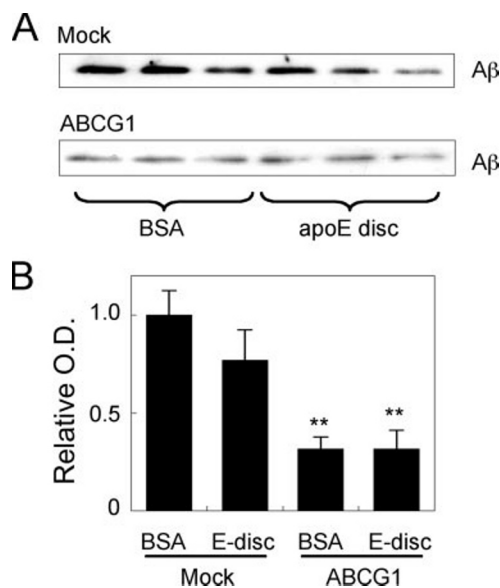
Cholesterol plays an important role in normal neuronal function and in neurodegeneration associated with Alzheimer disease (2, 60, 61). The data presented here indicate that apoE discs stimulate neuronal cholesterol efflux and thereby reveal a novel physiological function for these complexes that have been shown by several groups to be present in the central nervous system (15, 32–38). Previous studies indicate that reconstituted apoE discs (containing PL and cholesterol comparable with the discs used in the present work) promote cellular cholesterol efflux through scavenger receptor class B type I (62). This pathway is not likely to contribute to neuronal cholesterol

efflux as we could find no evidence for scavenger receptor class B type I expression in either primary neurons or neuronal cell lines<sup>5</sup>; a finding consistent with previous work (63).

Our data indicate that efflux of unmodified cholesterol from neurons to apoE discs exceeds the amount of cholesterol released through a pathway that relies on conversion of cholesterol to a more polar product, such as 24-OH-Ch, that could exit the cell membrane by diffusion (*i.e.* transporter independent). Interestingly, deletion of the cholesterol 24-hydroxylase gene (*Cyp46a1*) in mice resulted in a 40% reduction in cholesterol synthesis in the brain in the absence of reported neurological abnormalities (23). In light of our present studies, the absence of neuronal cholesterol accumulation in *Cyp46a1* null mice may be due to the maintenance of neuronal cholesterol efflux through the ABCA1/G1-apoE disc pathway.

The transcription of ABCG1 (as well as ABCA1 and apoE) is induced by the nuclear hormone receptors LXR and retinoid X receptor when appropriate ligands such as 22-hydroxycholesterol and 9-*cis*-retinoic acid are present (64). Recently 24-OH-Ch was shown to up-regulate LXR-mediated ABCG1 and apoE expression in astrocytes (65). Taken in the context of the data presented here, it seems plausible that even low concentrations of 24-OH-Ch produced by neurons could play an important role in regulating neuronal cholesterol efflux by stimulating neighboring astroglia to synthesize apoE discs that subsequently promote the efflux of non-modified cholesterol through the pathway we have proposed. Importantly, because the induction of astrocyte *ABCG1* and *APOE* gene expression is significant at low (1 to 5  $\mu$ M) oxysterol concentrations (65),

## Neuronal Cholesterol Efflux and A $\beta$ Production



**FIGURE 9. Impact of apoE discs on A $\beta$  peptide generation from CHO-APP cells +/- transient transfection with ABCG1.** CHO-APP cells were mock transfected or transfected with ABCG1 as described in the legend to Fig. 7 and incubated in the presence of 0.1% (w/v) BSA or 0.1% (w/v) BSA plus apoE discs (15  $\mu$ g of apoE/ml). After 16 h the cell culture medium was collected and analyzed for A $\beta$  by Western blotting (A) and the signal intensity of the Western blots was quantified (B). Data are representative of two experiments performed in triplicate. Data in B are means of triplicates with the S.E. represented by the error bars. \*\*,  $p < 0.01$ .

acceleration of neuronal cholesterol efflux via the ABCA1/G1-apoE disc pathway would not require 24-OH-Ch at levels that are neurotoxic, *i.e.* in the order of 50  $\mu$ M (21, 22).

A final novel finding in the present work concerns the ABCG1-mediated reduction of APP processing to generate A $\beta$  peptide. The possible impact of ABCG1 on cellular clearance of A $\beta$  was ruled out by adding exogenous A $\beta$  peptides, in either the presence or absence of apoE discs, to the CHO or CHO-ABCG1 cells. In these experiments, apoE discs accelerated the clearance of both A $\beta$ -(1–40) and A $\beta$ -(1–42), in agreement with the known association of A $\beta$  with lipidated apoE (66) and the proposed clearance of such complexes via the low-density lipoprotein receptor and low-density lipoprotein receptor-related protein family members (31). Importantly, ABCG1 expression did not modify A $\beta$  clearance; indicating that ABCG1 predominantly regulates A $\beta$  generation.

Our observation that ABCA1 also suppresses A $\beta$  generation is consistent with a previous report that used mouse Neuro2a cells expressing human APPsw (Swedish familial Alzheimer disease-specific amino acid substitutions K595N and M596L) transiently transfected with human ABCA1 (67). Also in general agreement with this previous work, we found that addition of apoE discs to the CHO-APP cells transiently transfected with ABCG1 did not result in a further reduction in A $\beta$  generation (as compared with CHO-APP cells exposed to apoE discs in the absence of ABCG1 overexpression). Interestingly, in a recent study using CHO cells expressing APP751 and human presenilin-1, lipid-free apoA-I was shown to inhibit A $\beta$ -(1–40) generation when the cells were treated with the LXR agonist TO901317 to induce ABCA1 expression (68). It therefore remains possible that modulation of membrane lipid composi-

tion and stimulation of cholesterol efflux both reduce A $\beta$  generation; depending on the experimental system or physiological conditions. This is consistent with our data indicating the lack of impact of ABCA2 expression on either cholesterol efflux to apoE discs or A $\beta$  peptide generation and implies that only ABC transporters that are capable of modulating membrane lipid distribution and cholesterol efflux will affect APP cleavage.

Transgenic animal studies may shed further light on this issue. Notably, treatment of transgenic mice expressing APPsw with the LXR agonist TO901317 resulted in a significant reduction in amyloidogenic APP processing (69). Given that ABCG1 gene transcription is also regulated by LXR and the fact that LXR is an important regulator of brain gene expression (70), it is possible that TO901317 may control APP processing through pathways that involve both ABCA1 and ABCG1. Other recent work from independent groups has examined cerebral A $\beta$  generation in amyloidogenic mouse models on an ABCA1 null background and the overall conclusion from this work is that deletion of ABCA1 led to increased A $\beta$  deposition in most of these animals (71–73). Experiments crossing ABCG1 null mice (74) with one or more of the amyloidogenic strains (previously shown to exhibit increased A $\beta$  deposition in the absence of ABCA1) will further clarify the role of ABCG1 in cerebral amyloidogenesis and potentially in Alzheimer disease.

In conclusion, our studies reveal a novel pathway regulating cholesterol efflux from neurons to apoE discs. Of the ABCA/G transporters detected in neurons so far, our data indicate that ABCA1 and ABCG1 regulate cholesterol efflux to apoE discs. We have also identified a novel role for ABCG1 in the regulation of APP processing to generate A $\beta$  peptides. These data shed new light on the mechanisms regulating neuronal cholesterol balance and may offer potential targets for Alzheimer disease therapeutic intervention.

*Acknowledgments*—Cecilia Chan is acknowledged for contributions to the preliminary data and Robyn Sharples is acknowledged for excellent technical assistance.

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