Differential expression and function of ABCG1 and ABCG4 during development and aging

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Abstract ABCG1 and ABCG4 are highly homologous members of the ATP binding cassette (ABC) transporter family that regulate cellular cholesterol homeostasis. In adult mice, ABCG1 is known to be expressed in numerous cell types and tissues, whereas ABCG4 expression is limited to the central nervous system (CNS). Here, we show significant differences in expression of these two transporters during development. Examination of β-galactosidase-stained tissue sections from Abcg1E250A/LacZ and Abcg4E250A/LacZ knockin mice shows that ABCG4 is highly but transiently expressed both in hematopoietic cells and in enterocytes during development. In contrast, ABCG1 is expressed in macrophages and in endothelial cells of both embryonic and adult liver. We also show that ABCG1 and ABCG4 are both expressed as early as E12.5 in the embryonic eye and developing CNS. Loss of both ABCG1 and ABCG4 results in accumulation in the retina and/or brain of oxysterols, in altered expression of liver X receptor and sterol regulatory-element binding protein 2 target genes, and in a stress response gene. Finally, behavioral tests show that Abcg4E250A mice have a general deficit in associative fear memory. Together, these data indicate that loss of ABCG1 and/or ABCG4 from the CNS results in changes in metabolic pathways and in behavior.—Bojanic, D. D., P. T. Tarr, G. D. Gale, D. J. Smith, D. Bok, B. Chen, S. Nusinowitz, A. Lövgren-Sandblom, I. Björkhem, and P. A. Edwards. Differential expression and function of ABCG1 and ABCG4 during development and aging. J. Lipid Res. 2010. 51: 169–181.

Supplementary keywords: oxysterols • liver X receptor • sterol regulatory-element binding protein-2 • central nervous system

The mammalian brain represents ~2% of body weight but contains 25% of total body cholesterol, making it the most cholesterol-rich organ in the body (1). Cholesterol homeostasis in the central nervous system (CNS) is unique because, unlike other tissues, it is separated from the general circulation by the blood-brain barrier that prevents the entry of cholesterol-carrying plasma lipoproteins into the CNS (1). Consequently, virtually all the cholesterol in the brain/CNS is thought to result from de novo synthesis (1). The importance of this synthesis can be gauged by the fact that a number of human diseases have been linked to abnormal sterol homeostasis in the CNS; these include cerebroside xanthomatosis and Smith-Lemli-Opitz syndrome (2, 3). Studies with mice defective in sterol homeostasis as a result of inactivation of cholesterol 24-hydroxylase or lathosterol 5-desaturase also identified critical roles for sterols in normal brain function (4, 5).

The retina, although technically part of the CNS, has the capacity both to synthesize cholesterol de novo and to acquire cholesterol from plasma lipoproteins (6). Within the retina, the photoreceptor cells (rods and cones) are the most enriched in cholesterol (7). Indeed, cholesterol is the major sterol of rod outer segment membranes, where it represents >22% of total lipids by weight (8). The importance of maintaining cholesterol concentration in photoreceptors is illustrated by the finding that rhodopsin activation and the visual transduction cascade are impaired when cellular cholesterol levels increase (7). Whether these changes result from an altered membrane environ-

This work was supported by grants from the National Institutes of Health (NIH 30368 and NIH 68445 to P.A.E.), Grant R01MH71779 (to D.J.S.) from the Lustb earth Fund, an HHMI Pritz Award (to P.A.E.), and the Swedish Science Council and Brain Power (to I.B.). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health.

Manuscript received 26 May 2009 and in revised form 9 July 2009.

Published, J R Med Libr Ser 2009, Vol 258, LR290

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This article is available online at http://www.jlr.org

Abbreviations: ABC, ATP binding cassette; CNS, central nervous system; BKO, double knockout; ERG, electroretinography; LTP, long-term potentiation; LXR, liver X receptor; TBST, TBS-T 1% Tween 20.

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§§ The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of one figure.

Journal of Lipid Research Volume 51, 2010 169
ment or from a direct interaction of cholesterol with rho-
dopin remains to be determined.

ABCG1 and ABCG4 are members of the superfamily of
ATP binding cassette (ABC) transporters. This family of
proteins utilizes ATP hydrolysis to transport a wide variety
of substrates across various cellular membranes (9).
ABCG1 and ABCG4 are closely related half-transporters
that share 69% identity (82% similarity) at the amino acid
level. When overexpressed in vitro, these two proteins can
form either homo- or heterodimers (10). Both mamma-
lian proteins have high amino acid similarity (~45%) to
the Drosophila white gene, the founding member of this
family of transporters. The Drosophila white gene forms ob-
ligate heterodimers with two other ABC transporters (scar-
et or brown) and plays an essential role in eye pigmentation
(11). However, recent studies have shown that neither
ABCG1 nor ABCG4 can functionally replace the white
gene since eye pigmentation was still defective in white
flies that overexpress either ABCG1 or ABCG4 (12).

Mammalian ABCG1 is expressed in many cell types (in-
cluding macrophages, endothelial and epithelial cells, T
and B cells, type II cells, astrocytes, and neurons) and in
numerous tissues, including the brain, eye, kidney, spleen,
lung, liver, and intestine (13–15). In contrast, several stud-
ies have demonstrated that ABCG4 expression is highly
restricted to the eye and to astrocytes and neurons of the
brain/CNS (15–18). One important difference be-
tween the two genes is their response to the nuclear recep-
tor liver X receptor (LXR); activation of LXR induces the
expression of Abcg1 mRNA and protein but has no effect
on the expression of Abcg4 (18).

Studies utilizing Abcg1−/− LacZ knockin mice estab-
lished a critical role for ABCG1 in cellular cholesterol ho-
meostasis in the lung; loss of ABCG1 resulted in massive
accumulation of macrophage foam cells that were loaded
with sterols and significant increases in pulmonary surfac-
tant (13, 19, 20). In addition, studies employing either
macrophages derived from Abcg1−/− mice or cells that
overexpress ABCG1 have shown that ABCG1 effluxes cel-
ular cholesterol to a variety of exogenous acceptors, in-
cluding HDL, LDL, liposomes, and cycloexeldrin (13, 14,
21). Not surprisingly, overexpression of the highly homol-
ogous ABCG4 protein in cultured cells also stimulates ste-
rol efflux to exogenous HDL (22–24).

Unlike ABCG1, ABCG4 is not expressed in tissues and
cells outside the CNS in adult mice. Insights into the po-
tential importance of ABCG4 and ABCG1 in the CNS have
come from studies with Abcg1−/− Abcg4−/− double knock-
out (DKO) and single knockout mice; the brains of DKO
mice contained increased levels of 27-hydroxysterol and
a number of cholesterol precursors, including des-
mosterol, lathosterol, and lanosterol (15). The changes in
sterol composition in the DKO mice were more severe
than in the single knockout mice, consistent with a role for
both ABCG1 and ABCG4 in sterol homeostasis in the brain
(15). More recently, it was suggested that ABCG4 may play
a role in the development of Alzheimer’s disease since
ABCG4 levels were reported to be increased in the brains
of patients with Alzheimer’s disease (25). Whether this
represents a direct or indirect link will require additional
studies.

In this study, we used several techniques to investigate the
expression and function of ABCG1 and ABCG4 during de-
velopment. Analysis of both Abcg1−/− LacZ and Abcg4−/− LacZ
knockin embryos identified, for the first time, cells that ex-
press ABCG4 in the absence of detectable ABCG1. We also
show that both ABCG1 and ABCG4 are highly expressed in
neurons of the adult retina and that expression is detect-
able in these cells as early as E15.5. Targeted disruption of
Abcg1 and/or Abcg4 led to the accumulation of various ste-
rol intermediates and oxysterols and to changes in the ex-
pression of LXR target genes and genes involved in
cholesterol and fatty acid biosynthesis, cholesterol oxida-
tion, and stress in both the retina and brains of these mice.
Despite these changes in the retina, electroretinograms
were normal. However, we report here that loss of ABCG4 is
associated with changes in contextual fear.

EXPERIMENTAL PROCEDURES

Animals

Abcg1−/− LacZ and Abcg4−/− LacZ mice were obtained from
Delagen as previously described (13, 18). Mice heterozygous
for gene deletion were backcrossed multiple times to C57BL/6 mice
(13, 18) and then intercrossed to generate homozygous single
knockout mice. Subsequently, Abcg1−/− Abcg4−/− double knock-
out mice were generated by intercrossing Abcg1−/− and Abcg4−/−
single knockout mice.

β-Galactosidase staining of embryos and tissue sections

Mouse embryos of gestational age 10.5, 12.5, and 15.5 days
were dissected in PBS and fixed for 4 h at 4°C in 4% paraformaldehyde/0.1 M phosphate buffer (pH 7.4). The em-
byros were rinsed three times in PBS and incubated for 16 h at
room temperature in the dark in 100 mM phosphate buffer, 2
mM MgCl2, 5 mM K2Fe(CN)6, 0.02% (v/v) Nonidet P-40, and 1
mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).
Following staining, embryos were washed three times for 15 min
each with PBS/0.1% Tween 20 and transferred to 4%
parafomaldehyde/0.1 M phosphate buffer (pH 7.4) and then
photographed using a Leica DC500 digital camera. For cell type-
specific staining, fixed embryos were cryoprotected, sectioned,
and stained for β-galactosidase activity as previously described
(15). Briefly, embryos were fixed and washed, as described above,
before being cryoprotected by incubation in 20% sucrose in PBS
at 4°C overnight. Embryos were snap-frozen in OCT and stored
at −80°C until sectioned. Ten micrometer sections were trans-
ferred to glass slides and stained for β-galactosidase activity
as described above for whole embryos. After overnight staining
and washing, slides were counterstained with nuclear fast red, rinsed
with water, dehydrated, and mounted under coverslips using a
xylene-based mounting medium.

In situ hybridization

Two 8 month old mice (C57BL/6) were nucleated, lenses
were removed, and eyecups were incubated in 4% paraformaldehyde/0.1 M phosphate buffer (pH 7.4) for 3.5 h at
4°C with constant rotation. Fixed retinas were cryoprotected by
emerision in 20% sucrose in PBS at 4°C for 1 h and snap-frozen
in OCT. Frozen sections (10 μm) were allowed to adhere to glass
slides, air dried for 1 h, and stored at −80°C until used. Sections
were bleached in 0.5% H₂O₂, permeabilized with proteinase K (1 µg/ml), treated with prehybridization buffer (50% formamide, 5× SSC at pH 4.5, 1% SDS, 50 µg/ml torula RNA, and 50 µg/ml heparin) for 30 min at 70°C, and hybridized with either Abg1 or Abg4 sense or antisense digoxigenin-labeled cRNA probes (1 µg/ml) overnight at 70°C. Sections were washed three times for 15 min each in Solution 1 (50% formamide, 5× SSC, pH 4.5, and 1% SDS) at 70°C, three times for 15 min each in Solution 2 (50% formamide and 2× SSC, pH 4.5) at 65°C, and three times for 10 min in TBS-1% Tween 20 (TBST) at room temperature. After blocking in 5% sheep serum/TBST for 1 h at room temperature, the sections were incubated with alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (1:2,000) in 1% sheep serum/TBST at room temperature for 2 h. The alkaline phosphatase reaction was performed in the presence of nitro blue tetrazolium in order to visualize the mRNA. The reaction was stopped with acidic PBS containing 1% Tween 20.

RNA isolation and analysis

Total RNA was isolated from retinas and half brains from 2 or 7 month old mice using TRIzol reagent (Invitrogen), and 1 µg of total RNA was treated with DNase and then reverse transcribed with random hexamers using SuperScript II (Invitrogen). Quantitative real-time PCRs were performed using the SYBR Green Supermix (Bio-Rad) using the MyIQ real-time PCR detection system (Bio-Rad). Results of quantitative PCR were evaluated by the comparative cycle number determined at threshold method and normalized to GAPDH or β-actin. Primer sequences are available upon request.

Sterol analysis in brain and retina

Levels of cholesterol, lathosterol, and oxysterols in the brain and retina of 8 month old mice were determined by isotope dilution-mass spectrometry as previously described using known standards (26–28).

Electron microscopy

Tissues for electron microscopy were processed as described (29).

Electroretinography

Six month old mice, overnight dark-adapted, were anesthetized with an intraperitoneal injection of normal saline containing ketamine (15 mg/g body weight) and xylazine (3 mg/g body weight). Electrorretinograms (ERGs) were recorded from the corneal surface of one eye after pupil dilation (1%, w/v, atropine sulfate in saline buffer) using a gold-loop electrode referenced to a similar gold wire in the mouth as described (30). Briefly, all stimuli were generated with a photic stimulator (model PS33 Plus; Grass-Telefactor) affixed to the outside of the dome at 90° to the viewing porthole. ERGs were recorded to a series of blue (Wavelength 47 A; Amax = 470 nm) flashes of increasing intensity, ranging from -4.7 to -0.44 log cd/s/m². Responses were sampled at 1 kHz, amplified (CP511 AC amplifier, x10,000; Grass-Telefactor), and digitized using an I/O board (PCI-1200; National Instruments) in a personal computer. Signal processing was performed with custom-written software. For each stimulus, responses were computer averaged with up to 50 records averaged for the weakest signals. A signal rejection window could be adjusted online to eliminate artifacts.

Fear conditioning

Learning and memory were assessed through a Pavlovian fear conditioning procedure using 7 month old mice as described (31, 32). Initial training was conducted in a modified Gemini Avoidance System (San Diego Instruments). The chamber had white plexiglass walls and a clear plexiglass ceiling (H, 25 cm; W, 22 cm; D, 18 cm). The floor of the chamber consisted of 12 stainless steel rods (2 mm diameter, 1.2 cm apart) connected to the shock generator. Tones were presented from a speaker mounted in one wall of the chamber. Indirect lighting (~200 Lux) was provided by a floodlight placed next to the apparatus. Mice were given a 3 min habituation period prior to a delay fear conditioning procedure consisting of 3 tone (2 kHz, 80 db, 15 s)/footshock (0.6 mA, 1 s) pairings delivered at 1 min intervals. Mice were removed from the chamber 2 min after the final pairing.

Context fear testing

One day after conditioning, mice began a contextual fear extinction series. On four consecutive days, at 24 h intervals, mice were returned to the conditioning chamber for a 6 min extinction test.

Cued fear testing

One day after the final contextual extinction session, mice were assessed for context generalization and cued fear. Testing was conducted in a novel rectangular chamber (L, 50 cm; W, 25 cm; H, 25 cm) with white laminated flooring and white plexiglass walls. After a 3 min baseline period, 6 CS (2 kHz, 80 db, 20 s), presentations were delivered with a 1 min ITI. Mice were removed from the chamber 2 min after the final cue presentation.

Behavioral analysis

For all test sessions, behavior was recorded from video cameras mounted above the apparatus then digitized at 15 frames per second by the EthoVision Pro tracking system (Noldus Information Technology). For each test, we quantified a range of behavioral endpoints, including velocity, path shape variables (turn angle and meander), place preference measures (thigmotaxis, corner preference), and immobility. All endpoints were estimated continuously by comparing pairs of consecutive samples. Velocity was defined as the distance moved by the animal’s center of gravity per unit time. Turn angle was defined as the average change in heading, in degrees, across two consecutive samples, where heading equaled the direction of movement relative to a reference line established over the preceding two samples. Meander was defined as the average turn angle per distance moved. Thigmotaxis for all tests was calculated as the percentage of time spent in the periphery of the apparatus, defined as the area within 5 cm of the wall.

Statistical analysis

Behavioral tests were performed on wild-type (n = 10), Abg1−/− (n = 7), Abg4−/− (n = 6), and Abg1−/−/Abg4−/− (n = 14) mice. All mice were 7 months old. To assess potential group differences, we performed one-way ANOVA on each individual endpoint. Fear conditioning endpoints were calculated independently for the preshock and postshock periods. Contextual fear endpoints were calculated for each 6 min test session. Contextual extinction rates were estimated through repeated-measure ANOVA on time course data (5 s bins). Cued fear endpoints were calculated independently for the 3 min baseline period and the CS presentation period. Significant group differences were characterized with posthoc analyses using Tukey-Kramer honestly significant difference test. A significance threshold of P < 0.05 was used for all tests.

Morris water maze

Morris water maze tests were performed on 14 wild-type and 14 Abg1−/−/Abg4−/− mice (7 months old) as described (33).
RESULTS

Differential expression of ABCG1 and ABCG4 during development

We previously characterized adult mice in which either the Abeg1 or Abeg4 gene was disrupted as a result of the insertion of the bacterial LacZ gene into exon 3 or exon 14, respectively (18). In these knockout/knockin mice, the promoters of the endogenous Abeg1 or Abeg4 genes control the expression of β-galactosidase (18). Importantly, the β-galactosidase protein is targeted to the nuclei as a result of the insertion of a nuclear localization signal (18). Thus, β-galactosidase-positive nuclei serve as a marker for the normal cellular expression and regulation of the corresponding endogenous ABCG gene.

To elucidate the expression pattern of ABCG1 and ABCG4 during fetal development, whole mouse embryos were obtained from pregnant Abeg1LacZ or Abeg4LacZ knockin mice, (abbreviated to AbegL or Abeg4L) at different stages of embryonic development and stained for β-galactosidase activity (Fig. 1A). Analysis of the stained embryos indicated that ABCG4 is expressed in the fetal liver at E10.5, E12.5, and E15.5 (Fig. 1A, panels a–c). Although β-galactosidase was undetectable in other embryonic tissues of the Abeg1L embryos at E10.5, we observed high expression in the developing eye and CNS at both E12.5 and E15.5 (Fig. 1A, panels b and c).

To better characterize the LacZ-expressing cells in the whole mounts of Fig. 1A, we also stained tissue sections obtained from E15.5 embryos, newborn pups (P0), and adult mice for β-galactosidase activity. The data indicate that ABCG4 is highly expressed in hematopoietic cells that populate the fetal liver at E15.5, but hepatic expression is barely detectable at P0 and absent from the livers of adult mice (Fig. 1B, panels a–c). Surprisingly, we also noted that ABCG4 is highly expressed in enterocytes that line the ileum at P0 (Fig. 2A). This expression of ABCG4 in enterocytes is transient since activity is undetectable in intestinal sections taken from Abeg4L adult mice (Fig. 2B).

The data of Figs. 1 and 2 identify significant differences in expression of β-galactosidase in Abeg1L and Abeg4L mice during development. For example, at E10.5, β-galactosidase expression was restricted to the olfactory pit in the Abeg1L embryo (Fig. 1A, panel d), with no discernable staining of the fetal liver. However, by E12.5 multiple tissues, including the CNS and eye stained intensely for β-galactosidase activity (Fig. 1A, panel e), although expression in the liver remained very low (Fig. 1A, panel f, white arrow). By E15.5, staining of the Abeg1L embryos was so intense and ubiquitous that only the eye and whiskers could be clearly discerned (Fig. 1A, panel f). We note that the expression of ABCG1 in the CNS and retina during development parallels that reported by Annicotte, Schoonjans, and Auwerx (34) for LXRβ, a nuclear receptor known to activate Abeg1.

Analysis of stained tissue sections confirmed the differences in expression of ABCG1 and ABCG4. For example, hepatic ABCG1 expression is limited to Kupffer cells and endothelial cells, a pattern of expression that persists from E15.5 through adulthood (Fig. 1B, panels d–f). We also

Fig. 1. Differential expression of ABCG1 and ABCG4 during mouse embryonic development. A: Whole mouse embryos with the indicated genotype were isolated at E10.5 (a and d), E12.5 (b and e), and E15.5 (c and f) and stained for β-galactosidase activity (blue-stained cells/tissues). The olfactory pit (O), liver (L), eye (E), whiskers (W), and CNS (C) are indicated. The white arrow indicates the relatively poorly stained liver of the Abeg1L embryo. B: Tissue sections from livers of the indicated mouse genotypes were stained for β-galactosidase activity. ABCG4 is highly expressed in fetal hematopoietic cells at E15.5 (a), barely detectable at PO (b), and absent from adult liver (c). ABCG1 is highly expressed in Kupffer cells (K) and endothelial cells (EC) of the mouse liver between E15.5 and adulthood (d–f).
show that ABCG1 expression in the intestine is limited to macrophages present in the lamina propria of both new born pups (P0) and adult mice and is absent from the enterocytes at all stages of development (Fig. 2c, d). Together, these studies identify, for the first time, cells (enterocytes and fetal hematopoietic cells) that express high levels of ABCG4 but little or no ABCG1. However, to date, no phenotype has been identified that is associated with the loss of ABCG4 from these cells.

We and others have previously utilized LacZ knockin mice to demonstrate that ABCG1 and ABCG4 are coexpressed in neurons of the CNS of adult mice (15, 18). Based on β-galactosidase-stained tissue sections, we had also noted that ABCG1, but not ABCG4, was highly expressed in the epithelial cells that form the choroid plexus and endothelial cells that line the blood vessels of the adult mouse brain (18). Unexpectedly, the current studies show that both ABCG4 and ABCG1 are expressed in the epithelial cells of the choroid plexus at E15.5 (Fig. 3a, c). However, the expression of β-galactosidase/ABCG4 observed in the choroid plexus of E15.5 embryos was transient, as it was undetectable in the corresponding tissue of adult knockin mice (data not shown) (18). Consistent with the intense staining of the CNS in developing whole-mount embryos (Fig. 1A), we show that neurons present in the dorsal root ganglion and spinal cord of both Abeg1-/- and Abeg4-/- mice stain positively for β-galactosidase activity at E15.5 (Fig. 3b, d). The effects of loss of ABCG1 and/or ABCG4 on neuronal function are discussed below.

Expression of ABCG1 and ABCG4 in the mouse retina

The data of Fig. 1A demonstrate that both ABCG1 and ABCG4 are highly expressed in the eyes of the developing embryos as early as E12.5. Analysis of sections taken from the eye (Fig. 4A, panel a) or retinas (Fig. 4A, panels b and d) shows that in early stages of embryonic development (E15.5), ABCG4 and ABCG1 are more highly expressed in the ganglion cells. However, in adult mice, the neurons present in the outer and inner nuclear layer of the retinas are particularly intensely stained (Fig. 4A, panels c and e). Indeed, we have noted that of all the tissues examined in the adult Abeg1-/- or Abeg4-/- knockout mice, β-galactosidase is most highly expressed in the neurons present in the retina (Fig. 4) and in the CA regions of the hippocampus (18).

We predicted that loss of functional ABCG1 or ABCG4 would disturb cellular steryl homeostasis and result in activation of LXR and its target genes (see below). Since β-galactosidase expression in the Abeg1-/- mouse is under the control of the endogenous regulatory elements (including multiple LXREs) that normally control transcription of the Abeg1 gene, we hypothesized that activation of LXR in these knockout mice would increase β-galactosidase expression and activity. Consequently, we also stained tissue sections from the retinas of heterozygous mice. As expected, the staining pattern for β-galactosidase activity in the retinas of heterozygous and knockout Abeg1 or Abeg4 mice was indistinguishable (data not shown). Nonetheless, we also performed in situ hybridization using retinas derived from adult wild-type mice where LXR activation would not be affected by loss of an ABC transporter; the data show clearly that both Abeg1 and Abeg4 mRNAs are highly expressed and localized to the neuronal cells of the adult retina of C57BL/6 mice (Fig. 4B, panels a and b). Little or no staining was observed with sense probes (Fig. 4B, panels c and d). Thus, studies with both wild-type, heterozygote, and knockout mice indicate that both ABCG1 and ABCG4 are highly expressed in neurons in the retina.
Effect of loss of ABCG1 and/or ABCG4 on the mouse retina

Loss of function of the ABC transporter ABCA4 (ABCR) from mice or humans is associated with various retinal defects (35). In mice, the observed defects depend on the genetic strain of mice; Abca4−/− mice on albino background, but not on a C57BL/6 background, show changes in organization of the rods and cones as determined by electron microscopy and changes in the response of the retinal cells to light as determined by electrotetroretinography (36, 37). Based on the latter studies, we determined whether loss of ABCG1 and/or ABCG4 affects retinal function. In attempts to identify altered function, we performed ERG under both dark- and light-adapted conditions, in order to distinguish between potential effects on rods versus cones, respectively. However, there was no significant difference between wild-type, Abcg1−/−, Abcg4−/−, or Abcg1−/−Abcg4−/− mice (Fig. 5A, B). Consequently, we conclude that the response of rods or cones to light are unaffected by loss of these two cholesterol transporters. In addition, detailed analyses of multiple electron micrographs obtained from both wild-type and Abcg1−/− retinas failed to identify any significant differences (Fig. 5C).

We also considered that loss of ABCG4 and/or ABCG1 might affect sterol homeostasis and gene expression in the retina. To test this proposal, we profiled retinal mRNAs obtained from wild-type and Abcg1−/−/Abcg4−/− (DKO) mice using a PCR-based Mouse Signal Transduction PathwayFinder (SuperArray). This approach identified a number of genes that were differentially expressed (data not shown). We then used quantitative RT-PCR to confirm these predictions. As shown in Fig. 6, a number of mRNAs that include the Lxr target genes Abca1 and Abcg1 are induced in retinas obtained from 2 month old DKO mice. In these knockout mice, Abcg1 mRNA levels were determined using primers that amplify exon 1 to exon 2. Importantly, since Abcg1−/− mice were generated by knocking in LoeZ into exon 3, both exon 1 and 2 are normally expressed from the endogenous promoter in both wild-type and Abcg1−/− mice.

In addition to altered expression of two Lxr target genes, the data in Fig. 6 show that loss of both ABCG1 and ABCG4 resulted in increased expression of Egr-1, a gene that is induced in response to various cellular stresses. Surprisingly, there was no significant difference in the level of the LDL receptor (Ldlr) mRNA or other genes involved in cholesterol homeostasis in the retinas of wild-type and DKO mice (Fig. 6) (data not shown). Nonetheless, taken together, these data indicate that loss of both ABCG1 and ABCG4 from the mouse retina results in activation of LXR and changes in gene expression associated with cellular stress. These data also suggest that sterol composition might be altered in the retinas of DKO mice. Consistent with this proposal, we show that lathosterol levels are significantly increased in retinas obtained from DKO mice (Table 1). However, the concentrations of a number of oxysterols, some of which are known to activate LXR, were not significantly different in retinas from the two genotypes (Table 1).
Fig. 5. Targeted disruption of Abcg1, Abcg4 or both genes in mice has no effect on retinal function and structure. ERG responses were recorded from Abcg1$^{+/+}$ (squares), Abcg4$^{+/+}$ (upwards triangles), Abcg1$^{-/-}$/
Abcg4$^{-/-}$ (downwards triangles), and wild-type (circles) mice at 6 months of age (n = 5 per group) under dark adaptation conditions (A) and under light adaptation conditions (B) as described in Experimental Procedures. C: Representative electron micrographs of the retinas from 8 month old wild-type and Abcg1$^{-/-}$/
mice reveal no significant structural difference. RPE, retinal pigment epithelium.

Effect of loss of ABCG1 and/or ABCG4 on gene expression in the brain

A recent report has shown that the brains of Abcg1$^{-/-}$/
Abcg4$^{-/-}$ mice contained increased levels of 27-
hydroxycholesterol and certain precursors of cholesterol (15). These latter data, together with the current studies
on the retinas from DKO mice (Figs. 4 and 6) suggest that deletion of ABCG1 and ABCG4 would result in changes in gene expression in the brain. To test this proposal and to assess whether the changes were age dependent, we quantified specific mRNAs in the brains of 2 and 7 month old DKO and wild-type mice.

The data show that mRNA levels of HMG-CoA reductase (Hmgcr), farnesyl dipiphosphate synthase (Fpps), and Ldr are repressed in the DKO brains in an age-dependent manner; mRNA levels were not significantly different at 2 months of age but were decreased significantly by 7 months (Fig. 7A). The mRNAs encoding lanosterol demethylase (Cyp51) and cholesterol 27-hydroxylase (Cyp27a1) were also repressed in the brains of the older DKO mice (Fig. 7A). The importance of the observed changes in Cyp27a1 mRNA levels is unclear, as we noted that the levels of this mRNA are extremely low in the brains of both genotypes. Importantly, not all Cyp genes were repressed as mRNA levels of Cyp46a1, encoding cholesterol 24-hydroxylase, were increased in the brains of the older DKO mice (Fig. 7A). These changes in gene expression suggest that sterol homeostasis was altered in the CNS following loss of ABCG1 and ABCG4.

The most striking changes in gene expression in the DKO brains involved specific LXR target genes; Abcg1 (as determined by quantification of exon1-2) and Abca1 were highly induced in both 2 and 7 month old DKO brains.
TABLE 1. Sterol levels in the retinas of wild-type and
A
glT-/-
A
gfl-/-
mice

Sterol                     Wild Type (n = 14)  A
glT-/- A
gfl-/- (n = 14)

Cholesterol          4.45 ± 0.3          5.18 ± 0.3
24(S)-OH-cholesterol  1.04 ± 0.1          1.24 ± 0.09
25-OH-cholesterol     ND                ND
27-OH-cholesterol     0.65 ± 0          0.96 ± 0
Lathosterol           155.1 ± 10        299.6 ± 13*

Retinas (total 56) were obtained from 14 wild-type and 14 DKO 8
month old mice. Seven retinas with the same genotype were combined
to generate four wild-type and four DKO samples (n = 4 samples per
genotype). Sterols were extracted and analyzed as described (26–28)
using isotope dilution-mass spectrometry. Data are presented as μg
(cholesterol) or ng (other sterols) per mg of protein. Values shown are
mean ± SEM. *P < 0.05, ND, not detectable.

(Fig. 7B). A third LXR target gene, Srebpl-c, was induced at
2 months, but there was no significant change in the brains
of the older DKO mice (Fig. 7B). Based on analysis of
the brain and retina, we conclude that transcription of both
A
gl and A
efl genes is more sensitive to activation by
LXR than Srebpl-c. Interestingly, the mRNA levels of steroyl
CoA desaturase 1 (Sed-1), a gene that is known to be ac-

Table 1 contains data on sterol levels in the retinas of wild-type and A
glT-/- A
gfl-/- mice. The table shows that the levels of cholesterol, 24(S)-OH-cholesterol, 25-OH-cholesterol, 27-OH-cholesterol, and lathosterol are significantly higher in A
gfl-/- mice compared to wild-type mice. These differences are statistically significant (P < 0.05).

The mRNA expression levels of genes involved in lipid metabolism were measured using quantitative RT-PCR. The results show a significant increase in the expression of Hmgcr, Fpps, Ldlr, Cyp51, Cyp27a1, and Cyp46a1 in DKO mice compared to wild-type mice.

Loss of ABCG1 and/or ABCG4 affects brain sterol composition

The findings that the expression of genes involved in 24- or 27-hydroxylation of cholesterol (Cyp46a1 and Cyp27a1, respectively) or in lanosterol demethylation (Cyp51) were differentially expressed in the brains of DKO mice (Fig. 7) suggested that loss of these transporters will affect sterol composition. Indeed, quantification of numerous sterols using isotope dilution-mass spectrometry shows that the brains from DKO mice contained increased concentrations of 24(S)-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, and lathosterol (Table 2). As expected, the changes in these sterols were greater in DKO mice compared to A
gfl-/- mice (Table 2). Additional preliminary analyses indicate that there is an increase in 7-dehydrocholesterol and testis meiosis-activating sterol, an intermediate between lanosterol and zymosterol, as well as a decrease in campesterol in the brains of DKO mice. (data not shown). We also noted that, when compared with wild-type or A
gfl-/- mice, total brain cholesterol levels (μg/mg wet weight) were reduced in the mice lacking ABCG4 (Table 2). This contrasts with the data of Wang et al. (15) who reported no change in cholesterol levels (μg/mg dry weight) in A
gfl-/- mice.

Functional changes associated with loss of ABCG1 and/or ABCG4

Changes in sterol levels and/or sterol precursors are known to affect synaptic plasticity (39). The latter is deter-
TABLE 2. Sterol levels are altered in the brains of AbegI<sup>−/−</sup>, Abegf<sup>−/−</sup>, and AbegI<sup>−/−</sup>Abegf<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th>Sterol</th>
<th>Wild Type (n = 5)</th>
<th>Abegf&lt;sup&gt;−/−&lt;/sup&gt; (n = 5)</th>
<th>AbegI&lt;sup&gt;−/−&lt;/sup&gt; (n = 5)</th>
<th>Wild-Type (n = 9)</th>
<th>AbegI&lt;sup&gt;−/−&lt;/sup&gt;Abegf&lt;sup&gt;−/−&lt;/sup&gt; (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>14.5 ± 0.2</td>
<td>14.3 ± 1</td>
<td>12.7 ± 0.7&lt;sup&gt;∗&lt;/sup&gt;</td>
<td>14.4 ± 0.3</td>
<td>15.5 ± 0.2&lt;sup&gt;∗&lt;/sup&gt;</td>
</tr>
<tr>
<td>24(S)-OH-cholesterol</td>
<td>33.5 ± 1.2</td>
<td>34.7 ± 1</td>
<td>31.7 ± 1.4</td>
<td>41.3 ± 1.6</td>
<td>47 ± 1&lt;sup&gt;∗&lt;/sup&gt;</td>
</tr>
<tr>
<td>25-OH-cholesterol</td>
<td>0.04 ± 0.01</td>
<td>0.05 ± 0.02</td>
<td>0.03 ± 0.004</td>
<td>0.01 ± 0.01</td>
<td>0.02 ± 0.01&lt;sup&gt;∗&lt;/sup&gt;</td>
</tr>
<tr>
<td>27-OH-cholesterol</td>
<td>0.3 ± 0.01</td>
<td>0.5 ± 0.01*</td>
<td>0.3 ± 0.01</td>
<td>0.33 ± 0.01</td>
<td>0.42 ± 0.01*&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lathosterol</td>
<td>18.74 ± 0.8</td>
<td></td>
<td></td>
<td></td>
<td>24.81 ± 1.3&lt;sup&gt;***&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Half brains were removed from 8 month old wild-type, Abegf<sup>−/−</sup>, and AbegI<sup>−/−</sup> mice (n = 5/group). Half brains were also obtained from 8 month old AbegI<sup>−/−</sup>Abegf<sup>−/−</sup> and wild-type mice (n = 9/group). Sterols were extracted and analyzed using isotope dilution-mass spectrometry (24-26). Data are presented as pg (cholesterol) or ng (other sterols) per mg of wet weight tissue. Values shown are mean ± SEM. <sup>∗</sup>P < 0.05, <sup>**</sup>P < 0.005, <sup>***</sup>P < 0.001; <sup>****</sup>P < 0.0005.

minded using long-term potentiation (LTP) or long-term depression to assess synaptic strengthening or synaptic weakening, respectively. Indeed, loss of cholesterol 24-hydroxylase has a dramatic effect on LTP that can be reversed in vitro by administration of the polyisoprenoid geranylergosterol (40). However, despite the finding that loss of both ABCG1 and ABCG4 leads to changes in brain sterol composition, there was no difference in LTP when the studies were performed using hippocampal slices from 8 month old wild-type and DKO mice (data not shown).

To assess whether loss of ABCG1 and/or ABCG4 affected other functions, we performed behavioral phenotyping on wild-type, single, and DKO 7 month old mice. Learning and memory were assessed through a Pavlovian fear conditioning procedure. This multiphase assay has been widely used to assess simple learning, such as contextual fear conditioning, a process that has been linked to hippocampal-dependent neuronal activity (41-43). In addition, Morris water maze tests were performed using 14 wild-type and 14 DKO mice. No significant differences were noted between these two genotypes in the latter test (data not shown).

Conditional fear phenotyping

Learning and memory were assessed through a Pavlovian fear conditioning procedure, a multiphase assay that characterizes various processes, including elemental and contextual associative learning, short-term memory, and memory extinction. General activity patterns were assessed prior to conditioning. All knockout and wild-type mice displayed comparable velocity (<sup>P</sup> < 0.8) and path shape profiles (<sup>P</sup> < 0.5; Fig. 8A) across the 3 min baseline period, when placed in a new environment (chamber), suggesting normal motor function. Similarly, all groups displayed equivalent preference for the periphery of the chamber (<sup>P</sup> < 0.5; data not shown), indicating comparable spatial processing in wild-type and single or DKO mice. Subsequent fear conditioning (three tone and foot shock pairings) produced robust shifts in activity. Analysis of the postshock period revealed lower levels of immobility in Abegf<sup>−/−</sup> compared with the AbegI<sup>−/−</sup> (<sup>P</sup> < 0.01) and wild-type mice (<sup>P</sup> < 0.07; Fig. 8B, left panel). As shown in Fig. 8B (right panel), Abegf<sup>−/−</sup> mice exhibited a significant difference in turn angle relative to both wild-type and AbegI<sup>−/−</sup> mice (<sup>P</sup> < 0.01) when tested at the same time. On the four consecutive days following training (days 2-5 in Fig. 8C), mice were returned to the conditioning chamber for 6 min to assess context fear. Abegf<sup>−/−</sup> mice displayed a significant decrease in freezing (increased mobility) compared with other genotypes, on day 2 (Fig. 8C, left panel) consistent with a persistent memory deficit (<sup>P</sup> < 0.01). This memory deficit, observed between Abegf<sup>−/−</sup> and wild-type or DKO mice, persisted from day 2 through day 4 (Fig. 8C, right panel). By the final test session (day 5), Abegf<sup>−/−</sup> mice did not differ significantly from the wild type (<sup>P</sup> < 0.3; Fig. 8C, right panel).

Context generalization was assessed in separate subsequent experiments by exposing mice to a novel chamber prior to cued fear testing (exposure to tone only). In contrast to the observed context fear deficits, Abegf<sup>−/−</sup> mice again displayed significantly reduced immobility relative to DKO mice (<sup>P</sup> < 0.05), yet were nearly identical to the wild type (<sup>P</sup> > 0.9; Fig. 8D, left panel). Interestingly, the level of immobility of AbegI<sup>−/−</sup> mice was much higher than that of the Abegf<sup>−/−</sup> mice, although the difference did not reach statistical significance (Fig. 8D, right panel). Subsequent cued fear testing produced high levels of immobility across the six tone presentations (Fig. 8D); however, the levels of immobility did not differ across all four genotypes (Fig. 8D, right panel). Path shape analysis revealed comparable profiles in all groups (<sup>P</sup> > 0.7; data not shown). Together, these studies identify a defect in associative fear memory in Abegf<sup>−/−</sup> mice. The absence of such changes in the DKO mice is unexpected and suggests that the loss of ABCG1 partially compensates for the loss of ABCG4. Whether such compensation is a result of cellular changes in other oxysterols or lipids will require additional experimentation. However, it is clear that the different behavior is not a result of gross differential expression of ABCG1 and ABCG4 in the amygdala as both genes are expressed in a similar pattern throughout this area of the brain (see supplementary Fig. 1).

DISCUSSION

Previous studies have shown that in adult mice, ABCG4 expression is limited to astrocytes and neurons of the CNS (17, 18). Although ABCG1 is highly expressed in these same cells in adult mice, ABCG1 is also expressed in many other tissues and cell types, including macrophages, Type II, endothelial, and epithelial cells (13, 14, 21). In an attempt to better understand the relationship between ABCG1 and ABCG4, we investigated the expression of both genes during mouse development using mice in which the endogenous genes were disrupted following insertion of the bacterial gene encoding β-galactosidase.

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This approach identified cells outside the CNS, namely, hematopoietic cells in the fetal liver and enterocytes in the fetal intestine, that express ABCG4 in the absence of detectable ABCG1 (Figs. 1 and 2). These results suggest that ABCG1 and ABCG4 play distinct roles during development. The data also suggest that expression of ABCG4 outside the CNS reflects a specific temporal requirement for this transporter. Nonetheless, the physiological importance of this differential expression of ABCG1 and ABCG4 remains to be determined.

In this study, we used both in situ hybridization and assays for β-galactosidase activity to demonstrate that ABCG1 and ABCG4 are both highly expressed in the developing and adult neural retina (Fig. 4). Moreover, both transporters share the same pattern of expression, suggesting that they may form heterodimers and/or homodimers that have overlapping roles in this tissue. Surprisingly, the disruption of both ABCG1 and ABCG4 has no effect on the retinal structure or function, as determined by electron microscopy and ERG, respectively (Fig. 5). These data contrast with the changes in structure and function of the retina that follows from disruption of ABCA4 (ABCR) in albino, but not C57BL/6, mice (37). ABCA4 normally transports an N-retinylidene-phosphatidyethanolamine complex across the disk membranes (36, 37). Despite the lack of functional changes, we do show that loss of both ABCG1 and ABCG4 from the retina results in the accumulation of a cholesterol precursor, lathosterol, in altered expression of a stress-induced gene and in increased expression of specific LXR target genes (Table 1, Fig. 6). Together, these data suggest that the retinas of these mice accumulate oxysterols that serve as ligands for the nuclear receptor LXR. This proposal is consistent with previous studies that have linked the accumulation of oxysterols in macrophages isolated from Abcg1−/− or Abcg1−/−Abca1−/− mice to increased apoptosis (19, 44, 45). Our inability to measure changes in oxysterol levels in the retinas of Abcg1−/−Abcg4−/− mice may simply demonstrate that very small changes in oxysterol levels within a subcellular compartment can have significant effects on gene expression.

Consistent with this latter proposal, we show that the brains of Abcg1−/−Abcg4−/− mice accumulate not only lathosterol, but also 24-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol (Table 2) and 7-dehydrocholesterol, desmosterol, and testis meiosis-activating sterol (data not shown). These results extend a recent report by Wang et al. (15), which showed that the brains of 4 month old Abcg1−/−Abcg4−/− mice accumulate desmosterol, lathosterol, and 27-hydroxycholesterol. These same authors also showed that primary cultured astrocytes isolated from the DKO mice were deficient in effluxing desmosterol and cholesterol to HDL, exhibited reduced cholesterol biosynthesis, but increased expression of ABCA1 and ABCG1 and secretion of apolipoprotein E (15).

Side-chain oxidized oxysterols are known to pass lipophilic membranes at rates orders of magnitude faster than cholesterol itself (46, 47), and the need for an active transport over cell membranes thus appears to be less important for oxysterols than for cholesterol. The result of the present work as well as that of the work by Wang et al. (15) support the contention that efflux of oxysterols may be facilitated by ABCG1 and ABCG4. Thus, there was a significant accumulation of the three side chain oxidized oxysterols 24(S), 25-, and 27-hydroxycholesterol in the brains of the Abcg1−/−Abcg4−/− mice. There was also an increase
in 24(S)-hydroxycholesterol in the retina of these mice, although the change did not quite reach statistical significance.

The accumulation of multiple oxysterols and desmosterol in the brains of the DKO mice (Table 2) (15) likely accounts for the observed increased expression of selective LXR target genes that include Abca1 and Srebp-1c. The finding that numerous genes in the cholesterol biosynthetic pathway are repressed in the brains of 7 month-old DKO mice is consistent with a decrease in expression of mature SREBP-2, a transcription factor known to regulate these same genes (48). These changes in cholesterogenic genes are age dependent as the mRNA levels of 2 month old DKO and wild-type mice are not significantly different (Table 2). Indeed, these age-dependent changes in the brain are reminiscent of the age-dependent accumulation of cholesterol, phospholipids, and inflammatory genes that have been shown to occur in the lungs of Abegf−/− mice (44, 49). The finding that aberrant expression of genes involved in cholesterol synthesis and uptake is more pronounced in the brains compared with the retinas of DKO mice suggests that cholesterol homeostasis in the brain is more dependent than the retina upon these two ABC transporters. Consistent with this proposal, cholesterol in the brain is known to be derived from de novo synthesis within the organ, since the brain is isolated from the systemic circulation by the blood-brain barrier that prevents passage of cholesterol from plasma lipoproteins into the brain (1). In contrast, cells in the retina can obtain cholesterol from both systemic circulation and de novo synthesis; however, the relative contribution of these two pathways to sterol homeostasis in the retina is not known (6). In addition, as a result of the blood-brain barrier, efflux of sterols from the brain to the blood may be impaired compared with sterol efflux from the retina. Such a difference might account for the more pronounced changes in sterol levels and altered expression of specific mRNAs (e.g., Ldlr) in the brain compared with the retina (Figs. 6 and 7).

The adverse effects that result from loss of functional ABCG1 and ABCG4 from the CNS might well be ameliorated in part by the increased expression of ABCA1 that would be expected to increase the efflux of sterols and phospholipids from astrocytes and neurons to apolipoprotein E-rich lipoproteins in the cerebrospinal fluid. However, it appears that increased expression of ABCA1 in the lung (15) or brain (Fig. 7) in response to deletion of ABCG4 and/or ABCG1 cannot adequately maintain cellular sterol levels. Whether this is because the ABCG1, ABCG4, and ABCA1 transport different sterols (15, 50) or whether it is simply a defect in the overall ability to efflux sterol mass remains unclear. Nonetheless, the data indicate that cells of the brain utilize numerous sterol-transport pathways, including those regulated by ABCG1, ABCG4, and ABCA1, to maintain critical intracellular sterol levels.

Cholesterol has long been thought to play a critical function in the CNS (39). Thus, the generation of viable mice that contain <3% of normal cholesterol as a result of deletion of desmosterol reductase, Dher24, the last enzyme in the cholesterol biosynthetic pathway, was remarkable (51). Nonetheless, various natural mutations in genes affecting sterol homeostasis in humans and/or gene knockouts in mice indicate that altered sterol homeostasis in the CNS often affects neuronal function (3). Surprisingly, results from this study identify only one physiological effect resulting from disruption of both Abegf and Abegf genes in mice. The data demonstrate a specific memory defect in Abegf−/− mice. The pronounced contextual fear deficits suggest that ABCG4 contributes significantly to processes mediating the acquisition and/or expression of contextual fear. The observed immobility deficits do not appear to stem from reduced pain sensitivity, as the Abegf−/− mice exhibited normal shock-induced activity bursts (P > 0.8). Nor are they the product of a general deficit in production of the fear response, as Abegf−/− mice exhibited normal immobility levels during tone presentations. Hyperactivity does not account for the deficit given the normal velocity and path shape profiles. Likewise, a general defect in the processing of contextual information is not a likely source of the deficit considering the normal place preference levels observed prior to conditioning. Rather, the phenotype exhibited by Abegf−/− mice suggests an effect for this gene on processes related to neuronal plasticity underlying long-term contextual memory storage.

The original observations that overexpression of ABCG1 or ABCG4 in cultured cells promotes the efflux of cellular cholesterol to HDL led to the proposal that ABCG1 might be involved in efflux of cellular cholesterol to HDL and possibly other plasma lipoproteins (24). However, numerous studies have now shown that plasma lipoprotein levels are unaltered in transgenic mice that overexpress ABCG1 from a human BAC or lack ABCG1 and/or ABCG4 as a result of disruption of the endogenous genes (49, 52, 53). Thus, the current evidence suggests that ABCG1 and ABCG4 function primarily to control intracellular sterol movement and homeostasis and play little, if any, role in regulating plasma lipoprotein levels. The current studies are consistent with this proposal and indicate that both ABCG4 and ABCG1 are important for neuronal cell function.

The authors thank Dr. A. Bäldin and laboratory members for critical reading of the manuscript.

REFERENCES


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