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The Role of Transporters ABCG1/4 and ABCA1 in Brain Cholesterol Metabolism*

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Abstract The brain is the most cholesterol-rich organ in the body and consists of 25% of total cholesterol. ATP-binding cassette (ABC) transporters play essential roles in cellular cholesterol efflux and homeostasis in the brain. Recently, ABCG1, ABCG4 and ABCA1 expression in the adult brain has been described. The absence of one or more of these transporters has been implicated in the development of neurodegenerative diseases. In this study, we characterized the mRNA and protein expression levels of ABCG1, ABCG4 and ABCA1 in the developing postnatal brain of normal C57BL/6J mice fed a chow diet. We studied the correlation between ABC transporters expression and cholesterol levels (free cholesterol, esterified cholesterol) in the brain and serum, to elucidate a potential role of these transporters in cholesterol metabolism in the brain and body during postnatal stages. We further investigated the changes of expression levels of ABCG1, ABCG4, ABCA1, cholesterol related genes and brain cholesterol levels in ABCG1$^{-/-}$, ABCG4$^{-/-}$ and ABCG1$^{-/-}$ ABCG4$^{-/-}$ double knockout mice. ABCA1 mRNA expression were detectable in multiple tissues, and ABCG1, ABCG4 were highly expressed in adult brain. ABCG1 and ABCG4 mRNA levels peaked at 42 days of age, while ABCA1 mRNA levels were near baseline. ABCG1 protein levels peaked at day 28 then decreased, while ABCG4 levels peaked at day 42. ABCA1 levels remained near baseline. Interestingly, circulating plasma and brain esterified cholesterol levels exhibited a biphasic distribution, which peaked at day 42. Loss of ABCG1 is compensated by increased ABCG4 and vice versa. Loss of both ABCG1 and ABCG4 results in altered expression of cholesterol synthesis related genes and cholesterol accumulation in the brain. The data suggest that ABCG1 and ABCG4, but not ABCA1 are important for transporter function in the developing brain. ABCG1 and ABCG4 play complementary roles in maintaining brain cholesterol homeostasis.

Key words ATP-binding cassette (ABC) transporters, brain development, cholesterol
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The brain is the most cholesterol-rich organ in the body and consists of ~25% of total cholesterol [1]. Almost all cholesterol acquired in the brain is derived from *de novo* biosynthesis because of the effective prevention of blood-brain barrier [2]. Thus, the isolated cholesterol pool in the brain demands a precise mechanism for homeostasis of the cholesterol content. The ATP-binding cassette (ABC) transporter superfamily has been reported to play crucial roles in cellular cholesterol efflux, which is essential for the cholesterol homeostasis in the brain [3]. ABCG1, ABCG4 and ABCA1 have been recently demonstrated to be expressed in the brain [4-6]. The absence of ABCG1 is implicated in the disturbance of cholesterol homeostasis and could contribute to the neurodegenerative disease including Alzheimer's disease [7]. However, the role of ABCG1, ABCG4 and ABCA1 in the brain cholesterol equilibrium remains elusive. In addition, brain neurons switch from cholesterol biosynthesis during embryonic period to cholesterol uptake from astrocytes after birth [8]. It is therefore postulated that postnatal neurons may obtain

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astrocytes-secreted cholesterol *via* apolipoprotein E (Apo E) containing HDL particles[9]. The ATP-binding cassette (ABC) transporters mediate the transport of intracellular cholesterol to ApoE and ApoA1, the crucial process for HDL synthesis [10]. Therefore, we hypothesize that ABCG1, ABCG4 and ABCA1 may express in specific cell types of the brain and mediate cholesterol homeostasis in time-controlled manner.

In our study, we have characterized the mRNA and protein expression of ABCG1, ABCG4 and ABCA1 in postnatal developing and adult mouse brains. We studied the correlation between ABC transporters expression and cholesterol levels (free cholesterol, esterified cholesterol) in the brain and serum, to elucidate a potential role of these transporters in cholesterol metabolism in the brain and body. We further investigated the effects of ABCG1 and ABCG4 on the cholesterol levels and cholesterol synthesis related genes in the brain of ABCG1$^{-/-}$, ABCG4$^{-/-}$ and ABCG1 $^{-/-}$ ABCG4$^{-/-}$ double knockout mice. These studies will lead to a better understanding in neurological disease where aberrant cholesterol metabolism has been implicated.

1 Materials and methods

1.1 Reagents

Trizol was purchased from Invitrogen (Carlsbad, CA, USA). iScript™ cDNA Synthesis kit, iQ™ SYBR Green Supermix and Immun-blot™ PVDF membrane were purchased from Bio-Rad (Hercules, CA, USA). All primers were obtained from IDT (Coralville, IA, USA). BCA™ Protein Assay Kit was obtained from Pierce (Rockford, IL, USA). Rabbit polyclonal anti-ABCA1 antibody was purchased from Novus Biologicals (Littleton, CO, USA). Rabbit polyclonal anti-ABCG1 and anti-ABCG4 antibody was obtained from Open Biosystems (Huntsville, AL, USA). Monoclonal anti-β-tubulin antibody, peroxidase-conjugated anti-rabbit IgG, peroxidase-conjugated anti-mouse IgG and Kodak Biomax MS film were purchased from Sigma (St. Louis, MO, USA). ECL™ Western blotting detection reagents was purchased from GE Healthcare (Piscataway, NJ, USA). Total and free cholesterol kits were purchased from Wako Chemicals USA, Inc (Richmond, UA, USA).

1.2 Mice

C57BL/6J wild type (WT) mice were obtained from JAX, USA. Animals were kept under natural lighting conditions, and they were allowed standard chow diet food and water ad libitum. Animals were fasted 6 h before dissection. The protocol was approved by the Institutional Animal Care and Use Committee in Nationwide Children's Hospital. Brain and serum were harvested from neonatal (1-, and 7-day-old), pubertal (14-, 21-, 28-, 35-, 42-day-old) and adult (12-week-old) mice. Six mice per age group were used except for the neonatal period, where 10 animals were utilized. The tissues were not pooled but treated individually. ABCG1$^{-/-}$, ABCG4$^{-/-}$ and ABCG1$^{-/-}$ ABCG4$^{-/-}$ mice were kindly provided by Dr. Matthew Kennedy's laboratory in Nationwide Children's Hospital.

1.3 Real-time quantitative PCR analysis of ABCG1, ABCG4 and ABCA1 mRNA expression

Total RNA was isolated from the tissues by using Trizol per manufacturer's protocol. After isolation, 2 μg of total RNA was reverse transcribed into cDNA at 42℃ for 1 h by using Oligo (dT) and 1 μl of iScript Reverse transcriptase (iScript™ cDNA Synthesis kit) in a 20 μl final volume. The mRNA expression of ABCG1, ABCG4 and ABCA1 were measured using primers designed for mouse by Primer 3 software (Premier Biosoft International, Palo Alto, CA, USA) (Table 1). Other genes expression (Hmgr, Fpps, Cyp51 and Acat1) were analyzed by using primers shown in Table 3. All these primers were purchased from IDT. Corp. The Real-time quantitative PCR (Real-time PCR) reactions were performed using iQ™ SYBR Green Supermix according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA) and 0.5 μmol/L of each primer. In all Real-time PCR reactions, a negative control corresponding to reverse transcriptional reaction without the reverse transcriptase enzyme and a blank sample were carried out (data not shown). Amplification of the housekeeper gene peptidylprolyl isomerase A (PpiA) cDNA was used as internal control to quantify the expression of a given gene in Real-time PCRs [11]. Quantification was performed with the Optical System software, version 1.0 (Bio-Rad). Products of reaction were visualized by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide and photographed under a UV transilluminator.

1.4 Immunoblots analysis of ABCG1, ABCG4 and ABCA1 protein expression

Proteins were measured according to the manufacturer's instructions (BCA™ Protein Assay kit) and BSA as a standard. Samples were prepared for electrophoresis by boiling them in the corresponding

volume of sample buffer 2× (8 mol/L urea, 70 mmol/L Tris-HCl pH 6.8, 3% SDS, 0.005% Bromopheonol blue, and 5% β-mercaptoethanol) for 5 min. Proteins (50 μg) were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electro-transferred onto nitrocellulose membranes. Membranes were blocked with 3% blotting grade blocker in Tris-buffered saline (TBS: 140 mmol/L NaCl, 50 mmol/L Tris-HCl, pH 7.4) for 60 min at 37 ℃ and then incubated with anti-ABCG1 (1 ∶ 50 dilution), anti-ABCG4 (1 ∶ 50 dilution), anti-ABCA1 (1 ∶300 dilution) or monoclonal anti-β tubulin (1 ∶ 5 000 dilution) overnight at 4℃. Membranes were thoroughly rinsed and incubated with peroxidase-conjugated anti-rabbit IgG (1 ∶3 000 dilution) or peroxidase-conjugated anti-mouse IgG (1 ∶5 000 dilution). Antigen-antibody complexes were revealed by chemiluminescence and the membranes were exposed to Kodak biomax MS film.

1.5 Free cholesterol (FC) and esterified cholesterol (EC) levels assays in mouse brain and serum

Free and esterified cholesterol levels of mouse brain and serum were determined. Samples were prepared by a modified Folch method[11-12]. In brief, the brain was homogenized with a 2 ∶1 Chloroform-methanol mixture. Extracts were washed by addition of 0.9 mol/L NaCl solution. The lower chloroform layer containing the lipids was transferred to a wheaten vial and dried down under Nitrogen. The samples were resolubilized in 250 ml 95% ethanol and quantified using a kit for total and free cholesterol per manufactures protocol (Wako Chemicals, USA, Inc. Richmond, VA, USA). Esterified cholesterol was determined by subtracting the free fraction from the total cholesterol fraction.

1.6 Analysis of data and statistical analysis

The immunoreactive bands in Western blots obtained for each time interval studied were scanned with a laser scanner. The intensity of the immunoreactive band was quantified by Scion image program (Scion Corporation, Frederick, MD, USA). The values were normalized to the 1 day values for developmental studies. The data are expressed as the $\bar{x} \pm s$. Student's *t*-test was used for comparisons between two groups. Differences were considered statistically significant as $P < 0.05$. Data for Real-time PCR were also expressed as the $\bar{x} \pm s$. All analyses were carried out with Sigma plot 9.0 software (SPSS Inc., Chicago, IL, USA).

2 Results

2.1 ABCG1 and ABCG4, but not ABCA1, mainly expressed in adult brain

The mRNA expression profile of ABCG1, ABCG4 and ABCA1 were evaluated by Real-time PCR using total RNA isolated from mouse different tissues in adulthood. Peptidylprolyl isomerase A (PpiA), a house keeper gene, was used as internal control since it is expressed constitutively and constantly in all samples [11]. ABCG1, ABCG4 and ABCA1 primers (Table 1) amplified a single product with an expected size of 120 bp, 120 bp and 125 bp by Real-time PCR (data not shown). A negative control corresponding to RT reaction without the reverse transcriptase enzyme was carried out and showed no PCR product amplification (data not shown).

Table 1 Specific primers designed for mouse ABCG1, ABCG4 and ABCA1

Gene	GenBank accession numbers	Primers		Product size/bp
		Sense	Anti-sense	
ABCG1	NM_009593	5′ GGGGAAAGGTCTCCAATCTC 3′	5′ TGTTCTGATCCCCGTACTCC 3′	120
ABCG4	NM_138955	5′ GCGTGGTTACCAACCTGATT 3′	5′ TGGGGTTCAGGTCTCCATAC 3′	120
ABCA1	NM_013454	5′ AACAGTTTGTGGCCCTTTTG 3′	5′ CACAATCAGGCTGAAGACCA 3′	125
PpiA	NM_008907	5′ GCATACAGGTCCTGGCATCT 3′	5′ CTTCCCAAAGACCACATGCT 3′	121

ABCG1, ABCG4 and ABCA1 expression were detectable in multiple tissues, including testis, brain, spleen, kidney, liver, white adipose tissue (WAT), brown adipose tissue (BAT), adrenal gland, muscle and lung (Figure 1). ABCG1 was highly expressed in brain and next in lung. Spleen, kidney, WAT, BAT,

and adrenal gland contained low but appreciable levels of ABCG1 mRNA expression (Figure 1a). The ABCG1 mRNA expression in the testis, liver and muscle were detectable. On the other hand, ABCG4 was strongly expressed in the brain (Figure 1 b). The ABCG4 mRNA expressed in testis, kidney, liver, and lung were at very low levels. Surprisingly, we detected moderate ABCG4 mRNA expression in WAT, BAT, adrenal gland, and muscle, which has not reported before. As previous described, ABCA1 mRNA broadly expressed of in many tissues with prominent mRNA expression in liver, BAT, adrenal gland, and lung [13] (Figure 1c).

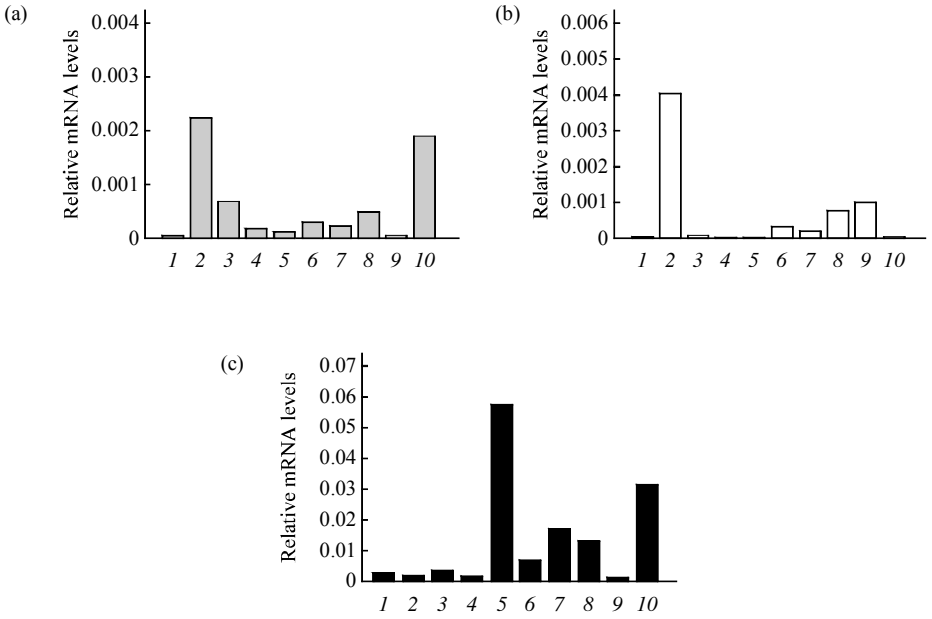

Fig. 1 mRNA expression profile of ABCG1, ABCG4 and ABCA1 in adult mouse tissues

Total RNA was extracted from adult C57BL/6J mouse tissues on chow diet. After the reverse transcription, amplification was carried out with 0.5 μmol/L of mouse ABCG1, ABCG4 or ABCA1 primers (Table 1). The histograms show: (a) ABCG1, (b) ABCG4 and (c) ABCA1 mRNA expression in several mouse different tissues. All data were normalized to the internal reference PpiA and expressed as fold increase relative to normalized reference value. The relative values are comparable in groups. *1*: Testis; *2*: Brain; *3*: Spleen; *4*: Kidney; *5*: Liver; *6*: WAT; *7*: BAT; *8*: Adrenal gland; *9*: Muscle; *10*: Lung.

2.2 ABCG1 and ABCG4 mRNA levels peaked at 42 days of age, while ABCA1 mRNA levels were near baseline

To determine the pattern of ABCG1, ABCG4 and ABCA1 expression in the brain during development, we measured their mRNA levels in mouse brain at various time points during postnatal development. The mRNA expression of ABCG1, ABCG4 and ABCA1 obtained from neonatal (1-, and 7-day-old), pubertal (14-, 21-, 35-, and 42-day-old), and adult (>55 days) mice brain were studied by Real time Q-PCR analyses. ABCG1 or ABCG4 mRNA expression differed greatly from ABCA1 in mice brain during development (Figure 2, n=6). ABCG1 and ABCG4 mRNA levels tended to increase with development and reached the highest level at 42 days with the fold value of 26.7 ($^{+}P < 0.05$, 42 versus 1 day) or 2.75 ($^{*}P < 0.01$, 42 versus 1 day) (Figure 2a, n=6). In contrast to ABCG1, ABCG4 mRNA levels tended to elevate from 1 to 14 days then significantly decreased from 14 to 21days ($^{+}P < 0.04$, 21 versus 14 day) before rising again from 21days to adulthood (Figure 2b, n=6). Interestingly, the levels of ABCA1 mRNA expression were low in the developmental brain and remained at this level throughout all the developmental at time points tested (Figure 2c, n=6).

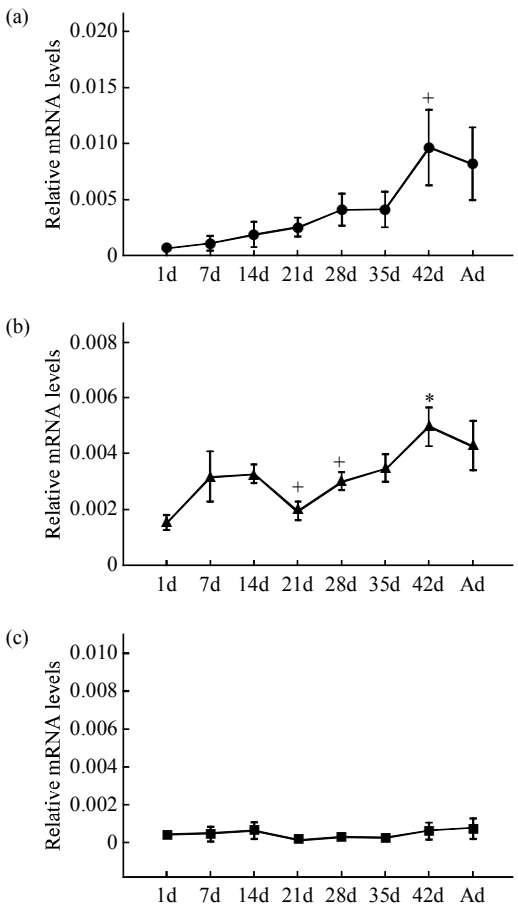

Fig. 2 mRNA expression profile of ABCG1, ABCG4 and ABCA1 in mouse brain during postnatal development

Real time Q-PCR was performed for ABCG1 (a), ABCG4 (b) and ABCA1(c) genes in mouse brain during postnatal development. All data were normalized to the internal reference PpiA and expressed as fold increase relative to normalized reference value. (a) The increase between 42 and 1 day ($^{+}P < 0.05$, n=6) was significant. (b) The decrease from 14 to 21 days ($^{+}P < 0.04$, n=6) and the increase from 21 to 28 days ($^{+}P < 0.05$, n=6) and the elevation between 42 and 1 day ($^{*}P < 0.01$, n=6) were significant. d: Days old. Ad: Adult. The data were obtained from six independent experiments for each age group. Neonatal: 1d, 7d and 14d; Pubertal: 21d, 28d, 35d and 42d; Adult: 12-week-old mice.

2.3 ABCG1 protein levels peaked at day 28 then decreased, while ABCG4 levels peaked at day 42, and ABCA1 levels remained near baseline

Representative Western blots of ABCG1, ABCG4 and ABCA1 are shown in Figure 3a. Protein expression of ABCG1 (74 ku), ABCG4 (74 ku) and ABCA1 (220 ku) varied during postnatal development in C57BC/J mice (Figure 3b, n=6). ABCG1 protein levels rose from 1 to 28 days after birth ($^{+}P < 0.05$, 28 versus 1 day) before dropping from 28 days to adulthood. In contrast, ABCG4 protein levels significantly increased from 21 to 28 days ($^{+}P < 0.03$) and reached the maximal levels from 42 days to adulthood ($^{*}P < 0.01$ 42 versus 1 day). Surprisingly, ABCA1 protein levels were low and relatively unchanged throughout development.

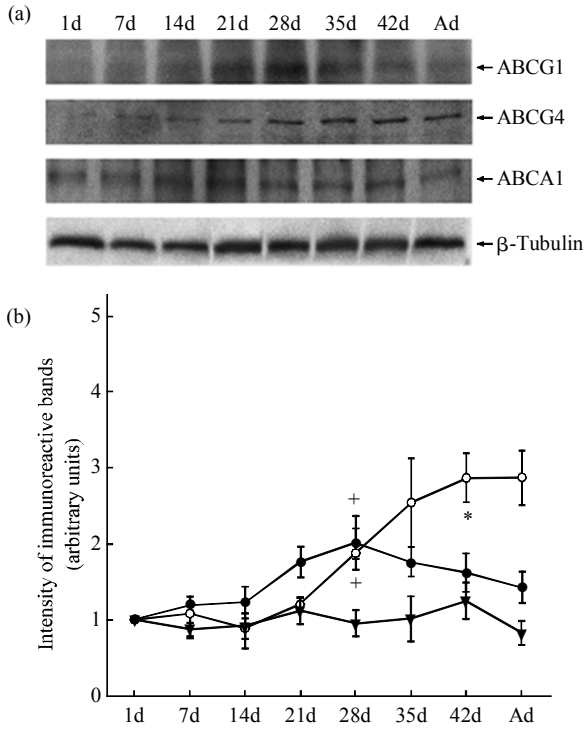

Fig. 3 Protein expression profile of ABCG1, ABCG4 and ABCA1 in mouse brain during postnatal development

50 μg of protein from mice developmental brain were subjected to 12% of SDS-PAGE and Western blot analyses were performed by using anti-ABCG1, ABCG4 and ABCA1 antibodies. Representative Western blots results showing that 74 ku ABCG1, 74 ku ABCG4 and 220 ku ABCA1 were present in mouse brain weekly throughout development (a). The immunoreactive bands were scanned and their intensities were quantified using the Scion Image program (b). All data were normalized to the internal control β-tubulin amounts. The intensities of the bands obtained in six independent experiments were relative to ABCG1, ABCG4 and ABCA1 protein levels at 1 day (1 fold). The values shown of ABCG1 (●—●), ABCG4 (○—○), and ABCA1 (▼—▼) are $\bar{x} \pm s$. The increase of ABCG1 protein levels between 28 and 1 day ($^{+}P < 0.05$, n=6) was significant. And the elevation of ABCG4 protein levels from 21 to 28 days ($^{+}P < 0.03$, n=6), and between 42 and 1 day ($^{*}P < 0.01$, n=6) were significant. d: Days; Ad: Adult.

2.4 Circulating plasma and brain esterified cholesterol levels exhibited a biphasic distribution, which peaked at day 42

In order to evaluate the potential roles of ABCG1,

ABCG4 and ABCA1 in the brain cholesterol metabolism during development, we measured the levels of free cholesterol (FC) and esterified cholesterol (EC) in the brain and serum. The developmental FC and EC level variations in the brain and serum are shown in Figure 4.

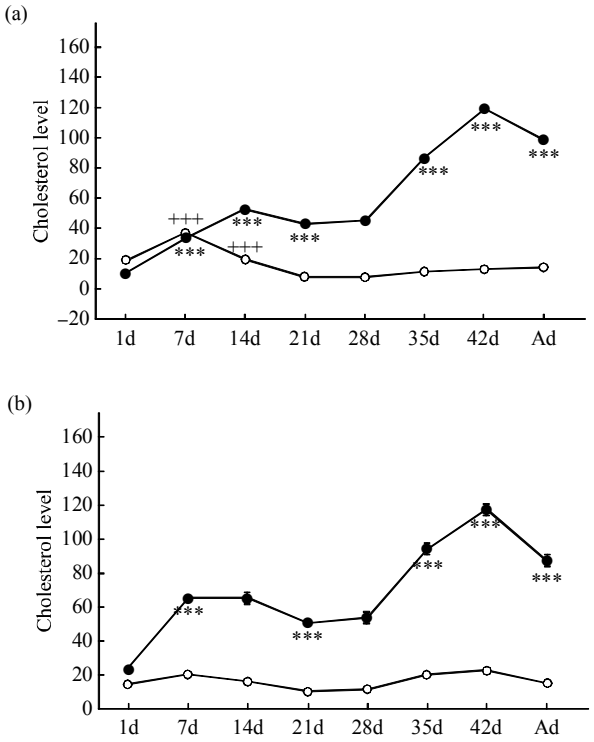

Fig. 4 Free cholesterol (FC) and esterified cholesterol (EC) levels in mouse brain and serum during postnatal development

WT C57BC/6J mice brain and serum were isolated. Free and esterified cholesterol were determined after isolation as described in materials and methods. (a) EC (●—●) and FC (○—○) are expressed in μg cholesterol/mg total protein for mouse brain. The values shown are $\bar{x} \pm s$ of four independent experiments. The increase of FC levels from 1 to 7 days (***P < 0.002, n=4) was significant. The increase of EC levels from 1 to 14 days (***P < 0.0001, n=4) and from 28 to 42 days (***P < 0.00001, n=4), and the decrease of EC levels from 14 to 21 days (***P < 0.005, n=4) and from 42 to adulthood (***P < 0.00005, n=4) were significant. (b) EC (●—●) and FC (○—○) are expressed in mg cholesterol/dl serum. The values shown are $\bar{x} \pm s$ of four independent experiments. The increase of EC levels from 1 to 7 days (***P < 0.00001, n=4) and from 28 to 42 days (***P < 0.002, n=4) were significant. The decrease of EC levels from 14 to 21 days (***P < 0.003, n=4) and from 42 days to adulthood (***P < 0.0004, n=4) were significant. d: Days; Ad: Adult.

In the brain, FC values were near 19 μg cholesterol/mg total protein at day 1 of postnatal (Figure 4 n=4) and rose to 37 μg cholesterol/ mg total protein by 7 days (^{+++}P < 0.002, 1 versus 7 day) before dropping to base line levels through adulthood (14 μg cholesterol/mg total protein). As expected, EC values were more than three times the FC values in the brain. Surprisingly, the increase in EC levels from 1 to14 days (***P < 0.0001) was followed by a significant decrease (***P < 0.005) from 14 to 21 days and then a marked increase from 28 days to adulthood and peaking by day 42 with the value of 119 μg cholesterol/mg total protein (***P < 0.00001, 42 versus 35 day) (Figure 4a, n =4). The EC levels was dropped in adulthood (***P < 0.00005).

Serum FC and EC levels was similar to that in the brain (Figure 4b, n =4). However, serum FC levels were low and constant throughout all development at time points. Serum EC values were 24 mg cholesterol/dl serum at day 1, peaking at 118 mg cholesterol/dl serum by day 42. Serum EC values showed a biphasic profile similar to that of EC levels in brain except that the change from 7-to-14 days was not statistically significant (P > 1).

2.5 ABCG1 and ABCG4 expressed reciprocally in Abcg1 or Abcg4 knockout mice (Abcg1$^{-/-}$ or Abcg4$^{-/-}$)

Since distinct temporal expression of ABCG1 and ABCG4, but not ABCA1, is seen in brain during postnatal developmental stage, we further investigated the changes of ABCG1 and ABCG4 expression in the brain of ABCG1$^{-/-}$ and ABCG4$^{-/-}$ mice. As assessed by quantitative Real-time PCR, diminished level of ABCG1 mRNA, but elevated level of ABCG4 mRNA was found in ABCG1$^{-/-}$(Figure 5a). Similar reciprocal relationship was also observed in ABCG4$^{-/-}$, represented by decreased ABCG4 expression but increased ABCG1 expression in the brain (Figure 5b).

Western blot analyses using anti-ABCG1 and ABCG4 antibodies revealed increased expression level of ABCG4 protein and concomitantly diminished level of ABCG1 protein in ABCG1$^{-/-}$ mice (Figure 5c). In contrast, ABCG4$^{-/-}$ mice contained high levels of ABCG1 protein and very low levels of ABCG4 protein (Figure 5d).

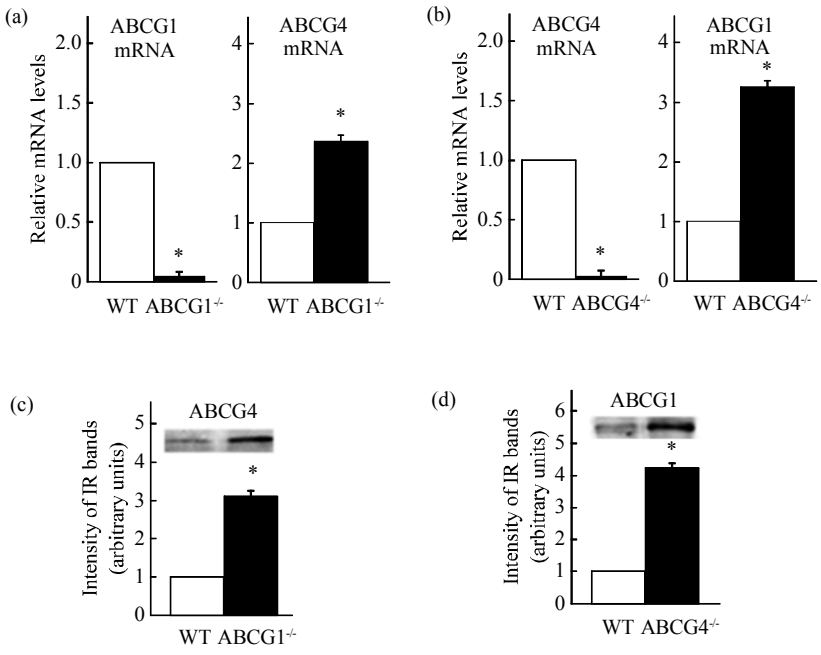

Fig. 5 Complementary expression of ABCG1 and ABCG4 in ABCG1$^{-/-}$ and ABCG4$^{-/-}$ mice

(a, b) Real time RT-PCR was performed for ABCG1 and ABCG4 genes in ABCG1$^{-/-}$ and ABCG4$^{-/-}$ mice. All data were normalized to the internal reference PpiA and are shown relative to the expression of each gene product in control wild-type (WT) mice (n=10, *P < 0.001 *vs*. WT). (c, d) Western blot analysis confirmed reciprocal expression of ABCG1 and ABCG4. 50 μg of protein from the brain of ABCG1$^{-/-}$, ABCG4$^{-/-}$ mice and WT mice were loaded to 12% SDS-PAGE and Western blot analyses were performed using anti-ABCG1 and ABCG4 antibodies. Semi-quantification of immunoreactive band intensity were performed and a representative blot was shown. Data are shown as $\bar{x} \pm s$ of three independent experiments (*P < 0.001 *vs*. WT).

2.6 Brain free and esterified cholesterol levels increased in ABCG1$^{-/-}$ ABCG4$^{-/-}$ mice

To elucidate the effects of ABCG1 and ABCG4 in the regulation of cholesterol homeostasis, we measured the levels of free and esterified cholesterol in the brain of ABCG1$^{-/-}$, ABCG4$^{-/-}$ and ABCG1 ABCG4 double knock out mice(ABCG1$^{-/-}$ ABCG4$^{-/-}$). We found that free and esterified cholesterol levels remain normal in the brain of the mice with either ABCG1 gene or ABCG4 gene was deleted (ABCG1$^{-/-}$ or ABCG4$^{-/-}$). However, significantly increased levels of free and esterified cholesterols were found in the brain from ABCG1$^{-/-}$ ABCG4$^{-/-}$ mice, which lack both ABCG1 and ABCG4 genes (Table 2). These findings suggest that ABCG1 and ABCG4 compensate for each other in the regulation of cholesterol homeostasis, and only loss of both transporters leads to cholesterol accumulation in the brain.

Brain tissues were dissected out from ABCG1$^{-/-}$, ABCG4$^{-/-}$ and ABCG1$^{-/-}$ ABCG4$^{-/-}$ mice and brain free and esterified cholesterol were measured. Free cholesterol and esterified cholesterol are expressed in μg cholesterol/mg total protein for mouse brain. The values shown are $\bar{x} \pm s$. Significant increase of free cholesterol and esterified cholesterol levels was noticed in the brains of ABCG1$^{-/-}$ ABCG4$^{-/-}$ compared

Table 2 Free cholesterol and esterified cholesterol levels in ABCG1$^{-/-}$, ABCG4$^{-/-}$ and ABCG1$^{-/-}$ ABCG4$^{-/-}$ brains

Cholesterol	Wild Type (n=5)	ABCG1$^{-/-}$ (n=10)	ABCG4$^{-/-}$ (n=10)	Wild Type (n=5)	ABCG1$^{-/-}$ ABCG4$^{-/-}$ (n=10)
Free cholesterol	14.9 ± 0.6	15.4 ± 0.8	15.2 ± 0.3	14.2 ± 0.6	25.9 ± 0.8*
Esterified cholesterol	85.7 ± 1.2	89.4 ± 1.0	91.2 ± 2.3	86.1 ± 0.9	109.2 ± 2.7*

to ABCG1$^{-/-}$ or ABCG4$^{-/-}$ (*P < 0.001, n=10 in each group). There is no significant change of cholesterol levels between ABCG1$^{-/-}$ and WT or ABCG4$^{-/-}$ and WT (P > 0.05; n=5 in WT group).

2.7 Changes of genes expression in ABCG1$^{-/-}$ ABCG4$^{-/-}$ mice brains

Brain cholesterol levels were significantly decreased in ABCG1$^{-/-}$ ABCG4$^{-/-}$ mice, we further investigated the changes of cholesterol synthesis related genes in the brain. Real time RT-PCR was applied to amplify the gene expression using the specific primers listed in Table 3 and quantification analysis was performed.

Table 3 Specific primers designed for mouse HMGR, FPPS, CYP51, ACAT1 and ABCA1

Gene	GenBank accession numbers	Primers		Product size/bp
		Sense	Anti-sense	
HMGR	NM_008255	5′ CACAAGCTGGAAACTGGTGA 3′	5′ GAAGAAGTAGGCCCCCAATC 3′	197
FPPS	AF_309508	5′ TGTACATGGCAGGCATTGAT 3′	5′ GAGGAGAGGCTCGTAGCAGA 3′	201
CYP51	NM_013454	5′ CCTTCACTCTCAGCCTCGTC 3′	5′ GCCCACCATGGTAAAGCTAA 3′	203
ACAT1	NM_144784.3	5′ CCAGATGTGGTGGTGAAAGA 3′	5′ ATTCGTGCCAATGGCTTAAC 3′	200
ABCA1	NM_020010	5′ AACAGTTTGTGGCCCTTTTG 3′	5′ CACAATCAGGCTGAAGACCA 3′	125

Our data show that mRNA level of HMG-CoA reductase (HMGR) and farnesyl diphosphate synthase (*FPPS*), which involves in the biosynthesis of free cholesterol, were remarkably decreased in ABCG1$^{-/-}$ ABCG4$^{-/-}$ mice. The mRNAs encoding lanosterol demethylase (CYP51) was also dramatically reduced in the brains of ABCG1$^{-/-}$ ABCG4$^{-/-}$ mice. Striking changes of gene expression of Acyl-coenzyme A: cholesterol acyltransferase1 (ACAT1), a specific esterified cholesterol enzyme, was significantly decreased in the brains of ABCG1$^{-/-}$ ABCG4$^{-/-}$ mice (Figure 6). Consistent with defective efflux and cholesterol accumulation, other ATP-binding cassette transporter, ABCA1 expression was significantly increased in ABCG1$^{-/-}$ ABCG4$^{-/-}$ brain (Figure 6). These changes in genes expression suggest that cholesterol homeostasis was altered in the brain following lack of ABCG1 and ABCG4.

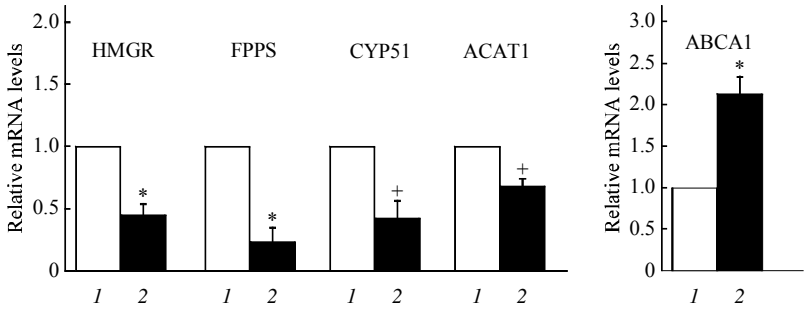

Fig. 6 mRNA expression levels of HMGR, FPPS, CYP51, ACAT1 and ABCA1 in the brains of ABCG1$^{-/-}$ ABCG4$^{-/-}$ mice

Real-time PCR was performed for HMGR, FPPS, CYP51, ACAT1 and ABCA1 genes in the brains of ABCG1$^{-/-}$ ABCG4$^{-/-}$ and WT mice. All data were normalized to the internal reference PpiA and expressed as fold increase relative to normalized internal control. The data were obtained from four independent experiments (*P < 0.01; ^{+}P < 0.05; n=4 in each group). *1*: WT; *2*: ABCG1$^{-/-}$ ABCG4$^{-/-}$.

3 Discussion

In this study, we have demonstrated that the ABC transporters ABCG1, ABCG4 and ABCA1 mRNAs are expressed in a variety of tissues of adult male C57BL/6J mice on a chow diet. When adjusted to

comparable levels, we were particularly struck by the data, which shows that ABCG1 and ABCG4 mRNA levels were several fold higher than ABCA1 mRNA levels in the adult brain (see Figure 1). We were interested in understanding the function of these transporters in the developing brain; therefore we determined the mRNA and protein levels during postnatal development. We were surprised to see that the three ABC transporter mRNA expression levels vary distinctly during postnatal brain development (see Figure 2). ABCG1 mRNA levels rose steadily with expression at P42 and stayed elevated throughout adulthood. ABCG4 mRNA levels exhibited a biphasic expression pattern with peaks in the prepubescent phase (P7～P14), and again at P42 into adulthood, similar to ABCG1. ABCA1 mRNA levels were at baseline from P1 through adulthood. Interestingly, ABC transporters protein levels did not correlate to their mRNA expression levels (see Figure 3). In particular, ABCG1 protein levels peaked at P28 then decreased, even though ABCG1 mRNA levels were elevated. ABCG4 protein levels were baseline through P21 and then climbed rapidly, peaking at P42. The protein levels did not show the same biphasic expression levels as the mRNA. Additionally, when serum and brain sterol levels were measured, esterified cholesterol levels in both the brain and serum had a biphasic distribution (see Figure 4). Interestingly, from P1～P7 in the brain, free cholesterol levels were higher than esterified cholesterol levels.

In contrast to a previous study that ABCA1 mRNA was only detected in the early postnatal brain[4], we found that it is expressed in the mouse brain throughout the postnatal development stages as well as adult period. Because ABC transporters are assumed to mediate cholesterol efflux process, ABCA1 expressed in the developmental brain is likely responsible for cholesterol efflux. However, the relatively low expression of ABCA1 mRNA and protein throughout postnatal brain development implies that ABCA1 may not play a major role in cholesterol efflux, resulting in the prevention of cholesterol loss before the blood-brain barrier is fully developed. In contrast, ABCG1 and ABCG4 appear to be the majorly expressed transporters in the brain during postnatal development and are likely to be important for cholesterol efflux.

As already noted, the mouse brain volume increased remarkably during the first three weeks of postnatal life [14]. Contributing to the dramatic developmental growth of the mouse brain is an elevation in lipids, which is required in the process of neurons differentiation or myelinogenesis. Since ABC transporters play crucial roles in brain lipid transport which is necessary for efficient neurons differentiation or oligodendrocytes myelination, it is predicted that the unique expression of ABC transporters may vary dramatically during mouse brain development [2]. Our studies support this hypothesis and are the first to indicate that ABCG1 and ABCG4 protein levels are expressed widely in the brain throughout development; and the expression pattern varied with the developmental time period. Elevated FC and EC levels in the brain by 7 days after birth, combined with increasing mRNA/protein levels of ABCG1 and ABCG4 in brain during neonatal development, suggests that ABCG1/ABCG4 are associated with cholesterol release. Moreover, high levels of ABCG1 and ABCG4, but not ABCA1, mRNA levels in adult brain suggest that ABCG1 and ABCG4 have roles in the regulation of lipid homeostasis during the late developmental period.

As previously demonstrated, astrocytes produce cholesterol in the brain and synthesize apoE containing nascent HDL to removes cholesterol from brain [15]. ABCG1 has been implicated in removing cholesterol from astrocytes and neurons *via* the interaction of the transporters with HDL particles [14–17]. Thus, we hypothesized that ABCG1 and ABCG4 may have an important role in apolipoprotein-dependent cholesterol efflux from astrocytes and neurons and our data in the developing brain support this theory.

In the present study, we also found the significantly decreased ABCG4 mRNA levels in brain by 21days, which is accompanied with the diminished esterified cholesterol level. Considering the previous observation that the rate of accumulation of sterol pool markedly decreased after three weeks of birth [1], the neuronal differentiation and synapse development tended to reduce as the animal matures, we assumed that such an effect of the elimination in ABCG4 transcription may reflect the decreased need of cholesterol, which would compromise the brain development.

Furthermore, ABCG4 mRNA and protein levels are remarkably elevated after 28 days during

development, which is accompanied with the slight decrease of ABCG1 protein levels. The evidence of complementary expression of ABCG1 and ABCG4 proteins during late development suggest that ABCG1 and ABCG4 could functionally compensate for one another in redistribution of cholesterol levels in the brain. However, ABCG1 and ABCG4 protein expression are not coincident with the mRNA expression during development. One could expect that another regulatory mechanism of the transporters exists at the translational levels. However, this remains to be determined.

Serum free cholesterol levels were low through all developmental time points, which is consistent with what have previously been demonstrated in adult mice [11]. In addition, esterified cholesterol levels increased steadily throughout development reaching previously described levels by adulthood. Unexpectedly however, there was a biphasic distribution with a minor peak from 7～14 days.

Brain esterified cholesterol levels showed a similar biphasic distribution with a minor peak at day 14 and a major peak at adulthood. Brain free cholesterol levels were consistent with serum FC levels from 21d to adulthood. However, FC levels were elevated to similar EC levels from 1～7 days and then slowly dropped to normal levels by day 21. What is causing this elevated FC levels is not clear and under investigation. Our results appear to be consistent with one report which describes cholesterol levels markedly decreased after three weeks of birth (Dietschy and Turley, 2004). However, this may be due to the fact that they were measuring rates of accumulation and we are measuring circulating sterol levels. The unexpected EC and FC levels in the postnatal developing mouse will require further investigation to elucidate the mechanism responsible for this observation.

The reciprocal expression pattern of ABCG1 and ABCG4 was further examined in knockout mice of ABC transporters. Deficiency of individual transporters is compensated by up-regulation of other transporters and disturbance of cholesterol homeostasis has not been observed in single knockout mice of either ABCG1$^{-/-}$ or ABCG4$^{-/-}$, suggesting that ABCG1 and ABCG4 transporters have overlapping functions in the regulation of cholesterol efflux. However, loss of both ABCG1 and ABCG4 from the brain results in the accumulation of cholesterol and altered expression of genes involved in the biosynthesis of free cholesterol. Although brain mitochondrial cholesterol overloading has been reported to cause neuronal apoptosis in a varieties of the central nervous system (CNS) diseases with gene mutations or genetic animals models which affect cholesterol homeostasis [18-21], no significant pathological or morphological changes were found in the of the brain of ABCG1$^{-/-}$ ABCG4$^{-/-}$ (data not shown). Upregulation of ABCA1 secondary to the deficiency of ABCG1 and ABCG4 in double knockout mice may increase cholesterol efflux from the brain using alternative cholesterol transport pathways and ameliorate neuronal stress caused by cholesterol accumulation. More pronounced elevation in cholesterol levels and defects in the CNS are expected to be seen in the mice with combined deficiency of ABCG1, ABCG4 and ABCA1.

In conclusion, distinct temporal expression of ABCG1 and ABCG4, but not ABCA1, is seen in the postnatal development of the adult brain. This may reflect their distinct cellular functions in the developing and adult brain, presumably to regulate lipid homeostasis in the brain and redistribute the lipid in different cells in the brain. ABCG1 and ABCG4 function primarily to control intracellular cholesterol movement and homeostasis in the brain.

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References

[1] Dietschy J M, Turley S D. Thematic review series: brain Lipids. Cholesterol metabolism in the central nervous system during early development and in the mature animal. J Lipid Res, 2004, **45**(8): 1375-1397

[2] Dietschy J M, Turley S D. Cholesterol metabolism in the brain. Curr Opin Lipidol, 2001, **12**(2): 105-112

[3] Bjorkhem I, Meaney S. Brain cholesterol: long secret life behind a barrier. Arterioscler Thromb Vasc Biol, 2004, **24**(5): 806-815

[4] Tachikawa M, Watanabe M, Hori S, *et al*. Distinct spatio-temporal expression of ABCA and ABCG transporters in the developing and adult mouse brain. J Neurochem, 2005, **95**(1): 294-304

[5] Hirsch-Reinshagen V, Zhou S, Burgess B L, *et al*. Deficiency of ABCA1 impairs apolipoprotein E metabolism in brain. J Biol Chem, 2004, **279**(39): 41197-41207

[6] Wang N, Yvan-Charvet L, Lutjohann D, *et al*. ATP-binding cassette transporters G1 and G4 mediate cholesterol and desmosterol efflux

to HDL and regulate sterol accumulation in the brain. Faseb J, 2008, **22**(4): 1073−1082

[7] Wollmer M A, Sleegers K, Ingelsson M, *et al*. Association study of cholesterol-related genes in Alzheimer's disease. Neurogenetics, 2007, **8**(3):179−188

[8] Pfrieger F W. Cholesterol homeostasis and function in neurons of the central nervous system. Cell Mol Life Sci, 2003, **60**(6):1158−1171

[9] Mauch D H, Nagler K, Schumacher S, *et al*. CNS synaptogenesis promoted by glia-derived cholesterol. Science, 2001, **294**(5545): 1354−1357

[10] Oram J F, Heinecke J W. ATP-binding cassette transporter A1: a cell cholesterol exporter that protects against cardiovascular disease. Physiological Reviews, 2005, **85**(4): 1343−1372

[11] Kennedy M A, Barrera G C, Nakamura K, *et al*. ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation. Cell Metab, 2005, **1**(2): 121−131

[12] Zhang F, Lavan B, Gregoire F M. Peroxisome proliferator-activated receptors as attractive antiobesity targets. Drug News Perspect, 2004, **17**(10): 661−669

[13] Wellington C L, Walker E K, Suarez A, *et al*. ABCA1 mRNA and protein distribution patterns predict multiple different roles and levels of regulation. Lab Invest., 2002, **82**(3):273−283

[14] Abildayeva K, Jansen P J, Hirsch-Reinshagen V, *et al*. 24 (S)-hydroxycholesterol participates in a liver X receptor-controlled pathway in astrocytes that regulates apolipoprotein E-mediated cholesterol efflux. J Biol Chem, 2006, **281**(18):12799−12808

[15] LaDu M J, Gilligan S M, Lukens J R, *et al*. Nascent astrocyte particles differ from lipoproteins in CSF. J Neurochem, 1998, **70**(5): 2070−2081

[16] Karten B, Campenot R B, Vance D E, *et al*. Expression of ABCG1, but not ABCA1, correlates with cholesterol release by cerebellar astroglia. J Biol Chem, 2006, **281**(7): 4049−4057

[17] Wahrle S E, Jiang H, Parsadanian M, *et al*. ABCA1 is required for normal central nervous system ApoE levels and for lipidation of astrocyte-secreted apoE. J Biol Chem, 2004, **279**(39): 40987−40993

[18] Huang Z, Hou Q, Cheung N S, *et al*. Neuronal cell death caused by inhibition of intracellular cholesterol trafficking is caspase dependent and associated with activation of the mitochondrial apoptosis pathway. J Neurochem, 2006, **97**(1): 280−291

[19] Mukherjee S, Maxfield F R. Lipid and cholesterol trafficking in NPC. Biochimica et Biophysica Acta, 2004, **1685**(1−3): 28−37

[20] Yu W, Gong J S, Ko M, *et al*. Altered cholesterol metabolism in Niemann-Pick type C1 mouse brains affects mitochondrial function. J Biol Chem, 2005, **280**(12): 11731−11739

[21] Fernandez A, Llacuna L, Fernandez-Checa J C, *et al*. Mitochondrial cholesterol loading exacerbates amyloid beta peptide-induced inflammation and neurotoxicity. J Neurochem: The Official Journal of the Society for Neuroscience, 2009, **29**(20): 6394−6405

转运蛋白 ABCG1/4 和 ABCA1 对脑胆固醇的调节作用 *

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摘要　脑是富含胆固醇的器官，机体大约有 25%的胆固醇集中在脑组织中．ATP 结合盒超家族转运蛋白对脑组织中胆固醇的膜外转运和动态平衡起着重要的调节作用．研究发现，ATP 结合盒超家族转运蛋白亚体 ABCG1、ABCG4 和 ABCA1 在成体脑组织中存在不同程度的表达，一种或多种亚体的缺失可以导致神经退行性病变．然而，ATP 结合盒超家族转运蛋白亚体对脑发育过程中脑胆固醇动态变化的调节缺乏相关性的报道．在本研究中，从低胆固醇饮食喂养的 C57BL/6J 小鼠中获取出生后不同发育时期的脑组织，对 ABCG1、ABCG4 和 ABCA1 的 mRNA 与蛋白质表达水平进行测定，并对脑组织和血清中 ATP 结合盒超家族转运蛋白的表达水平与胆固醇水平的相关性进行研究．同时，使用 ABCG1、ABCG4单一基因敲除鼠和 ABCG1、ABCG4双基因敲除鼠，研究 ATP 结合盒超家族转运蛋白对与胆固醇合成的相关基因表达的影响以及对脑组织胆固醇代谢的调节作用．结果发现，ABCG1、ABCG4 和 ABCA1 在机体多个器官中均有表达，但 ABCG1 和 ABCG4 在小鼠脑组织中表达量最高．在脑组织发育过程中，ABCG1 和 ABCG4 mRNA 水平呈现明显的表达时效性，小鼠于出生后 42 天达到峰值，而 ABCA1 mRNA 的表达水平无明显变化．血清和脑组织中中酯化型胆固醇水平呈双高峰分布，也于出生后 42 天达到最高．基因敲除鼠模型显示，单一敲除 ABCG1 或者 ABCG4基因对脑组织胆固醇水平无明显影响，而 ABCG1 和 ABCG4基因的同时缺失导致脑胆固醇水平显著升高，并明显降低胆固醇合成相关基因的表达水平．本研究表明，在脑发育成熟过程中，ATP 结合盒超家族转运蛋白亚体 ABCG1 和 ABCG4，而非 ABCA1，以调节脑胆固醇的膜外转运；ABCG1 和 ABCG4 互补调控脑胆固醇的动态平衡．

关键词　ATP 结合盒超家族转运蛋白，脑发育，胆固醇

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