

# FA2H is responsible for the formation of 2-hydroxy galactolipids in peripheral nervous system myelin<sup>§</sup>

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**Abstract** Myelin in the mammalian nervous system has a high concentration of galactolipids [galactosylceramide (GalCer) and sulfatide] with 2-hydroxy fatty acids. We recently reported that fatty acid 2-hydroxylase (FA2H), encoded by the *FA2H* gene, is the major fatty acid 2-hydroxylase in the mouse brain. In this report, we show that FA2H also plays a major role in the formation of 2-hydroxy galactolipids in the peripheral nervous system. *FA2H* mRNA and FA2H activity in the neonatal rat sciatic nerve increased rapidly during developmental myelination. The contents of 2-hydroxy fatty acids were ~5% of total galactolipid fatty acids at 4 days of age and increased to 60% in GalCer and to 35% in sulfatides at 60 days of age. The chain length of galactolipid fatty acids also increased significantly during myelination. *FA2H* expression in cultured rat Schwann cells was highly increased in response to dibutyl cyclic AMP, which stimulates Schwann cell differentiation and upregulates myelin genes, such as UDP-galactose:ceramide galactosyltransferase and protein zero. These observations indicate that *FA2H* is a myelination-associated gene. FA2H-directed RNA interference (RNAi) by short-hairpin RNA expression resulted in a reduction of cellular 2-hydroxy fatty acids and 2-hydroxy GalCer in D6P2T Schwannoma cells, providing direct evidence that FA2H-dependent fatty acid 2-hydroxylation is required for the formation of 2-hydroxy galactolipids in peripheral nerve myelin. Interestingly, FA2H-directed RNAi enhanced the migration of D6P2T cells, suggesting that, in addition to their structural role in myelin, 2-hydroxy lipids may greatly influence the migratory properties of Schwann cells.—Maldonado, E. N., N. L. Alderson, P. V. Monje, P. M. Wood, and H. Hama. FA2H is responsible for the formation of 2-hydroxy galactolipids in peripheral nervous system myelin. *J. Lipid Res.* 2008. 49: 153–161.

**Supplementary key words** fatty acid 2-hydroxylase • fatty acid  $\alpha$ -hydroxylase • hydroxy fatty acids • galactosylceramide • sulfatide

In higher vertebrates, nerve conduction is greatly facilitated by myelin, a lipid-rich membrane that wraps around

the axon. Myelin is formed by oligodendrocytes in the central nervous system (CNS) and by Schwann cells in the peripheral nervous system (PNS). Myelin lipid compositions are very unique: ~25–30% of all lipids consist of galactosylceramides (GalCers) and sulfatides (3-sulfate esters of GalCer) in both CNS and PNS (1). As much as 60% of the amide-linked fatty acids in myelin galactolipids are hydroxylated at the C2 position (2-hydroxy fatty acids) (2, 3). No other mammalian tissues contain such high concentrations of 2-hydroxy fatty acids, suggesting an essential role for the 2-hydroxyl modification in maintaining myelin function.

Biophysical studies of 2-hydroxy galactolipids in model membranes have shown that the 2-hydroxyl group facilitates tight lipid packing via hydrogen bonds at the surface of the membrane (4–6). The 2-hydroxyl group also enhances carbohydrate-carbohydrate interactions between galactolipids on apposing membranes, presumably by altering the conformation of the carbohydrate head groups (7). These studies suggest that 2-hydroxy galactolipids play critical roles in lipid-lipid and lipid-protein interactions within myelin.

GalCer is synthesized from ceramide and UDP-galactose by the enzyme UDP-galactose:ceramide galactosyltransferase (CGT) (8). CGT-knockout mice, which cannot synthesize galactolipids, are able to form myelin but develop neurological abnormalities and have a short life span (9, 10). Detailed analyses of these mice revealed crucial roles for GalCer and sulfatide in myelination and axo-glial organization (11–13). Myelin of CGT-knockout mice is devoid of GalCer and sulfatide and instead contains glucosyl-

Abbreviations: CGT, UDP-galactose:ceramide galactosyltransferase; CNS, central nervous system; GalCer, galactosylceramide; GlcCer, glucosylceramide; PNS, peripheral nervous system;  $P_0$ , Protein Zero; Px, postnatal day x; RNAi, RNA interference; shRNA, short-hairpin RNA; siRNA, small interfering RNA.

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ceramides (GlcCers), which are not found in normal myelin (9, 10). Interestingly, the GlcCer in myelin of CGT-knockout mice contains 2-hydroxy fatty acids, suggesting a specific need for monoglycosylceramides with both non-hydroxy and 2-hydroxy fatty acids for proper myelin function and stability.

Recently, Fewou et al. (14) reported that CGT transgenic mice had unstable and uncompacted myelin and developed progressive hindlimb paralysis and demyelination. Although total myelin galactolipids in these mice were not altered significantly, the 2-hydroxy GalCer-to-nonhydroxy GalCer ratio was reduced. Therefore, it was concluded that the underlying cause of the unstable myelin was reduced 2-hydroxy GalCer.

The 2-hydroxyl group on the *N*-acyl chain of myelin galactolipids is incorporated during de novo biosynthesis of GalCer. In the biosynthesis pathway for 2-hydroxy galactolipids, fatty acid 2-hydroxylase (FA2H) is the major enzyme that catalyzes fatty acid 2-hydroxylation in the CNS (15). FA2H activity increases in the CNS during developmental myelination to provide precursors for the synthesis of myelin 2-hydroxy galactolipids. Biochemical studies of FA2H have been conducted almost exclusively in the CNS, and little is known about this enzyme in the PNS. The current study is aimed at establishing that FA2H is also responsible for the formation of 2-hydroxy galactolipids in PNS myelin. Furthermore, a novel function of 2-hydroxy lipids in cell migration was discovered during the study using RNA interference (RNAi).

## EXPERIMENTAL PROCEDURES

### Materials

Fetal bovine serum was purchased from Atlanta Biologicals (Norcross, GA). Bovine sulfatides, cerebrosides (kerasin, phrenosin), glucocerebrosides, and odd-chain fatty acids (C<sub>15</sub>–C<sub>21</sub>) were purchased from Matreya (Pleasant Gap, PA). Deuterated tetracosanoic acid[3,3,5,5-D<sub>4</sub>] was purchased from Larodan Fine Chemicals (Malmö, Sweden). Purified human NADPH:cytochrome P450 reductase and NADPH-regenerating system solutions were purchased from BD Biosciences (Bedford, MA). Recombinant heregulin-β<sub>177–244</sub> was a generous gift from Genentech, Inc. (South San Francisco, CA). Forskolin and dibutyl cAMP were purchased from Sigma (St. Louis, MO).

### Animals

Fisher and Sprague-Dawley rats were maintained in animal care facilities of the Medical University of South Carolina and the University of Miami Miller School of Medicine, respectively, with water and food ad libitum. Rats were treated in accordance with the Institutional Animal Care and Use Committee-approved procedures at these institutions.

### Cell cultures

Rat Schwann cells were obtained from sciatic nerves of 3 month old Fisher rats by the method of Morrissey, Kleitman, and Bunge (16) with modifications. Nerve segments were explanted in DMEM (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated FBS (Hyclone) and depleted of fibroblasts by sequential transplantation (two to three times) to new plastic dishes.

After 2 weeks, tissue explants were dissociated with 0.25% dispase (Roche) and 0.05% collagenase (Worthington), and the resulting cell suspension was plated on dishes coated with poly-L-lysine substrate (200 μg/ml). Cells were grown in medium containing DMEM-10% FBS supplemented with a mixture of mitogens [2 μM forskolin, 20 μg/ml bovine pituitary extract (Biomedical Tech., Stoughton, MA), and 2.5 nM recombinant heregulin-β<sub>177–244</sub> (Genentech)]. After 1 week in culture, the cells were trypsinized and the remaining fibroblasts were removed by a 30 min incubation with anti-Thy 1.1 antibodies (conditioned medium from mouse hybridoma cells; American Type Culture Collection) followed by the addition of rabbit complement (ICN). Primary Schwann cells from passages 1 to 4 (two to eight population doublings) were cultured on poly-L-lysine-coated dishes in medium containing mitogens. These cells were >98% Schwann cells based on immunostaining with anti-S100 (Dako), a Schwann cell-specific marker. To induce differentiation, Schwann cells were plated in 10 cm poly-L-lysine-laminin-coated dishes (2 × 10<sup>6</sup> cells/dish) in DMEM containing 1% FBS and stimulated for 3 days with 1 mM dibutyl cAMP (Sigma). Under this condition, myelin-associated genes are induced and Schwann cells acquire a differentiated phenotype (17).

Rat Schwannoma-derived D6P2T cells (18) were purchased from the American Type Culture Collection and grown in DMEM containing 10% FBS. Cells were harvested at ~60–70% confluence.

### Isolation of galactolipids from rat sciatic nerve

Rats at postnatal day 4 (P4), P7, P10, P15, P20, P30, and P60 were euthanized according to guidelines established by the Institutional Animal Care and Use Committees. Sciatic nerves were exposed through a longitudinal incision in the thigh and excised immediately. Whole nerves were homogenized in PBS using a PT1200E Polytron with a 7 mm generator. GalCer and sulfatides (2-hydroxy and nonhydroxy) were isolated as described by Coetzee et al. (10) with minor modifications. Lipids were extracted with 19 volumes of chloroform-methanol (2:1, v/v) (19). The extracts were washed with 0.2 volume of 0.9% NaCl, and the lower phase was collected and dried under an N<sub>2</sub> stream. Dry lipid extracts were subjected to mild alkaline hydrolysis (0.5 N KOH for 10 min at 50°C) to remove glycerolipids. TLC plates (Silica Gel 60; Merck) were saturated with solvent vapor [chloroform-methanol-water (70:30:4, v/v)] for 45 min before development. Lipid spots were visualized under ultraviolet light after spraying with primuline solution [0.005% primuline in acetone-water (80:20, v/v)] and identified using the corresponding standards. GalCer and sulfatide were separately removed from the plates, and internal standards (C<sub>15</sub>, C<sub>17</sub>, C<sub>19</sub>, and C<sub>21</sub> fatty acids) were added before alkaline hydrolysis (4 N KOH overnight at 80°C). Lipids were acidified by the addition of glacial acetic acid, and free fatty acids were extracted three times with 3 ml of diethyl ether. Combined ether extracts were brought to dryness under N<sub>2</sub>.

### Fatty acid determination by GC-MS

Fatty acid methyl esters were prepared by adding 1 ml of methanolic HCl to each sample, incubated at 65°C for 45 min, and brought to dryness under N<sub>2</sub>. To prepare trimethylsilyl derivatives of hydroxyl groups, 100 μl of Tri-Sil Reagent (Pierce Biotechnology, Rockford, IL) was added and incubated for 30 min at room temperature. Derivatized samples were applied to a GC-2010 gas chromatograph (Shimadzu Scientific, Columbia, MD) with the injector in splitless mode. The injection port and transfer line were maintained at 250°C. Analytes were fractionated on a Restek RTX-5 column (5% diphenyl and 95% dimethyl polysiloxane; 0.25 mm inner diameter, 0.25 μm D.F., 30 m). The initial oven temperature was 110°C and was increased to 300°C

at 10°C/min. Mass spectra data were obtained on a Shimadzu GC-MS-QP2010 mass spectrometer after electron-impact ionization. Peaks of the target analytes and internal standards were processed using the GC-MS Lab Solutions software (Shimadzu Scientific). Calibration curves were constructed by plotting peak area ratios of the target analytes to their respective internal standards against concentration.

### Fatty acid 2-hydroxylase assay

FA2H activity was determined in rat sciatic nerves and in D6P2T cells using the method described previously by Alderson, Walla, and Hama (20). Briefly, crude sciatic nerve or D6P2T homogenates (50 µg of protein) were added to an assay mixture containing 2.7 mM Tris-HCl, pH 7.6, 1.20 mM NADP<sup>+</sup>, 3.3 mM glucose 6-phosphate, 3.3 mM MgCl<sub>2</sub>, 0.2 unit of glucose 6-phosphate dehydrogenase, and 1 µg of human NADPH:cytochrome P450 reductase, in a total volume of 1.4 ml. The substrate, 1 µg (2.7 nmol) of [3,3,5,5-D<sub>4</sub>]C<sub>24</sub> fatty acid (stock solution: 10 µg/ml in 1.5 mM α-cyclodextrin), was added at time zero. After gentle mixing by swirling, the assay mixture was incubated with shaking (100 rpm) at 37°C for 180 min. At the end of the incubation, 1 pmol of C<sub>23</sub> fatty acid was added to each sample as an internal standard, and samples were acidified by the addition of glacial acetic acid (20 µl). Fatty acids were extracted three times with diethyl ether (2 ml), and combined diethyl ether extracts were brought to dryness under N<sub>2</sub>. Fatty acids were derivatized and quantified as described above.

### Quantitative RT-PCR

Whole sciatic nerves of P4, P7, P10, P15, P20, P30, and P60 rats were homogenized in PBS using a PT1200E Polytron with a 7 mm generator. Total RNA was isolated using the Qiagen RNeasy Lipid Tissue kit. Total RNA from cultured Schwann cells was isolated using the Qiagen RNeasy kit. RNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc.). cDNA was generated using the Bio-Rad iScript cDNA Synthesis kit. Real-time quantitative PCR was performed on a Bio-Rad MyiQ single-color real-time PCR detection system. The primers used for each gene were as follows: for rat *FA2H*, rFA2H-F1 (cca tta cta cct gca ctt tgg) and rFA2H-R1 (tct gga atg agg gtg tgg a); for rat *CGT*, rCGT-F1 (ggt cat ggg tcc agc tgg tg) and rCGT-R1 (ctg gcc ggc ttt gtt agg); for rat Protein Zero (*P<sub>0</sub>*), rP0-F1 (ctg cac tgc tcc ttc tgg t) and rP0-R1 (cct tgg cat agt gga aga tgg); for rat 18S rRNA, r18S-F1 (ggc ccg aag cgt tta ctt) and r18S-R1 (cgg ccg tcc ctc tta atc). PCR was performed on a 96-well plate with a reaction mixture containing 15 µl of iQ SYBR Green Supermix (Bio-Rad), cDNA template, and 200 nM each of forward and reverse primers in a total volume of 30 µl. All reactions were performed in triplicate. The thermal cycling conditions were set at 95°C for 3 min followed by 40 cycles of two-step amplification (10 s at 95°C and 45 s at 57°C). Data were analyzed with MyiQ software. The abundances of *FA2H*, *CGT*, and *P<sub>0</sub>* mRNA were normalized against 18S rRNA contents using the ΔΔC<sub>t</sub> method (21).

### Construction of short-hairpin RNA expression plasmids

Four small interfering RNAs (siRNAs) against rat *FA2H* were designed using the Ambion (Austin, TX) siRNA Target Finder and screened for the ability to reduce cellular 2-hydroxy fatty acids. The most effective sequence (aag aga tta ttc act tgt ggt) was selected for the construction of a short-hairpin RNA (shRNA) expression plasmid. The control shRNA expression plasmid was constructed based on the sequence of Control siRNA 5 from Ambion. Each insert contained a *Bam*HI linker, sense strand, loop sequence (ttc aag aga), antisense strand, RNA Pol III terminator, and a *Hind*III linker. Double-stranded DNA inserts

were prepared by annealing 63 base complementary synthetic oligonucleotides and cloned into *Bam*HI and *Hind*III sites of pSilencer 5.1-U6 Retro (Ambion). The correct inserts were confirmed by sequencing.

### Transfection of D6P2T cells

D6P2T cells were transfected with double-stranded siRNA (2 µg) or shRNA expression plasmids (2.5 µg) using the Nucleofector kit T (amaxa, Gaithersburg, MD). Transfections were performed according to the manufacturer's instructions (Program T-20). Briefly, cells were harvested at 60–70% confluence by trypsin-EDTA treatment. Approximately 2 × 10<sup>6</sup> cells were transfected for each treatment condition and immediately plated in 75 cm<sup>2</sup> flasks to propagate for 48 h. Transfection efficiency was monitored for each experiment using pmaxGFP and visualization with fluorescence microscopy. Typical experiments averaged ~80–90% transfection efficiency with high viability.

### [<sup>14</sup>C]acetate labeling in D6P2T cells

D6P2T cells were transfected with 2.5 µg of control shRNA or FA2H-shRNA expression plasmids using the Nucleofector kit T (amaxa). After 48 h, puromycin (1 µg/ml) was added to the culture medium and resistant cells were selected for 2 weeks. Stably transfected cells were grown to ~50% confluence and harvested for fatty acid analysis or metabolically labeled with [<sup>14</sup>C]acetate (2 µCi/ml) for 48 h. Labeled cells were harvested by trypsin-EDTA treatment and washed with PBS. Cellular lipids were extracted with 19 volumes of chloroform-methanol (2:1, v/v) (19). The extracts were washed with 0.2 volume of 0.9% NaCl, and the lower phase was collected and dried under an N<sub>2</sub> stream. Dried lipid extracts were subjected to mild alkaline hydrolysis (0.5 N KOH for 10 min at 50°C) to remove glycerolipids. A small aliquot was removed from each sample for determination of total lipid <sup>14</sup>C counts to confirm equal <sup>14</sup>C incorporation. TLC plates (Silica Gel 60) were immersed in 1% sodium tetraborate (in methanol) for 15 s and air-dried overnight, followed by heating at 110°C for 30 min before sample application. Nonhydroxy and 2-hydroxy GalCer standards were added to each sample as carriers, and equal <sup>14</sup>C counts were spotted on borate-impregnated TLC plates (see supplementary Fig. 1). TLC plates were saturated with solvent vapor [chloroform-methanol-water (70:30:4, v/v)] for 45 min before development. Lipid spots were visualized under ultraviolet light after spraying with primuline solution [0.005% primuline in acetone-water (80:20, v/v)] and identified using corresponding standards. Nonhydroxy and 2-hydroxy GalCer spots were removed from the plates separately, and <sup>14</sup>C incorporation was measured by liquid scintillation counting.

### Migration assay

D6P2T cells were transfected with control (Ambion Control siRNA 5) or FA2H (target sequence, aagagattattcactgtggt) siRNA. Cells were harvested at 72 h after transfection, and migration was measured by a method based on the Boyden chamber principle. Equal numbers (50,000) of viable cells were placed on the top surface of 24-well cell culture inserts (polyethylene terephthalate membrane, 8 µm pore; Falcon No. 353097) with 300 µl of serum-free DMEM. Each insert was placed in a well containing 500 µl of DMEM with 10% FBS and placed in a CO<sub>2</sub> incubator at 37°C for 4 h. Cells remaining on the top surface of the inserts were removed with a cotton swab, and migratory cells on the bottom surface were stained and counted using light microscopy. Migration assays were also performed with D6P2T cells stably transfected with control shRNA or FA2H-shRNA expression plasmids.



## RESULTS

### FA2H expression is upregulated in sciatic nerve during myelination

We previously demonstrated that *FA2H* is expressed in oligodendrocytes in the CNS (15). We further demonstrated that *FA2H* gene expression was upregulated ~400-fold during postnatal myelination in mouse brain in parallel with other myelin-associated genes. To show that FA2H is also involved in PNS myelination, we first examined the expression of *FA2H* during postnatal myelination of the PNS by quantitative RT-PCR. mRNAs for other well-characterized PNS myelin genes, *CGT* and *P<sub>0</sub>*, were also determined. *CGT* encodes UDP-galactose:ceramide galactosyltransferase, which catalyzes the synthesis of GalCer. FA2H was presumed to function in the same biosynthetic pathway as *CGT*; therefore, the two genes were expected to be coordinately expressed during myelination. *P<sub>0</sub>* is the major structural protein of PNS myelin and serves as a good marker for myelination. As shown in **Fig. 1**, *FA2H* was expressed in sciatic nerve at all ages. *FA2H* mRNA increased significantly after P7 and reached the highest level at P20 (~6-fold the level at P4), which coincides with the period of active myelination in rat sciatic nerve. During this same period, *CGT* and *P<sub>0</sub>* increased 3.7-fold and 6.3-fold, respectively. By P60, the mRNA levels of the three genes were decreased to 25–38% of the levels measured at P20. The temporal changes in *FA2H* expression closely paralleled those of the two well-established myelin genes, suggesting a role in PNS myelination.

*FA2H* expression was also determined in primary cultures of Schwann cells isolated from adult rat sciatic nerve. Adult-derived Schwann cells expanded in vitro display features of undifferentiated cells (16). However, in the absence of axons, the cells can be induced to reacquire a differentiated phenotype (i.e., high myelin gene expression) by exposure to agents increasing the intracellular levels of cAMP (17). As shown in **Fig. 2**, *FA2H* was expressed at very low levels in quiescent, nondifferentiated Schwann cells

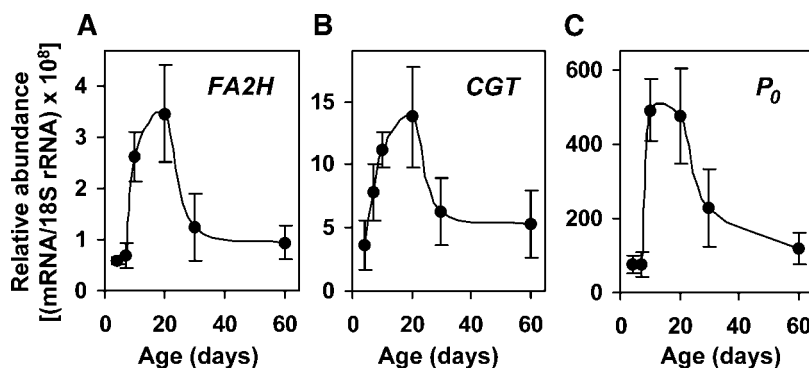
compared with the relatively high basal levels of *CGT* and *P<sub>0</sub>* expression. Upon dibutyryl cAMP-stimulated differentiation, *FA2H* mRNA was increased 86-fold, whereas *CGT* and *P<sub>0</sub>* expression was increased 3- to 4-fold. These results indicate that *FA2H* is highly inducible in Schwann cells in response to signals for differentiation, as are other myelin-associated genes.

### FA2H activity in sciatic nerve increases during myelination

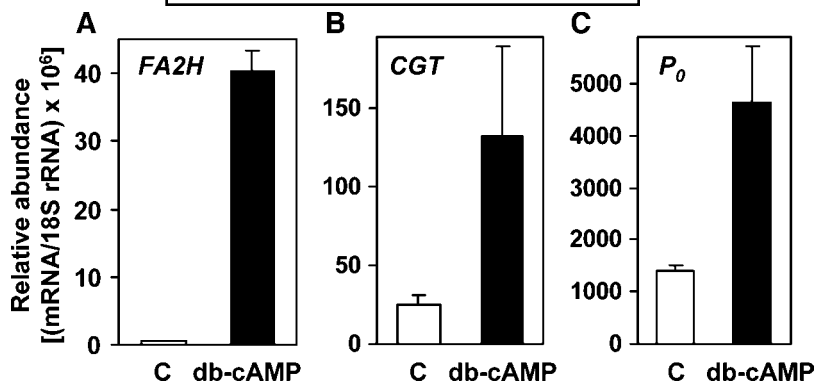
The high expression of *FA2H* in myelinating sciatic nerve and differentiated Schwann cells is indicative of FA2H being involved in the biosynthesis of myelin 2-hydroxy galactolipids. To show that *FA2H* expression correlates with FA2H enzymatic activity, FA2H activity was determined in sciatic nerve during postnatal myelination using the method developed in our laboratory (20). This assay specifically measures the conversion of the exogenous substrate, deuterated C<sub>24</sub> free fatty acid, to deuterated C<sub>24</sub> 2-hydroxy fatty acid. Consistent with the increase in *FA2H* mRNA, FA2H activity increased progressively to P20, reaching ~700% of activity levels measured at P4 (**Fig. 3**). These data provide evidence supporting a role for FA2H in the synthesis of 2-hydroxy galactolipids in the PNS.

### 2-Hydroxy galactolipids in sciatic nerve increase during developmental myelination

The fatty acid compositions of myelin galactolipids have been studied extensively in the mammalian CNS. Relative contents of very long-chain fatty acids (>C<sub>20</sub>) and 2-hydroxy fatty acids in brain galactolipids increase during developmental myelination. Although the high galactolipid contents in the mammalian PNS are known, developmental changes in the fatty acid compositions of galactolipids are not fully defined. If FA2H was responsible for the synthesis of 2-hydroxy galactolipids in the PNS, 2-hydroxy fatty acid contents in galactolipids would be expected to increase in parallel with the sharp increase in *FA2H* expression and activity during myelination of the sciatic nerve.



**Fig. 1.** Fatty acid 2-hydroxylase (*FA2H*) expression is upregulated during developmental myelination of rat sciatic nerve. Total RNA was isolated from rat sciatic nerves, and *FA2H* (A), UDP-galactose:ceramide galactosyltransferase (*CGT*) (B), and Protein Zero (*P<sub>0</sub>*) (C) mRNA levels were determined by quantitative RT-PCR. Data are normalized to 18S rRNA levels. Means  $\pm$  SD are shown [ $n = 5$  for postnatal day 7 (P7), P15, P20, P30, and P60;  $n = 4$  for P30]. The data at P4 represent a pool of sciatic nerves from four rats in triplicate measurements.



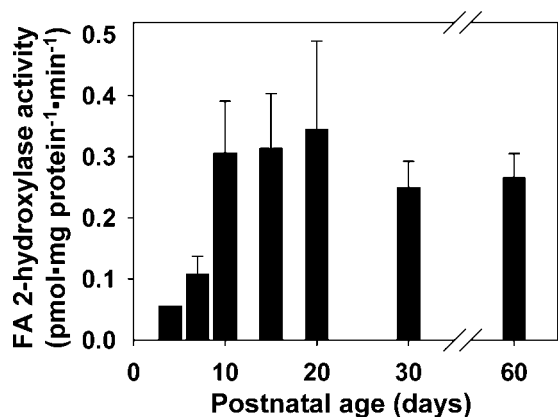
**Fig. 2.** *FA2H* expression in primary Schwann cells is highly upregulated upon differentiation. Schwann cells were isolated from adult rat sciatic nerve. Total RNA was isolated from proliferating control cells (C) or cells treated with 1 mM dibutyl cAMP (db-cAMP). *FA2H* (A), *CGT* (B), and *P<sub>0</sub>* (C) mRNA levels were determined by quantitative RT-PCR. Data are normalized to 18S rRNA levels. Means  $\pm$  SD are shown.

Therefore, we quantified the nonhydroxy and 2-hydroxy fatty acids associated with GalCer and sulfatides in sciatic nerve during postnatal myelination (Figs. 4, 5). The changes in relative nonhydroxy and 2-hydroxy fatty acid contents are shown in Fig. 6.

There were two distinct features in the changes of GalCer-associated fatty acids from P4 to P60 (Fig. 4). First, 2-hydroxy fatty acids were minor components in GalCer in neonates and increased markedly as animals matured, whereas total nonhydroxy GalCer contents (per protein) remained relatively unchanged. 2-Hydroxy fatty acids continued to increase after the peak myelination period, and 2-hydroxy C<sub>22</sub> and C<sub>24</sub> fatty acids were the predominant species at P30 and P60 (Fig. 4, right). Second, fatty acid chain length (both nonhydroxy and 2-hydroxy) shifted from long-chain (C<sub>16</sub>–C<sub>20</sub>) in neonates to predominantly very long-chain (C<sub>22</sub>–C<sub>26</sub>) in adults (Fig. 4, left). Although fatty acids in GalCer were almost exclusively C<sub>16</sub> and C<sub>18</sub> at P4, very long-chain fatty acids (C<sub>22</sub>–C<sub>26</sub>) continually increased and were major species at P30 and P60. Interest-

ingly, there was little 2-hydroxy C<sub>26</sub> fatty acid even though nonhydroxy C<sub>26</sub> was present at all ages. Unsaturated fatty acids (C<sub>18:1</sub> and C<sub>24:1</sub>, both hydroxy and nonhydroxy) were below detectable levels. The shift from nonhydroxy to 2-hydroxy fatty acids is evident when the relative fatty acid contents are calculated as percentage of total GalCer-associated fatty acids (Fig. 6A). The proportion of 2-hydroxy fatty acids increased from 5% at P4 to 62% at P60.

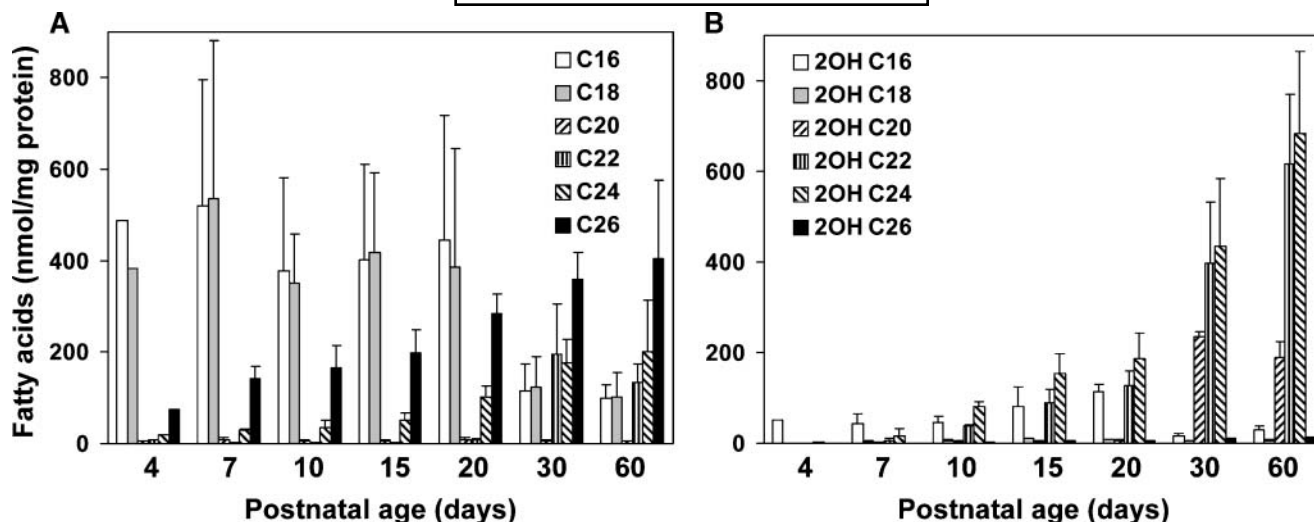
The developmental changes in sulfatide-associated fatty acids were similar to those of GalCer-associated fatty acids, with some interesting differences (Fig. 5). As in GalCer, there was little 2-hydroxy fatty acid in sulfatide at P4. The increase in 2-hydroxy fatty acid contents in sulfatide was less dramatic than that in GalCer, and nonhydroxy fatty acids were still predominant at P60. The most notable difference was that nonhydroxy C<sub>26</sub> content did not increase significantly, as in GalCer. Unsaturated fatty acids (C<sub>18:1</sub> and C<sub>24:1</sub>, both hydroxy and nonhydroxy) were undetected in sulfatide as well. The relative abundance of 2-hydroxy fatty acids changed from 4.5% at P4 to 34% at P30 and remained at the same level at P60 (Fig. 6B). These percentages are comparable to TLC-based measurements in mice (22).



**Fig. 3.** FA2H activity in postnatal rat sciatic nerve. Crude nerve tissue homogenates (50  $\mu$ g of protein) were used for FA2H assays. Means  $\pm$  SD are shown (n = 5 for P15, P20, P30, and P60; n = 3 for P7; n = 2 for P10). The data at P4 represent a pool of sciatic nerves from four animals.

### FA2H is required for the synthesis of 2-hydroxy GalCer

The data shown above indicate a strong correlation between *FA2H* expression/activity and the increase in 2-hydroxy galactolipid contents in postnatal rat sciatic nerve. To provide direct evidence for the involvement of FA2H in 2-hydroxy galactolipid synthesis, FA2H-directed RNAi was performed in the cell line D6P2T. Originally isolated from a Schwannoma, D6P2T cells maintain many characteristics of Schwann cells, including the synthesis of 2-hydroxy galactolipids and myelin proteins (18, 23). For stable RNAi, D6P2T cells were transfected with a FA2H-specific shRNA expression plasmid or a control shRNA expression plasmid. The retroviral elements within the plasmid vector facilitate efficient integration into the host genome, conferring puromycin resistance to the cells. By culturing transfected cells in the presence of puromycin for 2 weeks, polyclonal stably transfected cells were ob-



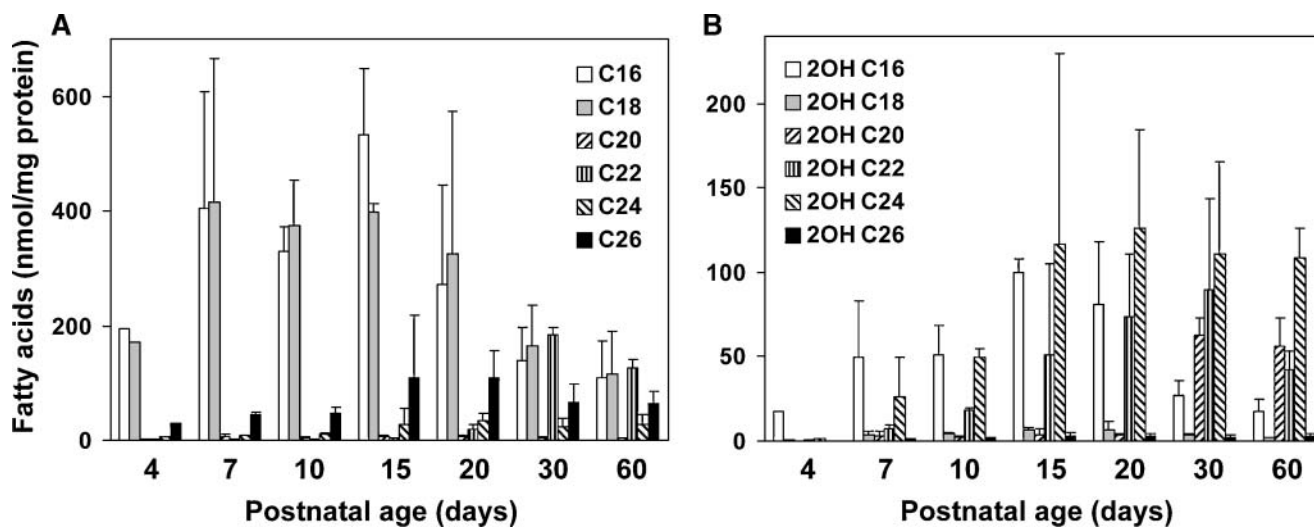
**Fig. 4.** Fatty acid compositions of rat sciatic nerve galactosylceramide (GalCer). Total lipids were extracted from sciatic nerve. GalCer were purified by TLC, and fatty acid compositions were determined by GC-MS. Means  $\pm$  SD of three to four animals are shown, except at day 4, which represents a pool of sciatic nerves from four animals. In all samples, C<sub>18:1</sub> and C<sub>24:1</sub> fatty acids were not detectable. Graph A shows nonhydroxy fatty acids and graph B shows 2-hydroxy fatty acids.

tained. FA2H shRNA expression resulted in an  $\sim$ 40% reduction in total cellular 2-hydroxy fatty acids, with no effect on nonhydroxy fatty acids (Fig. 7A). The effects on total 2-hydroxy fatty acids were very similar to the effects seen with FA2H siRNA transfection (Fig. 8). Therefore, the reduction of 2-hydroxy fatty acids is not likely an artifact caused by the integration of the plasmid into the genome or by selection with puromycin. Subsequently, cellular lipids were metabolically labeled with [<sup>14</sup>C]acetate for 48 h to measure GalCer produced in these cells. As shown in Fig. 7B, 2-hydroxy GalCer was reduced 36% by FA2H shRNA expression, with no effect on nonhydroxy

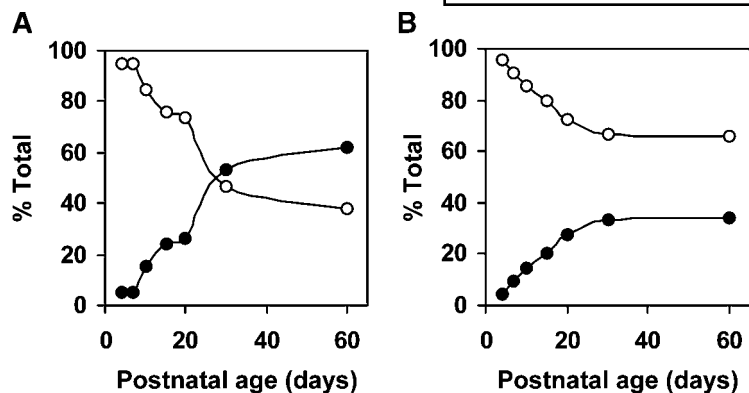
GalCer. These data provide direct evidence that FA2H is required for the formation of 2-hydroxy GalCer.

#### 2-Hydroxy lipids may play a role in the regulation of cell migration

Enhanced expression of surface lipid antigens present in myelinating cells, such as GalCer, is a key feature of cAMP-mediated Schwann cell differentiation (17). Our results further indicate that these changes in myelin lipid contents also correlate with the enhanced expression of FA2H in differentiated Schwann cells (Fig. 2). Because 2-hydroxy lipids likely have a role in the tight packing of lipids re-



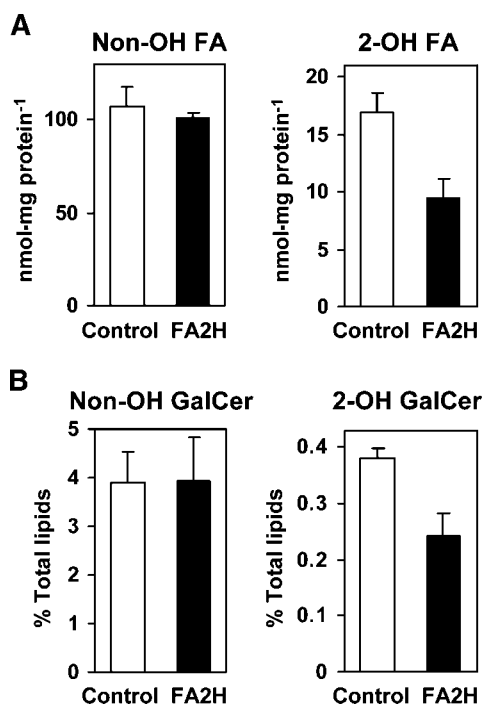
**Fig. 5.** Fatty acid compositions of rat sciatic nerve sulfatide. Total lipids were extracted from sciatic nerve. Sulfatides were purified by TLC, and fatty acid compositions were determined by GC-MS. Means  $\pm$  SD of three to four animals are shown, except at day 4, which represents a pool of sciatic nerves from four animals. In all samples, C<sub>18:1</sub> and C<sub>24:1</sub> fatty acids were not detectable. Graph A shows nonhydroxy fatty acids and graph B shows 2-hydroxy fatty acids.



**Fig. 6.** Relative contents of 2-hydroxy and nonhydroxy galactolipids of postnatal rat sciatic nerve. The 2-hydroxy (closed circles) and nonhydroxy (open circles) fatty acid contents were expressed as percentages of total fatty acids in purified GalCer (A) and sulfatide (B).

quired for membrane compaction during myelin sheath formation, a possible consequence of altered levels of *FA2H* expression would be a change in cell membrane fluidity and therefore cell migration capability. Therefore, we tested whether *FA2H*-directed RNAi alters migratory processes in D6P2T cells. Transfection of *FA2H* siRNA decreased *FA2H* activity by 60% compared with transfection of control siRNA (Fig. 8A). Accordingly, total 2-hydroxy fatty acids were decreased by ~60% compared with controls (Fig. 8B). As mentioned above, these results are in close agreement with results obtained with shRNA expression

(Fig. 7A). Surprisingly, we found that cell migration in response to serum was enhanced by ~2.8-fold with *FA2H* siRNA treatment compared with control siRNA-transfected cells (Fig. 8C). The migration of cells treated with transfection reagent without siRNA was indistinguishable from that of control siRNA-treated cells. Very similar enhancement of cell migration was also observed with *FA2H* shRNA expression (data not shown). These observations suggest that, in addition to their structural role in the compaction of myelin membranes, 2-hydroxy lipids may also greatly influence the migratory properties of Schwann cells.



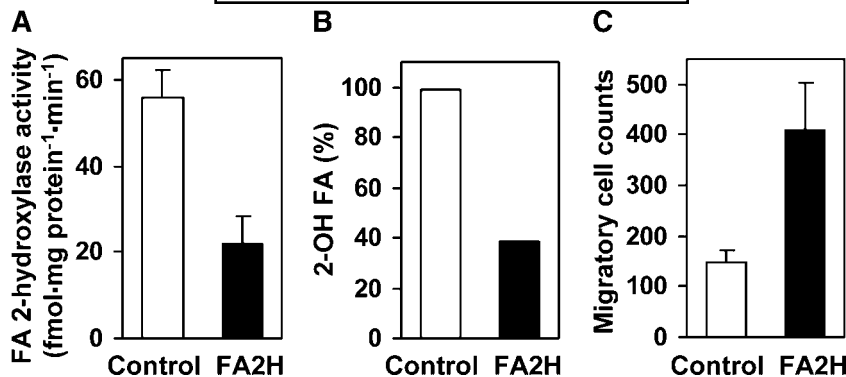
**Fig. 7.** *FA2H*-directed RNA interference (RNAi) reduces cellular 2-hydroxy fatty acids and 2-hydroxy GalCer. D6P2T cells were transfected with control short-hairpin RNA (shRNA) or *FA2H* shRNA expression plasmids. Transfected cells were selected for puromycin resistance (1  $\mu$ g/ml) for 2 weeks. A: Total nonhydroxy and 2-hydroxy fatty acids were measured by GC-MS after hydrolysis of all cellular lipids. B: Cells were metabolically labeled with [<sup>14</sup>C]acetate for 48 h. Nonhydroxy and 2-hydroxy GalCers were isolated by TLC, and radioactivity was measured by liquid scintillation counting. Means  $\pm$  SD of triplicate samples are shown.

## DISCUSSION

Here, we provide evidence that *FA2H* is required for the formation of 2-hydroxy galactolipids in rat PNS myelin and that 2-hydroxy lipids may have a regulatory role in cell migration. Our data show that 1) *FA2H* expression in rat sciatic nerve is upregulated during developmental myelination; 2) *FA2H* is expressed in cultured Schwann cells and is highly upregulated in response to differentiation signals, such as cAMP; 3) the fatty acid compositions of rat sciatic nerve galactolipids change dramatically during myelination, such that increasing proportions of 2-hydroxy fatty acids and very long-chain fatty acids are incorporated; 4) the synthesis of 2-hydroxy GalCer in D6P2T Schwannoma cells is reduced by the expression of *FA2H* shRNA; and 5) cell migration is enhanced when cellular 2-hydroxy lipids are reduced as a consequence of *FA2H* siRNA or shRNA. Together, these data demonstrate the direct link between *FA2H* activity and the synthesis of 2-hydroxy galactolipids in the PNS. The data also indicate that 2-hydroxy lipids are not merely structural lipids of myelin, as thought previously, but may also regulate cell migration.

Previous studies showed that *FA2H* is highly expressed in human brain (24) and upregulated during myelination in mouse brain (15, 25). *FA2H* catalyzes the 2-hydroxylation of free fatty acids to provide precursors of myelin 2-hydroxy galactolipids (15). Biochemical characteristics of *FA2H* indicate that it is the same enzyme as the rat brain fatty acid  $\alpha$ -hydroxylase, studied extensively by Kishimoto, Akanuma, and Singh (for review, see Ref. 26). Previously, fatty acid  $\alpha$ -hydroxylase activities could not be detected in any rat tis-





**Fig. 8.** *FA2H*-directed RNAi enhances D6P2T cell migration. Cells were transfected with control or *FA2H* small interfering RNA and harvested after 72 h. A: *FA2H* activities in whole cell lysates. Means  $\pm$  range of two measurements are shown. B: Total 2-hydroxy fatty acids were measured by GC-MS after hydrolysis of all cellular lipids. The combined quantities of the three major 2-hydroxy fatty acids (2-hydroxy C<sub>16</sub>, C<sub>18</sub>, and C<sub>24</sub>) are shown. C: Transfected cells (50,000 per insert) were placed in cell culture inserts and allowed to migrate for 4 h before staining. Means  $\pm$  SD of triplicate cell countings are shown.

sues outside of the brain (27), and a question remained whether the same enzyme was responsible for the synthesis of myelin 2-hydroxy galactolipids in the PNS. The new method to measure *FA2H* activity provided improved sensitivity and enabled us to characterize *FA2H* in most biological samples, including tissue culture cells (20) and cultured human keratinocytes (28). To our knowledge, this is the first report on *FA2H* in the sciatic nerve. The data shown here indicate that the synthesis of 2-hydroxy galactolipids in PNS myelin is *FA2H*-dependent, as in the CNS.

In both CNS and PNS, 2-hydroxy fatty acid contents in galactolipids are very low at the onset of myelination and increase rapidly during developmental myelination. Fatty acid chain length also changes from long-chain (C<sub>16</sub>–C<sub>20</sub>) to very long-chain (>C<sub>20</sub>) fatty acids during myelination. Currently, the physiological significance of these transitions is not fully understood. Previous biophysical studies indicate that the 2-hydroxyl group of ceramide-linked *N*-acyl chains can facilitate lipid packing via additional hydrogen bonds. In addition, longer acyl chains would enhance hydrophobic interaction among lipids. Therefore, it is conceivable that the transition of the *N*-acyl chains of myelin galactolipids may confer progressively higher membrane rigidity during the course of myelination, which may be necessary for the proper organization of myelin proteins and myelin compaction. The recent findings by Fewou et al. (14) are consistent with this hypothesis. In CGT transgenic mice, total myelin galactolipid levels were not altered significantly, but the proportion of 2-hydroxy GalCer was reduced compared with that in wild-type mice. Myelin of these mice was unstable and uncompacted, and the mice developed progressive hindlimb paralysis and demyelination (14). These findings indicate that the fatty acid compositions of galactolipids are critical for myelin stability.

The biochemical basis for the increase in 2-hydroxy galactolipids during myelination is now explained, at least in part, by the upregulation of *FA2H* in oligodendrocytes and Schwann cells. The mechanism for the developmental changes in fatty acid chain length is less clear. In the cer-

amide biosynthetic pathway, several reactions are catalyzed by multiple enzyme isoforms. For instance, there are at least six isoforms of fatty acid elongases, encoded by *Elovl1*–*Elovl6*, each of which shows distinct tissue-specific expression (29). Similarly, there are at least six isoforms of dihydroceramide synthases (*Lass*/*CerS*) with distinct substrate specificities (30). The isoforms of these enzymes involved in the synthesis of myelin galactolipids are unknown. The remarkable diversity and developmental transition of galactolipid molecular species during myelination are likely achieved by the coordinated regulation of specific *Elovl* gene(s) and *Lass*/*CerS* gene(s) along with *FA2H*.

2-Hydroxy fatty acids are highly abundant in the nervous tissue as components of myelin galactolipids. A number of nonneural tissues also contain 2-hydroxy fatty acids as components of various sphingolipids, although at much lower concentrations. Examples include ceramide in the epidermis (31), sphingomyelin in kidney, intestine, and spermatozoa (32–34), and various glycosphingolipids in liver and intestine (35–37). In all of these tissues and organs, the physiological roles of 2-hydroxy lipids are not understood beyond a hypothetical role as structural lipids that confer physical stability to the membrane. Recently, we demonstrated that *FA2H*-dependent synthesis of 2-hydroxy ceramide/GlcCer is required for the proper formation and secretion of lipid lamellar bodies in differentiating human keratinocytes, providing the first evidence that 2-hydroxy lipids may regulate complex cellular processes (28). The current study also presents the intriguing possibility that 2-hydroxy lipids greatly influence cell migration. This may be attributable to their ability to alter membrane fluidity. Alternatively, 2-hydroxy lipids may influence the lipid/protein organization within sphingolipid- and cholesterol-rich microdomains, or lipid rafts. Lipid rafts play critical roles in neural development (38), and a number of myelin proteins involved in cell signaling and adhesion are associated with lipid rafts (39). Interestingly, the raft association of Plp, the major myelin protein in the CNS, is disrupted in the absence of GalCer, indicating that myelin



galactolipids play a major role in the proper organization of myelin proteins in lipid rafts (40). It is conceivable that 2-hydroxy lipids influence lipid raft constituents, thereby modulating cell adhesion and migratory properties. Our findings warrant further study of the regulation of cell migration by 2-hydroxy lipids. ■

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