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Acetoacetyl-CoA synthetase is essential for normal neuronal development

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ABSTRACT

Cholesterol and fatty acids are essential, abundant components of neuronal tissue. Acetoacetyl-CoA synthetase (AACS) is a ketone body-utilizing enzyme for the synthesis of cholesterol and fatty acids and is highly expressed in the brain. In this study, we investigated the regulation of AACS during neurite outgrowth to clarify the physiological role of AACS in neurogenesis. Messenger RNA levels and the expression of AACS were increased during neurite outgrowth in Neuro-2a cells. The expression of HMG-CoA reductase, a key enzyme of cholesterol biosynthesis, was also increased. ChIP assays showed that the amount of SREBP-2, a key transcription factor of cholesterol synthesis, interacted with the AACS promoter was increased during neurite outgrowth, and knockdown of SREBP-2 down-regulated the mRNA levels of AACS in Neuro-2a cells. The expression of AACS in the brains of mouse embryos was dramatically increased between E16.5 and E18.5. Moreover, knockdown of AACS in primary neurons caused decreases in the expression of MAP-2 and NeuN, which are markers of neuronal differentiation, as well as synaptopodin, a marker of spine apparatus. These results suggest that AACS is regulated by SREBP-2 and involves in the normal development of neurons.

1. Introduction

Cholesterol and fatty acids are critical constituents of cellular membranes and are found at particularly high concentrations in neural tissue [1]. They play a key role in embryonic and fetal development, especially in neuronal development [2,3]. The generation of acetyl-CoA in the cytosol is the first step in lipid biosynthesis. Therefore, acetyl-CoA is a crucial substrate for the cholesterol and fatty acids synthesis pathway. Cytosolic acetyl-CoA is produced by ATP-citrate lyase (ACL), which cleaves citrate to produce acetyl-CoA and oxaloacetate in the cytosol [4,5]. Acetyl-CoA is also produced from acetoacetyl-CoA by the action of cytosolic acetoacetyl-CoA thiolase [6].

Ketone bodies, including acetoacetate and β-hydroxybutyrate, are known to be significant energy sources for various tissues. This is particularly true for the brain, which has no substantial non-glucose-derived energy source [7]. Recent studies showed that ketone bodies are involved in insulin secretion and neonatal metabolic homeostasis, suggesting that they play an important role not only in the fasting state but also in the normal state [8,9]. Mitochondrial succinyl-CoA:3-ketoacid CoA transferase (SCOT; EC 2.8.3.5) is the enzyme thought to be responsible for ketone body utilization for energy production [10]. However, it has been shown that acetoacetate is activated to its CoA ester by a cytosolic acetoacetate-specific ligase, acetoacetyl-CoA synthetase (AACS, acetoacetate-CoA ligase, EC 6.2.1.16), for the direct production of acetyl-CoA, which is then used for the synthesis of physiologically important lipidic substances, such as cholesterol and fatty acids, in the cytosol [11]. We purified AACS as a discrete enzyme from the bacterial strain *Zoogloea ramigera* I-16-M [12] and from rat liver for the first time [13], and we demonstrated that its activity in rats is remarkably changed under certain physiological or pathological conditions, such as development [14], pravastatin administration [15], and streptozotocin (STZ)-induced diabetes [16].

Recently, we found that AACS mRNA was highly expressed in the brains of humans and rats and enriched in neuronal-like cells in the hippocampus and cortical regions [17,18], where the localization profile of AACS mRNA was different from that of SCOT mRNA. These results suggest that ketone body utilization via AACS is an important pathway in neural tissue. To clarify the physiological role of AACS in neurons, we examined the expression of AACS during neurite outgrowth and embryogenesis and the effects of AACS knockdown on neuronal development.

2. Materials and methods

2.1. Cell culture

Mouse Neuro-2a cells (JCRB No. IFO50081) were purchased from the Health Science Research Resources Bank (Japan). The cells

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were maintained in Eagle's minimal essential medium (E-MEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) at 37 °C in an atmosphere of 5% CO_2. Cells were seeded at 5.0×10^4 cells/mL, incubated for 24 h, and then treated with serum-free E-MEM containing retinoic acid (Sigma).

2.2. Analysis of RNA

Total RNA was purified from Neuro-2a cells using an RNeasy mini kit (Qiagen) or an Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare). Total RNA from Neuro-2a cells was analyzed by real-time RT-PCR as described previously [19]. cDNA and gene-specific primers were added to the SYBR Green PCR Master Mix (SYBR Premix Ex Taq, Takara) and subjected to PCR amplification in an Applied Biosystems StepOne (Applied Biosystems). The amplified transcripts were quantified using the standard curve method with β-glucuronidase (Gusb) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal controls. The real-time PCR primers were designed based on data from GenBank (according to accession numbers) using Primer Express software (Applied Biosystems).

2.3. Western blot analysis

Mouse hepatocytes were lysed in RIPA buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 1% sodium lauryl sulfate and 0.1% sodium deoxycholate]. Cell debris was removed by centrifugation at 14,000*g* for 15 min at 4 °C, and the resulting supernatant (cell lysate) was used for Western blot analysis. Protein concentrations in the cell lysates were measured using a Bio-Rad protein assay kit.

For Western blotting, 15 μg of protein was separated using SDS polyacrylamide gel electrophoresis (SDS–PAGE, 7.5% gel) and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were probed with specific antibodies and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. HRP was detected with Immobilon Western Chemiluminescent HRP Substrate (Millipore).

2.4. Microscopic procedures

Neuro-2a cells were grown on cover glasses. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min. The cells were permeabilized with 0.1% (v/v) Triton X-100 for 5 min and blocked with 3% (w/v) bovine serum albumin (BSA) for 30 min. After blocking, the cells were incubated overnight at 4 °C in Can Get Signal immunostain solution A (Toyobo) containing anti-AACS antibody. Then, the cells were washed three times with 0.1% BSA in PBS and incubated with Alexa Fluor 488 secondary antibody (Invitrogen) at room temperature for 1 h in the dark. After being washed in 0.1% BSA in PBS, the cells were covered with Mowiol mounting solution.

For DNA visualization, preparations were stained with propidium iodide (Calbiochem). After incubation with the secondary antibody, the cells were incubated with 1 mg/mL RNase in PBS. Then, the cells were stained with propidium iodide for 15 min. After being washed in 0.1% BSA in PBS, the cells were covered with Mowiol mounting solution. Digital images of fixed cells were taken with a Confocal Laser Scanning Microscope (Bio-Rad).

2.5. Chromatin immunoprecipitation (ChIP) assays

ChIP assays were carried out as described previously [20]. Briefly, Neuro-2a cells were fixed in normal culture medium with formaldehyde at a final concentration of 1% for 10 min at 37 °C. After centrifugation, the cells were suspended in 50 mM Tris–HCl (pH 8.0) containing 1% SDS, 10 mM EDTA and protease inhibitor cocktail (Sigma). Chromatin was sheared by sonication on ice. After centrifugation to remove the cell debris, the supernatant was diluted with 50 mM Tris–HCl (pH 8.0) containing 167 mM NaCl, 1.1% Triton X-100, 0.11% sodium deoxycholate and protease inhibitor cocktail (Sigma). Aliquots of the supernatant were incubated for 15 min at 4 °C with protein G-sepharose beads (GE Healthcare). Immunoprecipitation was carried out overnight at 4 °C with the following antibodies (1 μg): anti-normal rabbit IgG (Santa Cruz Biotechnology) and anti-SREBP-2 (Abcam). The immunoprecipitated DNA–protein complex was eluted with 200 μL of 10 mM Tris–HCl (pH 8.0) containing 300 mM NaCl, 5 mM EDTA and 0.5% SDS. DNA-protein cross-links were reversed at 65 °C for 4 h and then subjected to PCR amplification. The sequences of the PCR primers included the following: AACS, forward 5′-GAATGAACGAACGAACGAGG-3′ and reverse 5′-ACCACGCCCTCTTCTGTAAC-3′.

2.6. RNA interference

To knock down SREBP-2 gene expression, cells were transfected with a validated pool of siRNA duplexes directed against mouse SREBP-2 (Dharmacon). Neuro-2a cells were transfected with the indicated siRNAs (40 nM) using LipofectAMINE RNAiMAX Reagent (Invitrogen). After 24 h, total RNA was extracted and analyzed using real-time PCR.

2.7. Primary neuron

Mouse primary cortical cultures were prepared from gestational day 14 embryos as described previously with minor modifications [21]. Briefly, brains were dissected and digested for 20 min in 15 units papain (Worthington) and 0.01% DNase1 (Roche). Cells were cultured in Neurobasal medium (Miltenyi Biotec) containing 2% MACS Supplement B-27 (Miltenyi Biotec), 2 mM L-glutamine (Invitrogen) and penicillin/streptomycin (Invitrogen) on plastic dishes coated with poly-L-lysine. After 3 days, the cells were exposed to 4 μM Cytosine β-D-arabinofuranoside to inhibit the proliferation of non-neuronal cells.

2.8. Virus production and transduction

Lenti-X 293T cells (Takara) were seeded in 100 mm poly-L-lysine plates (Iwaki) and transfected with pGreenPuro shRNA vector (System Biosciences) and packaging mix (Invitrogen) according to the manufacturer's protocols. The media were replaced with 10% FBS/D-MEM at approximately 14 h post-transfection, and the viral supernatants were collected 48 h after transfection.

To knock down AACS expression, primary neurons were infected with lentiviruses encoding shRNA sequences against 2 different mouse AACS sequences (shAACS #1, GTTCAGTGGAATCGTCTAC; shAACS #2, CCGTGTGGTCGGCTATCTA) or with control viruses encoding shRNA sequences against pGL3 (shcontrol #1, CTTACGCTGAGTACTTCGA) and LacZ (shcontrol #2, ATCGCTGATTTGTGTAGTC). After 2 days, the culture media were replaced. Subsequently, the medium was changed every 5 days, and protein was extracted at the indicated time.

3. Results

3.1. Induction of a ketone body-utilizing enzyme during neurite outgrowth

Previously, we demonstrated that AACS is highly expressed in the brains of mice and rats and showed neuron-specific expression of the AACS gene in the cerebral cortex [18,20]. These results suggest the involvement of ketone body utilization in neuronal functions. To clarify the physiological role of AACS in neurons,

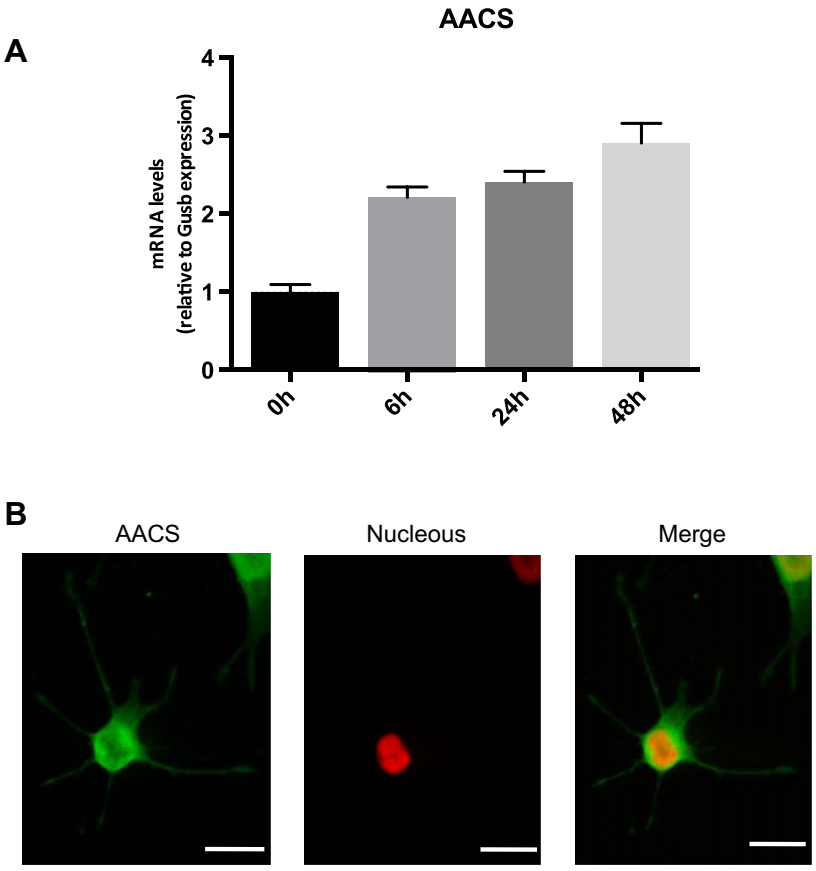

Fig. 1. Expression and localization of AACS during neurite outgrowth in Neuro-2a cells. (A) Cells were seeded and incubated for 24 h and then cultured in serum-free E-MEM containing 10 μM all-trans retinoic acid. The numbers indicate the hours at which the cells were harvested. AACS and Gusb were detected by real-time PCR. Gusb was used as an internal control. (B) Neuro-2a cells were stained at 48 h with anti-AACS and Alexa Fluor 488 secondary antibody (green). For the visualization of DNA, preparations were stained with propidium iodide (red) as described in Section 2. Scale bar = 50 μm.

we examined the time course of AACS gene and protein expression during neurite outgrowth in mouse Neuro-2a neuroblastoma cells. AACS gene and protein expression was markedly increased during neurite outgrowth (Figs. 1A and S1). Next, we analyzed the localization of AACS in Neuro-2a cells. Fig. 1B shows that AACS was localized near the nucleus and at the growth cone. These results suggest that AACS plays an important role in neurite outgrowth and neuronal function.

Next, we analyzed the mRNA levels of ketone body utilization-related genes during neurite outgrowth using real-time PCR. The expression of SCOT, which is responsible for ketone body utilization for energy production, was unchanged, suggesting that ketone body utilization during neurite outgrowth primarily contributes to the synthesis of lipid substrate rather than to energy production (Fig. S2). Then, we examined the expression of fatty acid synthesis-related genes, such as ACL, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). The expression of FAS was increased at the initiation step of neurite outgrowth; however, no marked difference in the gene expression of ACC-1, which is a rate-limited enzyme of fatty acid synthesis, ACC-2 or ACL was observed. In contrast, cholesterol synthesis-related genes, such as HMG-CoA reductase (HMGCR) and HMG-CoA synthase 1 (HMGCS1), were significantly increased during neurite outgrowth (Fig. 2). These results suggest that ketone bodies are mainly utilized for cholesterol synthesis during neurite outgrowth.

3.2. Identification of a transcription factor regulating AACS gene expression in Neuro-2a cells

To investigate the regulation of AACS gene expression during neurite outgrowth, we next assessed protein recruitment to the promoter region of AACS. ChIP assays revealed that interactions between SREBP-2 and the AACS promoter or the HMGCR promoter increased following neurite outgrowth (Fig. 3A). These results indicate that SREBP-2 is an important factor for gene regulation of AACS in Neuro-2a cells. Next, we examined whether SREBP-2 regulates AACS expression. The gene expression of SREBP-2 was effectively reduced by treatment with siSREBP-2 (Fig. S3). The knockdown of SREBP-2 resulted in a significant reduction in AACS (by 31%) and HMGCR (by 30%) mRNA levels (Fig. 3B) but not in ACL mRNA levels (Fig. S3). These results indicate that SREBP-2 regulates AACS expression during neurite outgrowth in Neuro-2a cells.

3.3. Protein expression of AACS during neurogenesis

To investigate protein expression of AACS in the brains of mouse embryos, we dissected mouse brains at embryonic day 14.5 (E14.5), E16.5 and E18.5. As shown in Fig. 4A, the protein expression of AACS was dramatically increased on E16.5 and E18.5. SCOT, the other ketone body-utilizing enzyme, was slightly increased at E16.5 and E18.5 compared to E14.5 (Fig. S4). The levels of fatty acid synthesis-related enzymes, such as ACL and FAS, were not altered during neurogenesis (Fig. S4). In contrast, the levels of HMGCR were dramatically increased during neurogenesis. To determine whether AACS has a role during neurogenesis, we examined the expression of neuronal markers in the brains of embryos (Fig. 4A). The expression of neuronal nuclei (NeuN) and microtubule-associated protein 2 (MAP-2) was increased at E18.5. Moreover, the expression of synaptopodin, an essential component of spine apparatus, was increased at E18.5. These results suggest that

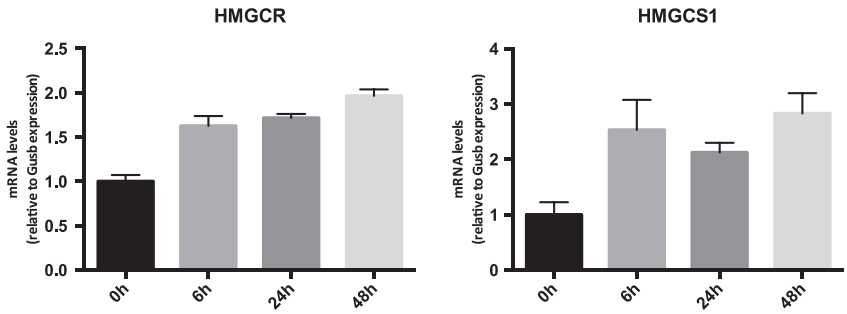

Fig. 2. Gene expression of lipogenic factors during neurite outgrowth in Neuro-2a cells. Cells were seeded and incubated for 24 h, and then cultured in serum-free E-MEM containing 10 μM all-trans retinoic acid. The numbers indicate the hours at which the cells were harvested. Gene expression of lipogenic enzyme s was detected by real-time PCR.

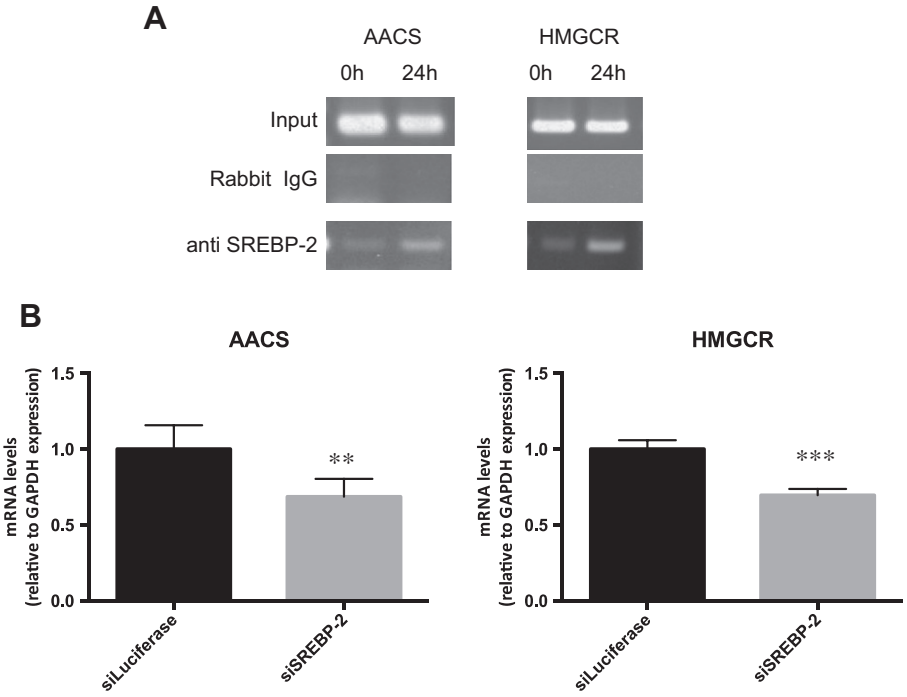

Fig. 3. Association of SREBP-2 with the promoter region of the AACS gene. (A) Cross-linked DNA was prepared from undifferentiated Neuro-2a cells (0 h) and differentiated Neuro-2a cells (24 h). ChIP assays were performed using the indicated antibodies, and recovered DNA was subjected to PCR amplification using primers specific to the proximal promoter region of the AACS gene or the HMGCR gene. A small aliquot was removed before immunoprecipitation and used for PCR amplification as an input control (Input). (B) The expression of AACS or HMGCR mRNA was analyzed by real-time PCR. Gene expression was normalized to that of GAPDH. The data are shown as the mean ± S.D. ($n = 4$). The average expression values in the control are indicated as 1.0. $^{**}P < 0.01$; $^{***}P < 0.001$ between siLuciferase (siLuc) and siSREBP-2-treated Neuro-2a cells.

AACS contributes to cholesterol synthesis in the embryo and has an important role in neurogenesis, especially dendritic outgrowth.

3.4. Effect of AACS knockdown on neuronal markers in primary neuron

To elucidate the physiological roles of AACS, we performed knockdown experiments using mouse primary neurons (Fig. 4B). Primary neurons were infected with lentivirus particles including short hairpin RNAs targeting AACS (shAACS#1 or shAACS#2) at 1 day in vitro (DIV). Western blotting showed that the expression of AACS was abolished in shAACS#1- and shAACCS#2-infected cells at 15 DIV. The protein expression levels of NeuN and MAP-2, which are markers of neurogenesis, were decreased in the shA-ACS groups compared to control groups. Moreover, the expression of synaptopodin, an essential component of spine apparatus, was decreased in shAACS-treated cells. These results suggest that AACS plays a pivotal role in neurogenesis.

4. Discussion

Fetal cholesterol originates mainly from endogenous de novo synthesis rather than from placental transfer during late gestation [22]. Ketone bodies can cross the placenta either by diffusion or by a low-specificity carrier-mediated process and can be used as fuels and lipogenic substrates by the fetus [22–24]. In the cerebellum, AACS mRNA expression was restricted primarily to glial cells, while in the cerebral cortex, it was restricted to neuronal cells. These results suggest that AACS fulfills an important role in neurogenesis and neuronal function. In this study, we found that the expression of AACS was increased during neurite outgrowth and was localized

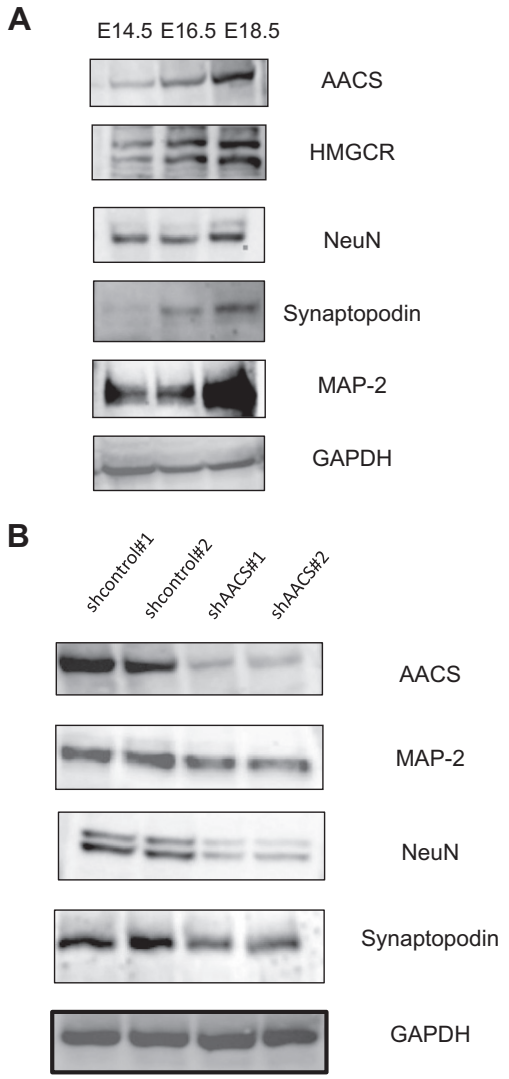

Fig. 4. Effects of shAACS on expression of neuronal markers in primary neurons. (A) Brain tissue was dissected from mouse embryos at E14.5, 16.5 or 18.5. Protein was extracted from the brain tissue, and the expression of AACS and other proteins was assessed by Western blotting. (B) Effects of shAACS on protein expression of neuronal markers in primary neurons. Primary neurons were infected with shcontrol- or shAACS-targeted lentivirus at 2 DIV. Protein was extracted from the cultures at 15 DIV. The expression of AACS, MAP-2, NeuN, synaptopodin and GAPDH was analyzed by Western blotting. GAPDH was used as an internal control.

near nuclei and at growth cones. Moreover, knockdown of AACS decreased expression of neuronal markers such as NeuN and MAP-2. These results indicate that ketone body utilization via AACS is an important pathway in neurogenesis.

The expression pattern of AACS was similar to that of HMGCR rather than that of ACC-1 during neurite outgrowth (Figs. 1, 2 and S2). These data imply that ketone bodies are utilized for cholesterol synthesis during neurite outgrowth. Moreover, knockdown of AACS significantly reduced the expression of NeuN, a marker of neural development, and slightly decreased the expression of MAP-2, a marker of dendritic outgrowth (Fig. 4B). Previous studies showed that cholesterol synthesis is important for neurite outgrowth and neuronal function [25–27]. Additionally, ketone bodies are incorporated into myelin and synaptosomal fractions [28]. Therefore, ketone body utilization via AACS may be important for action potential conduction or synaptic transmission. Furthermore, AACS protein was localized at the growth cone (Fig. 1B). The morphology of the growth cone is organized by actin formation, and small GTPases of the Rho family, such as Rac1 and Cdc42, are positive regulators that promote neurite outgrowth and growth cone protrusion [29,30]. Ketone body utilization via AACS may provide acetyl units for the mevalonate pathway, which in turn may be involved in the establishment of neuronal morphology.

ChIP assays and siRNA experiments revealed that AACS is transcriptionally regulated during neurite outgrowth by SREBP-2, a master regulator of cholesterol synthesis (Fig. 3A and B). Previous studies have shown that knockdown of SREBP-2 causes a decrease in the expression of markers of synapse formation, and reduction of SREBP-2 in the hypothalamus using shRNA results in increased feeding and weight gain [27]. We previously found that mRNA levels of AACS are reduced in the brains of mice treated with streptozotocin [18]. In addition, knockdown of AACS caused a marked reduction of synaptopodin (Fig. 4B), which is closely related to the formation of the spine apparatus and to hippocampal long-term potentiation [31]. These results suggest that ketone body utilization for cholesterol synthesis via AACS has an important role in neuronal function, especially synapse formation and appetite regulation.

In summary, we found that the expression of AACS was increased during neurite outgrowth and was transcriptionally regulated by SREBP-2. In addition, knockdown of AACS affected expression levels of neuronal markers, such as NeuN, synaptopodin and MAP-2. Our findings provide new insights into the importance of ketone body utilization during neuronal development.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.076.

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