

FABP7 expression in normal and stab-injured brain cortex and its role in astrocyte proliferation

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Abstract Reactive gliosis, in which astrocytes as well as other types of glial cells undergo massive proliferation, is a common hallmark of all brain pathologies. Brain-type fatty acid-binding protein (FABP7) is abundantly expressed in neural stem cells and astrocytes of developing brain, suggesting its role in differentiation and/or proliferation of glial cells through regulation of lipid metabolism and/or signaling. However, the role of FABP7 in proliferation of glial cells during reactive gliosis is unknown. In this study, we examined the expression of FABP7 in mouse cortical stab injury model and also the phenotype of FABP7-KO mice in glial cell proliferation. Western blotting showed

that FABP7 expression was increased significantly in the injured cortex compared with the contralateral side. By immunohistochemistry, FABP7 was localized to GFAP⁺ astrocytes (21% of FABP7⁺ cells) and NG2⁺ oligodendrocyte progenitor cells (62%) in the normal cortex. In the injured cortex there was no change in the population of FABP7⁺/NG2⁺ cells, while there was a significant increase in FABP7⁺/GFAP⁺ cells. In the stab-injured cortex of FABP7-KO mice there was decrease in the total number of reactive astrocytes and in the number of BrdU⁺ astrocytes compared with wild-type mice. Primary cultured astrocytes from FABP7-KO mice also showed a significant decrease in proliferation and omega-3 fatty acid incorporation compared with wild-type astrocytes. Overall, these data suggest that FABP7 is involved in the proliferation of astrocytes by controlling cellular fatty acid homeostasis.

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Introduction

Polyunsaturated fatty acids (PUFAs) have important metabolic, structural and signal transducing roles. PUFAs are abundant in the developing brain and are essential for normal development of the brain. Several studies have suggested a negative correlation between dietary consumption of PUFAs and risk of several neuropsychiatric diseases such as multiple sclerosis (Esparza et al. 1995; Lauer 1994; Swank et al. 1952), Parkinson disease (de Lau et al. 2005), Alzheimer disease (Barberger-Gateau et al. 2002; Morris et al. 2003), schizophrenia (Fedorova et al. 2009; Amminger et al. 2010), depression, other psychotic and mood disorders and developmental disorders like

ADHD and autism (Muskiel and Kemperman 2006; Hedelin et al. 2010). Therapeutic effects of PUFAs have also been suggested even in these diseases, and PUFA supplements are currently recommended to these patients as alternative or complementary medicine (Peet et al. 2001; Nordvik et al. 2000; Weinstock-Guttman et al. 2005). Nevertheless, there is limited evidence to support preventive and therapeutic effects of PUFAs (Ross et al. 2007; Mehta et al. 2009).

Fatty acid-binding proteins (FABPs) are intracellular low-molecular-weight (14–15 kDa) polypeptides and are key molecules in the uptake, transportation and storage of long-chain fatty acids and in mediating their signal transduction and gene regulation activities. Twelve members of the FABP family have been recognized so far, which are differentially expressed in different organs, tissues and cell types. FABP7 (brain-type FABP), a strong binder of omega-3 PUFAs, is abundantly expressed in neural stem cells and astrocytes of the developing brain, suggesting a role in glial cell differentiation and proliferation of glial cells (Owada et al. 1996; Maekawa et al. 2011). However, the functional significance of FABP7 expression in cortical astrocytes under normal and pathological conditions remains unknown.

Reactive gliosis, whereby astrocytes and other types of glial cells undergo massive proliferation, is a common hallmark of numerous brain pathologies. However, the underlying mechanisms of gliosis are largely unknown. Reactive gliosis consists of a range of molecular and morphological changes, especially in astrocytes, including cellular hypertrophy, alteration in molecular expression, proliferation and scar formation. Recently, it was reported that docosahexaenoic acid (DHA), a ligand of FABP7, prevents degradation of neurons and astrocytes following brain ischemia and spinal cord injury by protection of neurons and astrocytes from cell degeneration (Belayev et al. 2011), suggesting a role for FABP7 in regulating CNS injury. Furthermore, there is an association of FABP7 with poor prognosis of malignant glioma (Godbout et al. 1998; Tso et al. 2006; Liang et al. 2005; Kaloshi et al. 2007), providing support for a role of FABP7 in astrocytes responses to injury. FABP7 is also abundantly expressed in neural stem cells and plays a role in maintenance of neuroepithelial cells during early developmental stages (Arai et al. 2005), and as such, may be important in adult neural cell progenitors, which share some characteristics of neural stem cells.

In the present study, we examined the detailed localization of FABP7 in the normal and stab-injured brain cortex of adult mice and determined its role in astrocyte proliferation *in vivo* and *in vitro* using FABP7 knockout (KO) mice (Owada et al. 2006). We found that FABP7 regulated the proliferative response of astrocytes to injury.

Furthermore, we observed novel expression of FABP7 in oligodendrocyte progenitor cells (OPCs).

Materials and methods

Animals

Three-month-old male C57BL/6 mice (14 wild-type and 14 FABP7-KO) (Owada et al. 2006) were used for brain cortex stab injury experiments. All experimental protocols were reviewed by the Ethics Committee for Animal Experimentation of Yamaguchi University School of Medicine and were performed according to the Guidelines for Animal Experimentation of the Yamaguchi University School of Medicine and Under Law and Notification requirements of the Japanese Government.

Stab injury model

Animals were anesthetized (Isoflurane; Abbott, IL, USA) using a small animal anesthetizer (Muromachi Co., Tokyo, Japan) and a 1-mm-deep stab wound was made on the right cerebral neocortex (Bregma, -1 to -2.5 mm. Latero-lateral, 1.5–2.5 mm) (Buffo et al. 2005) (see Supplementary Fig. 1a, e). Mice were killed at 3 ($n = 7$ for wild-type mice, $n = 7$ for FABP7-KO mice) and 7 ($n = 7$) days post lesion (DPL).

Cell culture

For primary cultured astrocytes, the cerebral cortices of newborn mice (wild-type and FABP7-KO) were dissected, and the meninges were carefully removed. Tissues were cut into small pieces in HBSS (Hanks buffer salt solution) supplemented with glucose, penicillin and streptomycin and further dissociated by trypsin treatment (0.25%) at 37°C for 20 min. Debris was removed by filtering through a 100- μ m mesh, and the cell suspension was centrifuged at 1,000 rpm and 20°C for 5 min. The supernatant was removed and the pellet was resuspended in DMEM supplemented with 10% FBS, penicillin, streptomycin and amphotericin B and then seeded at a density of 2×10^6 cells per T25 flask. Cells were incubated in a 5% CO₂ incubator at 37°C until cells became confluent. The culture flasks were shaken on an orbital shaker (BR-40LF; TAI-TEC, Koshigaya, Japan) at 200 rpm and 37°C for more than 14 h to detach loosely attached microglia and OPCs from the astrocytic bed. Immediately after shaking, the cells were washed three times with PBS and culture medium was refreshed. After 2-day incubation in 5% CO₂ at 37°C, flasks were washed with PBS for three times and treated with trypsin/EDTA/PBS for 5 min at 37°C. The

purity of astrocytes was confirmed to be >95% by GFAP immunostaining.

OPC culture was performed as described before (Nishiyama et al. 2010). OPCs in mixed cortical glial cultures (primary culture) and isolated OPCs in the presence of PDGF AA (secondary culture) were characterized immunocytochemically by NG2 or PDGFR α expression.

For establishment of FABP7 stably over-expressing cells, the coding region of mouse FABP7 gene was amplified by PCR and sub-cloned into the pcDNA3 mammalian expression vector. The constructed expression vector (pcDNA3 for mock, pcDNA3/FABP7) was transfected into NG108 cells (rodent neuroblastoma/glioma hybrid cells; a kind gift from Dr. K Fukunaga, Tohoku University) using Lipofectamine 2000 (Invitrogen Co., Carlsbad, CA, USA) following the manufacturer's instructions, and the cells were selected for 2 weeks in culture media supplemented with 0.5 mg/ml G418 (Sigma-Aldrich, St. Louis, MO, USA). The resistant clones were harvested, and the over-expression of the gene and protein was confirmed by RT-PCR and Western blot analysis, respectively.

Immunohistochemistry

Animals were transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate buffer (pH 7.4). Brains were removed, postfixed in 4% PFA (at least for 4 h) and cryoprotected with graded sucrose (10, 20 and 30%; each for approximately 10 h) at 4°C then stored at -80°C. Brain samples were cut into serial coronal 12- to 14- μ m-thick sections with 50- to 60- μ m intervals using a cryostat (Leica CM1850; Leica, Nussloch, Germany) and then mounted onto glass slides. Sections were rehydrated in PBS containing 0.01% Tween 20 (TPBS) for 10 min, permeabilized with 0.3% triton X-100 in PBS for 30 min, washed three times in TPBS over 15 min and then blocked in 5% skim milk in TPBS as blocking solution for 1 h at room temperature. When using the antibody of mouse origin, we used M.O.M Kit (Vector Laboratories, Burlingame, CA, USA) for masking the endogenous Fc receptors. Sections were incubated in combinations of the following primary antibodies for 16 h at 4°C: rabbit anti-FABP7 (0.5 μ g/ml) (Abdelwahab et al. 2003), rabbit anti-NG2, guinea pig anti-NG2 and rabbit anti-PDGFR- α (1:1000, 1:50 and 1:1000, respectively, kindly provided by W. Stallcup, Burnham Institute, <http://www.burnham.org>), rat anti-GFAP (1:200, Invitrogen), mouse anti-NeuN (1:100, Millipore, Billerica, MA, USA), mouse anti-MAP2 (1:500, Sigma-Aldrich), rat anti-CD11b (1:50, Chemicon, Temecula, CA, USA), mouse anti-BrdU (1:100, BD Pharmingens, San Diego, CA, USA) (see Supplementary Table 1).

After primary antibody incubation, sections were washed three times in TPBS for 30 min and incubated with combinations of the following secondary antibodies for 30 min at room temperature: anti-rabbit IgG-Alexa488, anti-rabbit IgG-Alexa 633, anti-mouse IgG-Alexa568, anti-rat IgG-Alexa 568, and anti-guinea pig IgG-Alexa 555 (1:1000 for all; Invitrogen Co.). 4',6-Diamidino-2-phenylindole (DAPI) (0.5 μ g/ml, Invitrogen Co.) was added as a nuclear marker. When required, Zenon rabbit IgG labeling reagents (Molecular Probes, Invitrogen Co.) labeled with Alexa Fluor 594 and Alexa Fluor 488 were used for double-staining of rabbit primary antibodies. Slides were cover-slipped using Fluoromount (DBS, CA, USA). For negative controls of the antibodies used in this study, we omitted the primary antibody reaction in the immunohistochemical procedure and confirmed the absence of the reaction under such condition. Furthermore, for FABP7 antibody, the authenticity was confirmed by the complete absence of the reaction in FABP7-KO mouse tissues/cells.

BrdU uptake assay

For proliferation assay after stab injury, single intraperitoneal injection (100 mg/kg) of BrdU (Sigma-Aldrich) was performed at 3 days after lesion (DPL3), and animals were killed after 2 h ($n = 4$ for wild-type, $n = 4$ for FABP7-KO mice) or 4 days after injection (DPL7: $n = 4$ for wild-type, $n = 4$ for FABP7-KO mice) (White et al. 2010). For cultured astrocytes, BrdU (10 μ M) was added to culture medium at 3 days after seeding when the astrocytes reached a subconfluent state, and was incubated for 5 h. BrdU⁺ cells were visualized immunocytochemically using an anti-BrdU antibody as described previously with slight modifications (White et al. 2010; Yang et al. 2009). Briefly, cells were incubated with 2 M HCl at room temperature for 30 min followed by washing twice with 0.1 M sodium borate (pH 8.5) over 10 min. After blocking with 5% goat serum at room temperature for 30 min, they were incubated with mouse anti-BrdU antibody (1:100) at 4°C overnight, and incubated with anti-mouse IgG-Alexa568 (1:1000) at room temperature for 1 h and then counterstained with DAPI. After several washes with PBS, they were mounted and cover slipped. Brain sections were pretreated by microwave heating in citrate buffer (pH 6) at 96°C for 10 min followed by incubation in 2 N HCl at 37°C for 11 min and stained as described above.

Morphometric analysis

Sections were observed by a confocal laser scanning microscope (LSM510 META; Carl Zeiss, Oberkochen, Germany) and images were obtained in a 0.1 mm² area (40 \times lens with 0.7 zoom, 317 \times 317 μ m), approximately

0.1 mm either lateral or medial to the edge of the lesion in the gray matter of the cortex (mostly layers III and IV, where astrocyte population includes only protoplasmic astrocytes) (Nag 2011). Corresponding areas in the contralateral cortex were also imaged. For BrdU assessment, counts were performed in 0.4 mm^2 areas ($20\times$ lens with 0.7 zoom, $635 \times 635 \mu\text{m}$) from the edge of the lesion in both the lateral and medial sides (see Supplementary Fig. 1b).

Western blot analysis

Mice were killed 3 days after stab injury. The ipsilateral cortex containing the stab injury and the corresponding contralateral cortex were dissected ($2 \times 2 \times 2 \text{ mm}$ blocks). Whole tissue lysate was prepared in $2\times$ SDS-PAGE sample buffer containing protease inhibitors (Roche Diagnostics, Mannheim, Germany). The lysates were electrophoresed on a 15% SDS-polyacrylamide gel and immunoblotted onto Immobilon-P^{SQ} PVDF membrane (Millipore). After blocking with skim milk, membranes were incubated with rabbit anti-FABP7 antibody ($0.125 \mu\text{g/ml}$) (Abdelwahab et al. 2003) and mouse anti- β -actin antibody (1:5000, Santa Cruz Biotech, Santa Cruz, CA, USA) overnight at 4°C followed by incubation with HRP-conjugated goat anti-rabbit antibody (1:1000, Chemicon) and goat anti-mouse antibody (1:1000, Chemicon). Detection of reactive bands was performed using the ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

^3H -thymidine incorporation assay

Primary cultured astrocytes were incubated in 24-well plates at the subconfluent status. The cells were then incubated in fresh medium containing 10% fetal calf serum and ^3H -thymidine ($1 \mu\text{Ci}$ per well; Amersham, Piscataway, NJ, USA) for 24 h at 37°C . After incubation, the cells were washed twice with $500 \mu\text{l}$ of ice-cold PBS (0.1 M ; $\text{pH } 7.4$), detached with trypsin–EDTA solution and collected on GF/C filters (Whatman, Clifton, NJ, USA). The filters were washed three times with 1 ml of 10% trichloroacetic acid and rinsed twice with 1 ml of absolute ethanol. The radioactivity contained on each filter was determined using a scintillation counter (LSC-5100; Aloca, Tokyo, Japan).

Fatty acid incorporation assay

Primary cultured astrocytes were incubated in 12-well plates. A $0.1 \mu\text{Ci/ml}$ of ^{14}C -linoleic acid or ^{14}C - α -linolenic acid (Amersham Pharmacia Biotech) was added to confluent cultured astrocytes. After incubation for 30–120 min, the cells were washed thoroughly with cold PBS and lysed with 0.1 M NaOH. Radioactivity was measured using a

β -scintillation counter. Radioactivity was normalized to the DNA content of the sample.

Cell titer assay

The cell titer was quantified using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) according to the manufacturer's manual. Briefly, after seeding the cells onto 96-well plates at the density of 1×10^4 cells/well, the coloring solution was added to the culture media at each time point (0, 1, 2, 3, 4 and 5 days after seeding). Following incubation for 2 h, chromogenic development was measured at 490 nm by spectrophotometer (Beckman Coulter, Fullerton, CA, USA). The experiment was done in quadruplicate and the mean value of optical density in each time point was calculated.

Statistical analysis

All data are shown as mean \pm SD. Statistical comparisons of means were made by Student's two-tailed unpaired *t* test or, for multiple comparisons, one-way ANOVA followed by the Tukey test. *P* values <0.05 were considered statistically significant.

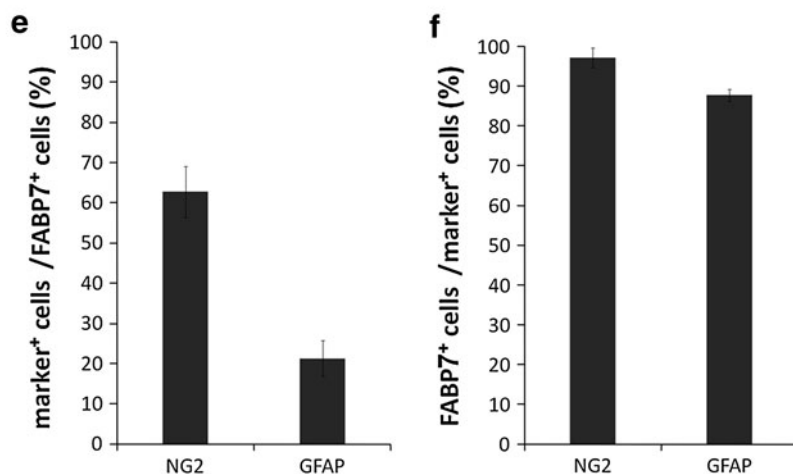
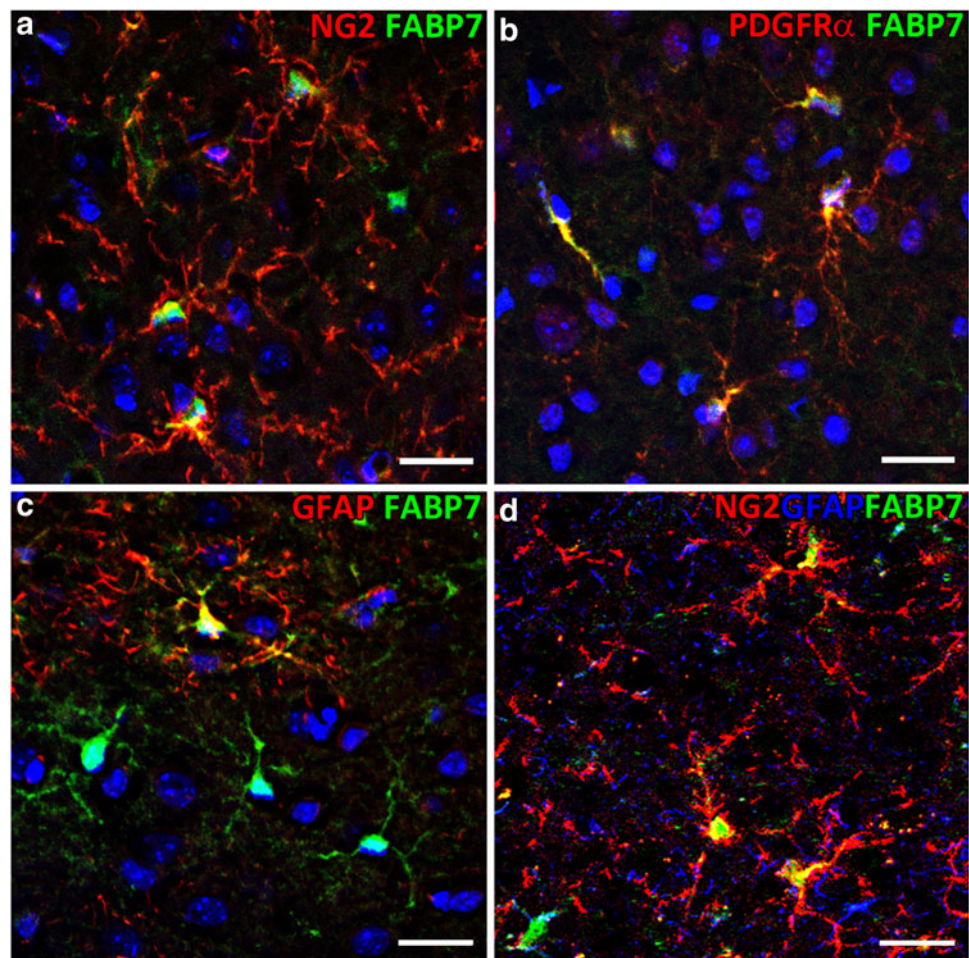
Results

Localization of FABP7 in normal cortex

In the normal (intact) cortex, FABP7⁺ cells exhibiting several cellular processes were evenly scattered throughout the cortex. In these cells, FABP7 immunopositive staining was observed in the nuclei and cytoplasm (Fig. 1). The majority ($62.7 \pm 6.3\%$, $n = 3$) of the FABP7⁺ cells were positive for NG2 proteoglycan, a marker for OPCs (termed polydendrocytes) (Nishiyama et al. 2009) (Fig. 1a, f). Confirmation of FABP7⁺ cells as OPCs was further performed by staining with PDGFR α , a specific marker of OPCs (Zhu et al. 2010; Gao et al. 2010; Ozerdem et al. 2001; Nishiyama et al. 2009) (Fig. 1b). Furthermore, $97.1 \pm 2.5\%$ of NG2⁺ OPCs in the intact cortex were FABP7⁺ (Fig. 1f). By contrast, $21.6 \pm 3.7\%$ of FABP7⁺ cells in the intact cortex were GFAP⁺ astrocytes (Fig. 1c) and $82.4 \pm 10.6\%$ of GFAP⁺ astrocytes were FABP7⁺. In the upper layers near the dorsal surface of the cortex where GFAP⁺ cells exhibit a higher population density, thicker and less branched processes and more intensive expression of GFAP (also known as interlaminar astrocytes, Nag 2011), GFAP⁺ astrocytes comprised near half of all FABP7⁺ cells, with the remainder being NG2⁺ OPCs (data not shown).

Fig. 1 Identification of FABP7 expressing cells in the normal adult cerebral cortex.

a–d Immunofluorescence micrographs showing expression of FABP7 in OPCs and astrocytes. **a** Expression of FABP7 (green) in NG2⁺ OPCs (red). **b** Expression of FABP7 in OPCs confirmed by colocalization of FABP7 (green) and PDGFR α (red). **c** Expression of FABP7 (green) in GFAP⁺ protoplasmic astrocyte. **d** Localization of FABP7 (green) in NG2⁺ OPCs (red) and GFAP⁺ astrocytes (blue). Note that the majority of FABP7⁺ cells are OPC. **e** Bar graph showing the percentage of NG2⁺ OPCs and GFAP⁺ astrocytes among total FABP7⁺ cells. **f** Bar graph showing the percentage of FABP7⁺ cells in NG2⁺ OPCs and GFAP⁺ astrocytes. Data in **e**, **f** are obtained from 0.1 mm² area. Scale bars 20 μ m



FABP7 was not colocalized with markers for neurons including NeuN (Supplementary Fig. 2a) and MAP2 (Supplementary Fig. 2b), or with markers for microglia including CD11b (Supplementary Fig. 2c). A 60% of cells immunopositive for Olig2 (a common marker for all oligodendrocyte lineage cells) did not express FABP7 (Supplementary Fig. 2d) suggesting down-regulation of

FABP7 during differentiation of OPCs to oligodendrocytes. We also performed a systematic search in the transcriptome databases of neural cells (Cahoy et al. 2008) and found that the FABP7 gene was statistically enriched in purified astrocytes and OPCs, confirming our results. In the same database, we found reciprocal enrichment of FABP7 in OPCs and FABP5 in oligodendrocytes.

Taken together, these data suggest that mainly OPCs and astrocytes comprise almost the entire population of FABP7 expressing cells in the intact adult cortex. To our knowledge, this is the first time FABP7 has been shown on OPCs. We also found strong expression of FABP7 on OPCs in primary and secondary OPC cultures (see Supplementary Fig. 3).

Localization of FABP7 in the stab-injured cortex

To evaluate changes in expression of FABP7 after cortical stab injury, the stab-injured cortices at DPL3 and the contralateral intact cortices were dissected for Western blotting. FABP7 protein expression was significantly increased in the injured cortex compared with the contralateral side (Fig. 2a). When assessed by immunohistochemistry, the population density of FABP7⁺ cells significantly increased (approximately 35%) compared with the intact cortex (Fig. 2b; 28.8 ± 1.9 cells at DPL3, 29.1 ± 1.3 cells at DPL7 vs. 19.1 ± 1.4 cells in the intact cortex; $p < 0.05$ and $p < 0.01$, respectively) in a 0.1 mm^2 area (0.1 mm lateral or medial to the edge of injury). In contrast to the intact cortex, the majority of FABP7⁺ cells (80.1 ± 0.2 and $82.8 \pm 5.3\%$ at DPL3 and DPL7, respectively) in the stab-injured cortex were GFAP⁺ (Fig. 2c, g), while almost all GFAP⁺ astrocytes (94.5 ± 0.8 and $97.0 \pm 0.01\%$ at DPL3 and DPL7, respectively) were positive for FABP7 (Fig. 2h). The number of GFAP⁺/FABP7⁺ cells significantly increased in number in the injured cortex (Fig. 2i; 23.3 ± 1.7 cells and 24.4 ± 0.1 cells at DPL3 and DPL7, respectively, vs. 4.1 ± 0.5 cells in intact cortex in a 0.1 mm^2 area), indicating a role for FABP7 in astrocyte proliferation.

A minor proportion of FABP7⁺ cells (35.2 ± 5.6 and $29.4 \pm 2.5\%$ at DPL3 and DPL7, respectively) in the stab-injured cortex co-expressed NG2 (Fig. 2d). The expression of FABP7⁺/NG2⁺ cells in the injured cortex was similar to that in the intact cortex and could be distinguished from NG2⁺ pericytes in the stab-injured cortex as characterized by their specific elongated morphology and their location around the vessels and close to the injury core. Furthermore, these cells co-expressed PDGFR α (Fig. 2e), indicative of OPCs rather than vascular pericytes.

While the total number of NG2⁺ cells significantly increased (approximately 30%) in the stab-injured cortex compared with the intact cortex (21 ± 1.1 and 19.1 ± 2.1 cells/ 0.1 mm^2 at DPL3 and DPL7, respectively, vs. 14 ± 1.9 cells/ 0.1 mm^2 in the intact cortex; Supplementary Fig. 4), the total number of NG2⁺/FABP7⁺ cells did not change (10.5 ± 2.8 and 9.2 ± 2.7 cells/ 0.1 mm^2 at DPL3 and DPL7, respectively, vs. 12.3 ± 2.2 cells/ 0.1 mm^2 in the intact cortex; Fig. 2i). Furthermore, the total number of PDGFR α ⁺ cells did not significantly differ between the

intact and injured cortex (16.4 ± 0.8 and 15.9 ± 4.4 cells/ 0.1 mm^2 at DPL3 and DPL7, respectively, vs. 14 ± 1.1 cells/ 0.1 mm^2 in the intact cortex; Supplementary Fig. 4). Similar to the intact cortex, in the stab-injured cortex, FABP7 expression was not seen in neurons positive for MAP2 or NeuN, or in microglia and/or monocyte-derived cells positive for F4/80 or CD11b (data not shown).

Based on these results, localization of FABP7⁺ in mainly NG2⁺ OPCs in the intact cortex is changed into mainly GFAP⁺ astrocytes in the stab-injured cortex, due to a profound increase in population density of GFAP⁺ astrocytes. Taken together, these results suggest an association between FABP7 and proliferation of reactive astrocytes after stab injury.

Role of FABP7 in proliferation of astrocytes after stab injury

For further evaluation of the role of FABP7 in proliferation of astrocytes, we examined whether FABP7-deficient astrocytes showed an altered proliferation in response to stab injury. We quantified the number of BrdU⁺ astrocytes and the total population of astrocytes in the cortices of wild-type and FABP7-KO mice after stab injury. BrdU was administered intraperitoneally to wild-type and FABP7-KO mice at 3 days after cortical stab injury, and the number of BrdU⁺ astrocytes was examined immunohistochemically at 2 h (DPL3; $n = 4$ for each genotype) and 4 days (DPL7; $n = 4$ for each genotype) after administration (Fig. 3a). In the contralateral side of DPL3 and DPL7 brain cortices, a few cells (1–2 cells per section) were labeled with BrdU, and these were PDGFR α ⁺/GFAP⁻ cells, presumably proliferating OPCs (Fig. 3b).

There were no differences in the total number of BrdU⁺ cells (including blood derived cells and activated microglia, reactive astrocytes, OPCs, pericytes and/or endothelial cells) between the stab-injured cortex of wild-type and FABP7-KO mice at either time points (Supplementary Fig. 5f). The total number of BrdU⁺ cells increased approximately twofold at DPL7, indicating that some of the cells that were in S-phase at DPL3 proceeded to mitosis or reentered the cell cycle until DPL7. Moreover, it is possible that some cells may have proliferated several times resulting in BrdU levels below the detectable limit.

At DPL3, there was no difference in the total number of BrdU⁺/GFAP⁺ cells between wild-type and FABP7-KO mice (8.6 ± 2 cells/ 0.4 mm^2 in wild-type vs. 6.3 ± 3 cells/ 0.4 mm^2 in FABP7-KO) (Fig. 3c, d, i), while at DPL7 there was a significant difference between two groups (29.3 ± 5.7 cells/ 0.4 mm^2 in wild-type vs. 18.3 ± 0.5 cells/ 0.4 mm^2 in FABP7-KO, $p < 0.05$) (Fig. 3e, f, i). Consistent with these data, the total number of GFAP⁺

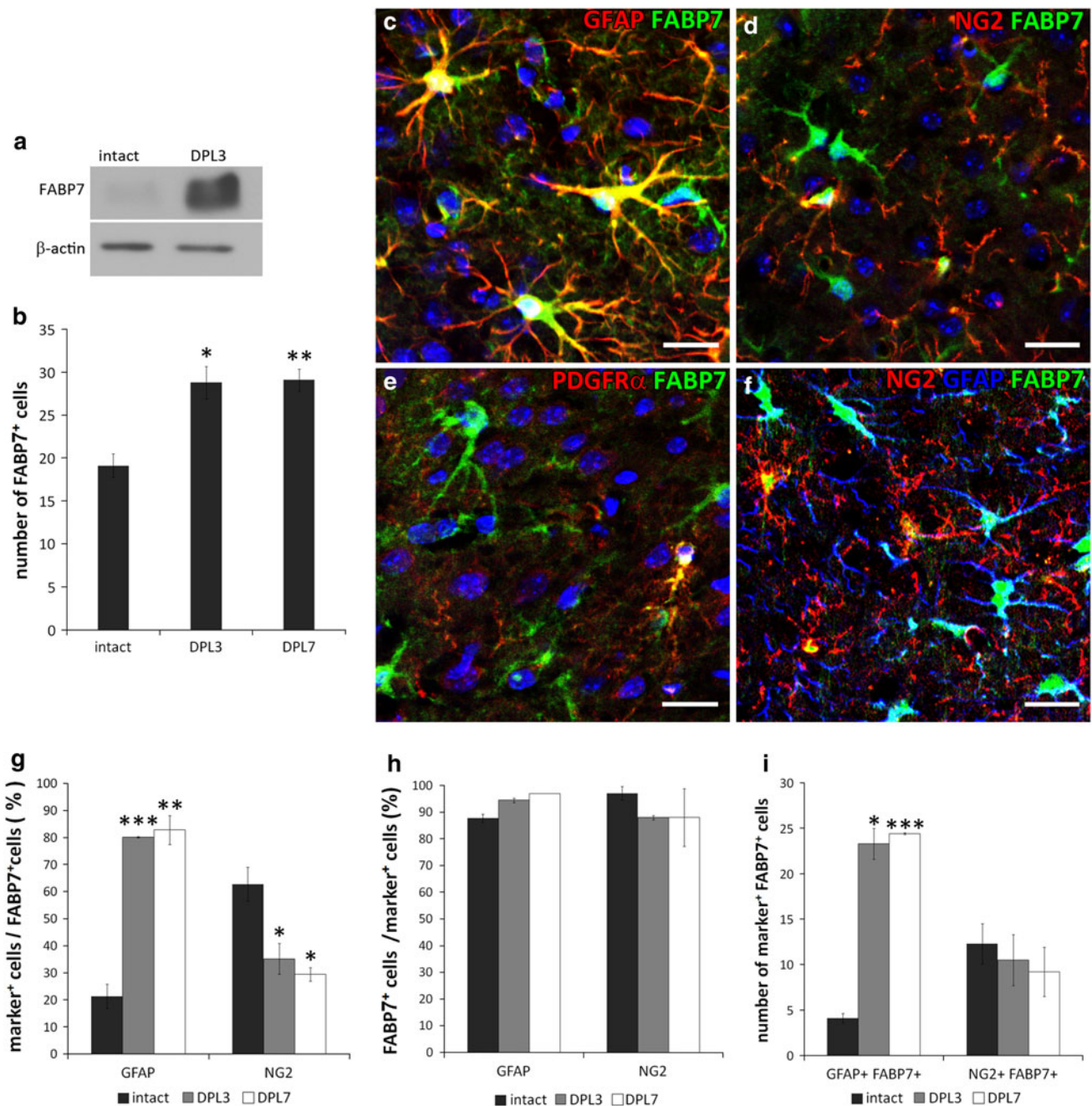


Fig. 2 FABP7 expression in stab-injured cortex. **a** Western blotting showing increased expression of FABP7 in stab-injured cortex compared with the intact cortex. **b** Bar graph showing increased population density of FABP7⁺ cells in injured cortex compared to intact cortex. **c–f** Immunofluorescence micrographs showing expression of FABP7 in OPCs and astrocytes after cortical stab injury. **c** Intensive expression of FABP7 (green) in hypertrophied GFAP⁺ reactive astrocytes (red). **d** Expression of FABP7 (green) in NG2⁺ OPCs (red). **e** Expression of FABP7 in OPCs after stab injury confirmed by colocalization of FABP7 (green) and PDGFR α (red).

f Localization of FABP7 (green) in GFAP⁺ astrocytes (blue) and NG2⁺ OPCs (red) after stab injury. Note that the majority of FABP7⁺ cells in stab-injured cortex are astrocytes. **g** Quantification of astrocytes and OPCs among FABP7⁺ cells in intact and injured cortex. **h** Quantification of FABP7⁺ cells among astrocytes and OPCs in intact and injured cortex. **i** Population density of FABP7⁺/GFAP⁺ astrocytes and FABP7⁺/NG2⁺ OPCs in intact and injured cortex. Data in **b**, **g**, **h**, **i** are obtained from 0.1 mm² area. DPL days post lesion. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. Scale bars 20 μ m

astrocytes was significantly decreased in FABP7-KO mice at DPL7 (125.7 ± 7.6 cells/0.4 mm² in wild-type vs. 106.7 ± 5.5 cells/0.4 mm² in FABP7-KO, *p* < 0.05)

(Fig. 3g, Supplementary Fig. 5a–d). The proportion of BrdU⁺ astrocytes among the total astrocyte population also significantly decreased at DPL7 ($25.67 \pm 5.1\%$ in wild-type

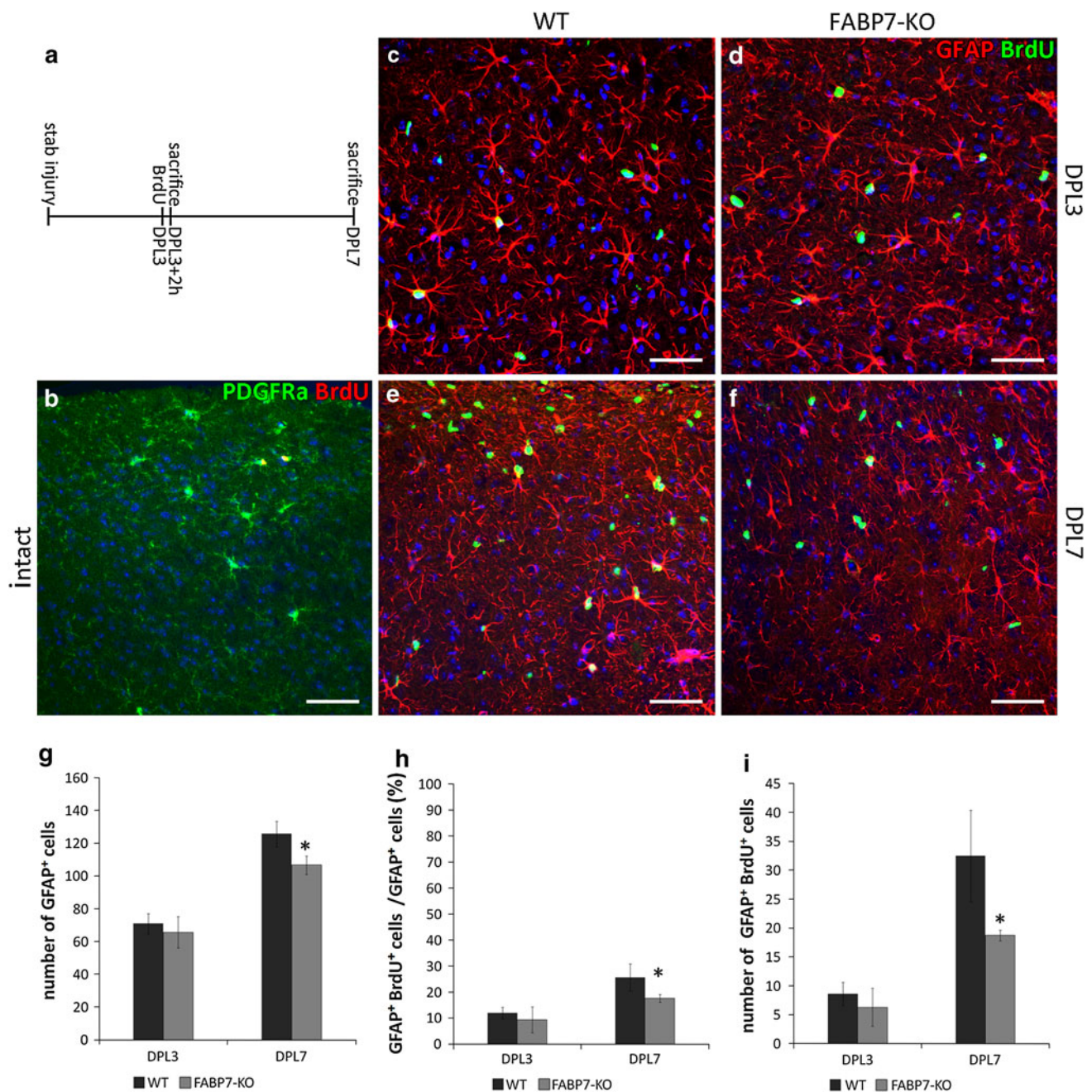


Fig. 3 Decreased proliferation of reactive astrocytes after cortical stab injury in FABP7-KO mice. **a** BrdU administration paradigm: single injection of BrdU was performed at 3 days after stab injury, and mice were killed 2 h or 4 days later. **b** Immunofluorescence micrograph showing that few BrdU⁺ (red) cells were detected in the intact cortex which were PDGFR α ⁺ (green) OPCs. **c–f** Representative immunofluorescence micrographs showing BrdU⁺ (green) cells and GFAP⁺ (red) astrocytes in stab-injured cortex in wild-type and FABP7-KO mice at 3 and 7 days after stab injury. Note the lower number of BrdU⁺/GFAP⁺

astrocytes in FABP7-KO mice compared with wild-type mice, especially in DPL7. **g** Bar graph showing the population density of GFAP⁺ astrocytes in the stab-injured cortex of wild-type and FABP7-KO mice. **h** Bar graph showing the proportion of BrdU⁺/GFAP⁺ astrocytes among total GFAP⁺ astrocytes in the stab-injured cortex of wild-type and FABP7-KO mice. **i** Bar graph quantifying the population density of BrdU⁺/GFAP⁺ astrocytes in stab-injured cortex of wild-type and FABP7-KO mice. Data in **g**, **h**, **i** are obtained from 0.4 mm² area. DPL days post lesion. * $p < 0.05$. Scale bars 50 μ m

vs. $17.55 \pm 1.5\%$ in FABP7-KO, $p < 0.05$) (Fig. 3h). These data show that astrocyte proliferation is decreased in FABP7-KO after stab injury compared with wild-type, suggesting a role of FABP7 in proliferation of astrocytes.

Role of FABP7 in astrocyte proliferation in vitro

FABP7 was abundantly expressed in primary cultured astrocytes at gene and protein levels (Supplementary Fig. 6a, b),

and in immunocytochemistry FABP7 was predominantly localized to the cell nuclei of cultured astrocytes (Supplementary Fig. 6c). To study the role of FABP7 in astrocyte proliferation, we examined BrdU and ^3H -thymidine incorporation in subconfluent primary cultured astrocytes from wild-type and FABP7-KO mice. The BrdU labeling index (proportion of BrdU⁺ astrocytes among all astrocytes) was significantly decreased (by approximately 30%, $p < 0.05$) in FABP7-KO astrocytes compared with wild-type (Fig. 4a, Supplementary Fig. 7c, d). Similarly, the uptake of ^3H -thymidine in FABP7-KO astrocytes was significantly decreased (by approximately 20%, $p < 0.05$) compared with wild-type (Fig. 4b). Delayed confluency was consistently observed in FABP7-KO astrocytes compared with wild-type (Supplementary Fig. 7a, b). These data further confirm the role of FABP7 in astrocyte proliferation. Next, we transfected FABP7 into NG108 cells, neuroblastoma and glioma hybrid cells, which show no endogenous FABP7 expression (Supplementary Fig. 7e). There was a significant increase in the proliferation of FABP7 transfected cells compared with mock-transfected cells (Supplementary Fig. 7f).

We also evaluated the uptake of α -linolenic acid ($^{18}\text{C}_3$, n-3) and linoleic acid ($^{18}\text{C}_2$, n-6) in FABP7-KO astrocytes. There was a decrease in incorporation of α -linolenic acid ($^{18}\text{C}_3$, n-3) in FABP7-KO astrocytes ($p < 0.05$; Fig. 4c), while no difference was observed in that of linoleic acid ($^{18}\text{C}_2$, n-6) (Fig. 4d). Taken together, these data suggest that FABP7 is involved in the proliferation of astrocytes by controlling fatty acid homeostasis (metabolism/signal transduction).

Discussion

FABP7 is abundantly expressed in neural stem cells and radial glia of the developing brain and has been associated with a number of brain diseases including psychotic disorders (Owada et al. 1996; Watanabe et al. 2007; Maekawa et al. 2011) or brain tumors (Godbout et al. 1998; Tso et al. 2006; Liang et al. 2005; Kaloshi et al. 2007). However, the underlying mechanisms of the actions of FABP7 are poorly understood, particularly in the adult brain under normal and pathologic conditions. Herein, we identified FABP7 expressing cells in the adult mouse cortex as both OPCs and astrocytes and found that FABP7 expression was markedly increased in the astrocytes after cortical injury. Furthermore, we examined the phenotype of FABP7-KO mice after cortical stab injury to study the role of FABP7 in gliosis and found that FABP7 played a critical role in regulating astrocyte proliferation *in vivo*.

Decreased proliferation of reactive astrocytes in FABP7-KO mice is one of the main findings in this study.

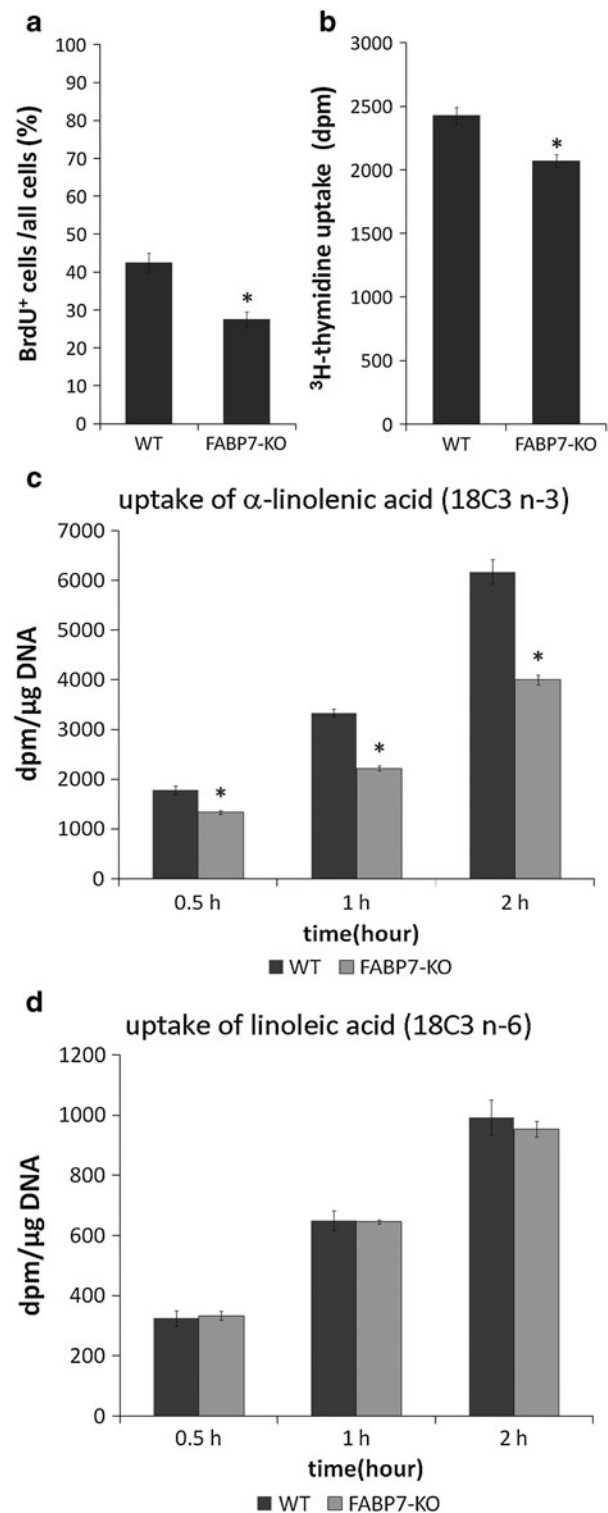


Fig. 4 Decreased proliferation and fatty acid incorporation in primary cultured FABP7-KO astrocytes. **a** Bar graph showing decreased BrdU⁺ cells at 3 days after passage in FABP7-KO cultured astrocytes compared with wild-type. **b** Bar graph showing decreased ^3H -thymidine uptake in FABP7-KO cultured astrocytes compared with wild-type astrocytes. Bar graphs showing incorporation of n-3 (**c**) and n-6 (**d**) fatty acids in wild-type and FABP7-KO cultured astrocytes. * $p < 0.05$

We previously reported that FABP7 was substantially expressed by GFAP⁺ astrocytes in the cortex of early postnatal developing brain (Owada et al. 1996), and that the number of GFAP⁺ cells (neural stem cells and early progenitors) and total BrdU uptake were decreased in the hippocampal dentate gyrus of the FABP7-KO mice at postnatal 4 weeks (Watanabe et al. 2007). However, no significant difference in the astrocyte population was detected in the normal adult cortex between wild-type and FABP7-KO mice in this study. One possible explanation is that the cellular demand of fatty acids was developmentally compensated in the FABP7-KO brain by the redundant molecule such as FABP5, another FABP expressed weakly in the astrocytes, while the dramatic increase in the fatty acid requirement for astrocyte proliferation during reactive gliosis, possibly for rapid remodeling of membrane lipid, was not fully covered. Another possibility is that the mechanism by which FABP7 controls astrocyte biology under pathological condition (gliosis process) is partly distinct from that in the normal brain development. However, this study demonstrated that the primary cultured FABP7-KO astrocytes showed the decrease in proliferation and the late confluency, suggesting that FABP7-KO mice may reach to the normal population of astrocytes in the *in vivo* cortex later than wild-type mice. This hypothesis should be further examined through careful quantification of cortical astrocytes in different developmental stages in FABP7-KO mice.

Primary cultured astrocytes from FABP7-KO mice showed a decrease in proliferation and a decrease in the cellular uptake of α -linolenic acid (¹⁸C₃, n-3), while there was no decrease in that of linoleic acid (¹⁸C₂, n-6). These data are consistent with the previous finding that FABP7 binds to n-3 PUFAs with high affinity (Balendiran et al. 2000), suggesting that FABP7 is involved in proliferation of astrocytes by controlling fatty acid homeostasis (metabolism/signal transduction). Although the role of n-3 PUFA in the astrocyte proliferation remains unknown, Tian et al. (2011) recently reported that dietary supplementation of the female mice during pregnancy and lactation period with n-3 PUFA results in increased numbers of GFAP⁺ astrocytes and increased expression of PPAR γ in the developing brain (Tian et al. 2011). Champeil-Potokar et al. (2004) reported an increased proliferation of primary cultured astrocytes supplemented with DHA by assessing DNA concentration. They also found an increased n-3 PUFAs and DHA composition of membrane phospholipids in astrocytes after supplementation with DHA (Champeil-Potokar et al. 2004). Although the molecular mechanism by which FABP7 regulates astrocyte proliferation remains to be studied, it has been recently shown that transfection of FABP7 in FABP7⁻ U87 cells (malignant glioma cell line) causes increased expression of growth factors such as FGF2 (Kipp et al.

2011), which is known to promote astrocyte proliferation (Stachowiak et al. 1997; Joy et al. 1997; Gomez-Pinilla et al. 1995). The same group also reported the morphological difference between FABP7⁺ and FABP7⁻ U87 cells (Mita et al. 2007), in contrast to our not noticing any difference in the morphology of cortical astrocytes in the adult brain (Owada et al. 2006) and in the primary cultured astrocytes between wild-type and FABP7-KO mice. Although the immortalized glioma cells and astrocytes are distinct in their biological characteristics, it is crucial and interesting to measure the expression levels of growth factors in the FABP7-KO astrocytes and further to examine whether decreased proliferation of reactive astrocytes in FABP7-KO mice has any impact on neuronal outcome including neuronal survival and/or regeneration after the brain damage.

We found that the major population of FABP7⁺ cells in the adult cortex was OPCs. OPCs are distributed throughout the gray and white matter and are the most prevalent cycling progenitors in the adult CNS (Dawson et al. 2003; Geha et al. 2010; Simon et al. 2011). OPCs are functionally and antigenically distinct from neurons, mature oligodendrocytes, astrocytes and microglia, and as such, are regarded as the fourth major glial population (Nishiyama et al. 2009). Several studies have reported that OPCs generate oligodendrocytes in the developing and mature CNS (Simon et al. 2011; Nishiyama et al. 2009) and that OPCs respond by proliferating, migrating, and differentiating into myelinating oligodendrocytes following demyelination (Levine et al. 2001; Levine and Reynolds 1999; Islam et al. 2009; Franklin 2002; Franklin et al. 2002; Watanabe et al. 2002). We also found that only a subpopulation of oligodendrocyte lineage cells expressed FABP7, suggesting that FABP7 is downregulated during differentiation of OPCs to oligodendrocytes. This finding was confirmed by our analysis of the transcriptome database of isolated neural cells (Cahoy et al. 2008), suggesting a role of FABP7 in OPC differentiation. Failure of OPCs to differentiate into myelinating oligodendrocytes results in failure of spontaneous remyelination, which is important in the pathophysiology of demyelinating disorders such as multiple sclerosis (Chang et al. 2002; Kuhlmann et al. 2008). Of interest, activation of RXR γ was reported to stimulate differentiation of OPCs to enhance remyelination (Huang et al. 2011), while FABPs can regulate gene expression by activation of nuclear receptors such as PPARs and RXRs (Kitajka et al. 2004; Schroeder et al. 2008). There are also several reports addressing the effects of n-3 PUFA on stimulation of the myelinogenesis process (DeWille and Farmer 1992; Martinez and Vazquez 1998; van Meeteren et al. 2006; Salvati et al. 2008). Our transcriptome database search of spontaneous remyelination (Huang et al. 2011) showed that FABP7 is upregulated during remyelination, suggesting a possible role of FABP7

in the process of OPC differentiation and remyelination. Although the role of FABP7 in OPC biology remains to be elucidated, the confirmation of strong expression of FABP7 in the primary and secondary cultured OPCs in our study suggests an important role and may enable us to do further detailed studies.

In contrast to several previous studies that reported increased number of OPCs after stab injury (Rhodes et al. 2006; Simon et al. 2011; Hampton et al. 2004; Buffo et al. 2005; Levine 1994; McTigue et al. 2001; Alonso 2005), we did not detect any significant changes in OPC numbers between normal and stab-injured cortex. This discrepancy can be partly explained by different sizes of the observation area and its distance from the edge of the injury as well as the different markers used for OPC labeling. Previous reports used mainly NG2 for identification of OPCs, which is also expressed by pericytes and activated microglia, in the injured cortex. In this study, quantification of OPC in the stab-injured cortex was confirmed by using PDGFR α positivities as the most specific marker for OPCs combined with co-expression of NG2 and FABP7. Interestingly, Hampton et al. (2004) showed that the total number of PDGFR α ⁺ cells remained constant after injury, while the number of NG2⁺ cells increased (Hampton et al. 2004). These results were, however, explained by proposing a NG2⁺/PDGFR α ⁻ subpopulation of OPCs, which is not consistent with the current understanding of the antigenic characteristics of OPCs.

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