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***Fosb* gene products contribute to excitotoxic microglial activation by regulating the expression of complement C5a receptors in microglia**

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Abstract

The *Fosb* gene encodes subunits of the activator protein-1 transcription factor complex. Two mature mRNAs, *Fosb* and *Fosb*, encoding full-length FOSB and FOSB proteins respectively, are formed by alternative splicing of *Fosb* mRNA. *Fosb* products are expressed in several brain regions. Moreover, *Fosb*-null mice exhibit depressive-like behaviors and adult-onset spontaneous epilepsy, demonstrating important roles in neurological and psychiatric disorders. Study of *Fosb* products has focused almost exclusively on neurons; their function in glial cells remains to be explored. In this study, we found that microglia express equivalent levels of *Fosb* and *Fosb* mRNAs to hippocampal neurons and, using microarray analysis, we identified six microglial genes whose expression is dependent on *Fosb* products. Of these genes, we focused on *C5ar1* and *C5ar2*, which encode receptors for complement C5a. In isolated *Fosb*-null microglia, chemotactic responsiveness toward the truncated form of C5a was significantly lower than that in wild-type cells. *Fosb*-null mice were significantly resistant to kainate-induced seizures compared with wild-type mice. *C5ar1* mRNA levels and C5aR1 immunoreactivity were increased in wild-type hippocampus 24 hours after kainate administration; however, such induction was significantly reduced in *Fosb*-null hippocampus. Furthermore, microglial activation after kainate administration was significantly diminished in *Fosb*-null hippocampus, as shown by significant reductions in CD68 immunoreactivity, morphological change and reduced levels of *Il6* and *Tnf* mRNAs, although no change in the number of Iba-1-positive cells was observed. These findings

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demonstrate that, under excitotoxicity, *Fosb* products contribute to a neuroinflammatory response in the hippocampus through regulation of microglial *C5ar1* and *C5ar2* expression.

Keywords

Neuroinflammation; Kainic acid; AP-1 transcription factors

Introduction

FBJ osteosarcoma oncogene B (FOSB), a FOS family protein, heterodimerizes with JUN family proteins to form activator protein-1 (AP-1) transcription factor complexes that regulate gene expression within multiple signaling pathways and functional outcomes (Chinenov and Kerppola, 2001; Jochum et al., 2001; Wagner and Eferl, 2005). Two mature mRNAs, *Fosb* and *Fosb*, are formed by alternative splicing of the *Fosb* gene, thus producing multiple protein products (Nakabeppu and Nathans, 1991; Metz et al., 1994; Ohnishi et al., 2011). Because there are two translation initiation sites (Sabatakos et al., 2008), each of the mRNAs is translated into two proteins (FOSB and vFOSB from *Fosb* mRNA, FOSB and 2 FOSB from *Fosb* mRNA, Fig. 1). The major translation products of the two mRNAs are FOSB and FOSB, which have been reported to differentially regulate target genes as subunits of distinct AP-1 complexes (Nakabeppu and Nathans, 1991; McClung and Nestler, 2003). However, their precise functions *in vivo* remain unclear.

FOSB, a lesser extent FOSB, are expressed in specific regions of normal brain, such as the nucleus accumbens, dorsal striatum, dentate gyrus of hippocampus and cerebral cortex (Ohnishi et al., 2011). *Fosb*-null mice exhibit depressive-like behaviors (Ohnishi et al., 2011) and adult-onset spontaneous epilepsy (Yutsudo et al., 2013), demonstrating the importance of *Fosb* gene products in neurological and psychiatric disorders. Moreover, FOSB and FOSB expression is dramatically induced in response to numerous stimuli, especially in the striatum, hippocampus, and cerebral cortex. Notably, chronic cocaine treatment or chronic seizures induce the selective accumulation of FOSB (Hope et al., 1994; Hiroi et al., 1998; Kelz et al., 1999), whereas excitotoxicity induced by kainate or forebrain ischemia induces expression of FOSB and FOSB (Mandelzys et al., 1997; Kurushima et al., 2005; Yutsudo et al., 2013). These observations indicate that FOSB and

FOSB have important roles during the neurodegenerative process as well as in normal brain function. To date, several down-stream targets of FOSB and FOSB have been identified: e.g., *Cdk5*, *GluR2*, *Nfkb1* and *Pdyn* in the striatum (Chen et al., 2000; Bibb et al., 2001; McClung et al., 2004; Nestler, 2008), and *Gal*, *Trh* and *Penk* in the hippocampus (Yutsudo et al., 2013). Expression and functions of these genes has been primarily studied in neurons, and little is known about functions of FOSB and FOSB in glial cells.

Recently, it has been reported that glial cell functions are altered in psychiatric disorders, including autism, schizophrenia, and depression (Cotter et al., 2001). Importantly, neuroinflammation is involved in not only these psychiatric diseases (Vargas et al., 2005; Monji et al., 2009; Anisman and Hayley, 2012), but also in neurodegenerative diseases and epilepsy (Liu and Hong, 2003; Block and Hong, 2005; Vezzani et al., 2013). We thus

hypothesized that *Fosb* gene products are involved in the regulation of glial cell functions. In this study, we focused on *Fosb* gene function in glial cells and found that expression levels of *Fosb* and *Fosb* mRNAs in microglia are equivalent to those in hippocampal neurons. We then explored downstream targets of *Fosb* gene products by microarray analysis of isolated wild-type and *Fosb*-null microglia.

Materials and Methods

Mice

Fosb^{+G} mice with a null-mutant allele (*Fosb*^G) and *Fosb*^{+d} mice with a *Fosb*^d allele encoding FOSB and 2 FOSB but not FOSB and vFOSB were established and maintained by backcrossing to C57BL/6J mice (Clea Japan Inc., Tokyo, Japan), as described previously (Ohnishi et al., 2008; Ohnishi et al., 2011). *Fosb*^{+FN} embryonic stem (ES) cell clones with a *Fosb*^{FN} allele encoding FOSB and vFOSB, but not FOSB or 2 FOSB, and with a neomycin resistant gene cassette (Ohnishi et al., 2008), were injected into blastocysts prepared from C57BL/6J mice. Established *Fosb*^{+FN} mice were crossed with mice carrying Meox2-cre, to obtain *Fosb*^{+F} mice carrying the *Fosb*^F allele encoding FOSB and vFOSB, but not FOSB or 2 FOSB, without the neomycin resistant gene cassette. The *Fosb*^{+F} mouse line was maintained by backcrossing to C57BL/6J mice. To yield homozygous mutant mice, *Fosb*^{+d} (N24 generation) or *Fosb*^{+G} (N20 generation) mice were intercrossed. *Fosb*^{+F} (N7 generation) mice, in which all chromosomes except chromosome 7 carrying the *Fosb* locus were confirmed to be replaced by those derived from C57BL/6J using the speed congenic method (Markel et al., 1997), were also intercrossed. Genotyping of each allele was performed as previously described (Ohnishi et al., 2008). Homozygous mutant mouse lines *Fosb*^{G/G} (*Fosb*-null), *Fosb*^{d/d} and *Fosb*^{F/F} were maintained by inbreeding. C57BL/6J mice were used as wild-type controls for all experiments. All animals were maintained in an air-conditioned, specific pathogen-free room with a time-controlled lighting system. The handling and killing of animals were carried out in accordance with nationally prescribed guidelines. Ethical approval for the study was granted by the Animal Care and Use Committee of Kyushu University, Fukuoka, Japan and of Mount Sinai, NYC, USA.

Primary culture of microglia and astrocytes

Primary microglial cells and astrocytes were prepared from the cerebral cortex of 2-day-old mice. Isolation of microglial cells has been described previously (Ifuku et al., 2007). Mixed glial cells were cultured in DMEM high glucose medium (Life Technologies Japan, Tokyo, Japan) supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 5 µg/ml insulin, in 75-cm² flasks coated with Poly-D-lysine (PDL) (Sigma-Aldrich Japan, Tokyo, Japan), and the medium changed every three days. On the 10th day, microglial cells were isolated by shaking (80 rpm, 37°C, 10 min), and floating microglial cells were collected. After centrifugation, microglia were resuspended in medium and cultured. Microglia were harvested after 24 h. The cell composition of the cultures was checked by immunostaining using anti-GFAP (Mouse monoclonal anti-GFAP, G-A-5, 1:1500, Sigma-Aldrich Japan), anti-NeuN (mouse monoclonal anti-NeuN, A60, 1:250, Merck Millipore, Billerica, MA, USA), and anti-Iba-1 (rabbit polyclonal anti-Iba-1, 1:1000, Wako, Osaka,

Japan). Cultures containing more than 95% Iba-1-positive cells were used (data not shown). For primary astrocyte cultures, mixed glial cells were cultured in PDL-coated dishes, and the medium changed every three days. On the 10th day, 8 μ M 1- β -D-arabino-furanosylcytosine (AraC) (Wako) was added to the medium. On the 13th day, 75 mM L-Leucine methyl ester (Sigma-Aldrich Japan) was added to the cultures for 60 min, to remove microglial cells (Hamby et al., 2006). Astrocytes were harvested 24 h after L-Leucine methyl ester treatment. Only GFAP-positive cells, and no F4/80 (rat monoclonal anti-F4/80 antibody, CI:A4-1, 1:1000, SEROTEC, Oxford, UK) positive cells, were observed (data not shown).

Primary culture of hippocampal neurons

Primary hippocampal neurons were prepared from E18.5 mouse embryos (Brewer et al., 1993). After isolation, hippocampal tissue was treated with trypsin-EDTA solution (0.006% trypsin, 25 μ M EDTA and 20 U/ml DNase I in Hanks' balanced salt solution). Trypsinization was stopped by the addition of 10% inactivated FBS. After centrifugation, cells were suspended in medium (Neurobasal medium with B-27; Life Technologies Japan, 100 U/ml penicillin, 100 μ g/ml streptomycin and 2 mM L-glutamine), and filtered through a 40 μ m cell strainer. Cells were cultured in PDL-coated dishes. Two days after plating, 5 μ M AraC, in medium, was added to the medium and cells were incubated for 2 days. Subsequently half of the medium was changed every 3 days. On the 8th day, half of the medium was changed and hippocampal neurons were harvested 24 h later. 80% of cells were NeuN-positive and 3% were GFAP-positive, and no Iba-1-positive cells were observed (data not shown).

Chemotaxis assay

The ChemoTx® Disposable Chemotaxis system (Neuro Probe, Inc, Gaithersburg, MD, USA) was used to examine the chemotaxis of cultured microglia *in vitro*. The lower wells were filled with serum-free DMEM with or without 120, 60, or 12 ng/ml C5a (R&D Systems, Inc., Minneapolis, MN, USA), 120, 60, or 12 ng/ml C5a^{desArg} (Hycult Biotech, Uden, the Netherlands), or 100 μ M ATP (Sigma-Aldrich Japan). After the framed filter (8- μ m pore) was placed over the filled wells, 4.8×10^4 cells were added to the upper wells. After 30-min incubation, the filter was washed in PBS and fixed in 100% methanol in addition to wiping away the cells in the upper well. Cells on the filter were stained with Giemsa stain solution (Wako), and digital images were acquired using an AxioImager microscope, equipped with an AxioCam HRc camera (Carl Zeiss Microimaging Japan, Tokyo, Japan) using a 20 x bright field objective. The number of microglial cells that had moved onto the filter was counted using Image J 1.42q (National Institutes of Health, Bethesda, MD, USA). Three independent experiments were performed.

Kainate treatment

Saline or 25 mg/kg kainate (Wako) dissolved in saline was administered to ten-week-old male mice by intraperitoneal injection. To estimate the degree of epileptiform seizure, we observed mice for 90 min after kainate administration, and scored the seizure according to previously described criteria: 0, no reaction; 1, arrest of motion; 2, myoclonic jerks of the head and neck with brief twitching movements; 3, unilateral clonic activity; 4, bilateral

forelimb tonic and clonic activity; and 5, generalized tonic-clonic activity with loss of postural tone and death from continuous convulsion (Yang et al., 1997; Kajitani et al., 2009). Mice were sacrificed 24 h after kainate administration for further analysis.

RNA extraction and quantitative real-time PCR (qPCR)

Total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Life Technologies Japan). Quantitative real time PCR was performed using the Thunderbird qPCR kit (Toyobo, Osaka, Japan) and a Thermal Cycler Dice® Real Time System Single (Takara Bio, Shiga, Japan). The CT method was used to calculate relative values, with *Gapdh* as an internal control, which did not alter under the experimental conditions used. Primer sets used in the present study are listed in Table 1.

Microarray analysis

Total RNA isolated with ISOGEN was cleaned up using RNeasy Mini kits (Qiagen KK, Tokyo, Japan). Expression profiles were determined using Affymetrix Mouse Gene 1.0 ST arrays (Affymetrix Japan, Tokyo, Japan), according to the manufacturer's instructions. The Ambion WT Expression Kit (Ambion, Austin, TX, USA) and the GeneChip WT Terminal Labeling and Controls Kit (Affymetrix) were used to generate amplified and biotinylated sense-strand DNA targets from entire expressed transcripts (100 ng of total RNA). Manufacturer's instructions were followed for the hybridization, washing and scanning steps. CEL files were generated and imported into GeneSpring GX software (version 11.5.1, Agilent Technologies, Palo Alto, CA, USA) for further data characterization, as described previously (Dan et al., 2008). Microarray data were deposited in the GEO database (accession number GSE40714).

Western blotting

Western blotting analysis was performed as described previously (Ohnishi et al., 2011). Cell pellets or hippocampal tissues were lysed in 2 x SDS sample buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 10% glycerol) and sonicated. After centrifugation (15,000 x g, 4°C, 15 min), protein concentrations of the supernatants were determined using the DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Samples were diluted in 1 x SDS sample buffer with 2-mercaptoethanol and bromophenol blue (BPB), boiled, separated on 12.5% SDS-polyacrylamide gels, and then transferred to Immobilon-P membranes (Merck Millipore). The membranes were washed with TBST buffer [10 mM Tris (pH 7.5), 150 mM NaCl and 0.1% Tween 20], and blocked in TBST buffer containing 5% BSA. Membranes were treated with rabbit monoclonal anti-FOSB (5G4) (1:1000; Cell Signaling Technology Japan, K.K., Tokyo, Japan) or mouse monoclonal anti-GAPDH (1:100000; Merck Millipore), and then horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:10000; Merck Millipore) or HRP-conjugated anti-mouse Ig (1:2500; BD Biosciences, San Jose, CA, USA). Membranes were washed in TBST buffer and bound antibodies were visualized using Immobilon® Western HRP Detection Substrate (Merck Millipore). Digitized images were obtained with the LAS1000 Plus Luminescent Image Analyzer (Fuji Film, Tokyo, Japan).

Immunostaining

Animals were deeply anesthetized with pentobarbital (100 mg/kg, i.p.) then perfused intracardially with saline followed by 4% paraformaldehyde in PBS. Brains were removed and immersed in 4% paraformaldehyde, followed by 20% and 30% sucrose in PBS, for 24 h each at 4°C. The brains were embedded in Tissue-Tek® O.C.T compound (Sakura Finetek Co. Ltd. Tokyo, Japan) and frozen. Serial coronal sections (40 µm thickness) were prepared using a cryostat and collected as free-floating sections. After incubating with Block Ace (DS Pharma Biomedical Co., Ltd., Tokyo, Japan) for 30 min at room temperature, sections were incubated with rabbit polyclonal anti-Iba-1 (1:1000), rat monoclonal anti-C5aR1 (1:100, BIO-RAD Laboratories), rat monoclonal anti-CD68 (1:50, BIO-RAD Laboratories), or mouse monoclonal anti-Ki67 antibody (1:100, Leica Microsystems GmbH, Wetzlar, Germany) at 4°C overnight, and then with an appropriate second antibody [Alexa Fluor® 488 Goat Anti-Rat IgG (H+L), Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L), Alexa Fluor® 594 Goat Anti-Rabbit IgG (H+L) Antibody; 1:300, Invitrogen] for 45 min at room temperature. The sections were further incubated with 0.05 µg/ml DAPI (Sigma-Aldrich Japan KK) for 10 min at room temperature, and mounted on slides with Vectashield (Vector Laboratories, Burlingame, CA, USA). Confocal immunofluorescence images were acquired using a laser scanning confocal microscope system, LSM510 META or LSM700 (Carl Zeiss MicroImaging Japan, Tokyo, Japan). The region of interest was selected in the CA3 region, including the stratum lucidum, and pyramidal layer. For C5aR1 and Iba-1 double immunostaining, individual plane images were acquired, while for CD68 or Ki67 and Iba-1 double immunostaining, projection images of z-stack series (Z step, 21 µm; 15 planes for low magnification, Z step, 30 µm; 30 planes for high magnification) were acquired. All sections from each experimental animal in the groups to be compared were processed in parallel. From each animal, six coronal sections, 160 µm apart (bregma -1.46 to -2.48 mm) were prepared from three mice from each group. To count CD68 positive microglial cells, 120 Iba-1 positive cells in each individual animal were selected without CD68 signals using Image J. Then, the expression levels of CD68 were evaluated in each Iba-1 positive cell, as Level 0 to Level 2: Level 0; completely CD68-negative, Level 1; exhibiting less than six CD68 positive dots in a single cell body, Level 2; carrying more than five CD68 positive dots or homogeneous immunoreactivity in a cell body. Three mice from each group were prepared for this analysis.

Morphological analysis of microglia

Confocal immunofluorescence images for Iba-1 were analyzed using Image J. Sixty microglial cells were analyzed in each mouse (n = 3). The images were converted to binary images, and the Iba-1 positive cell body area was measured. To count the number of processes in the Iba-1 positive cells, Iba-1 positive processes derived from each individual cell body were counted. The thickness of individual processes was measured at the boundary between each process and the cell body.

Counting Iba-1 positive cells using unbiased stereological counting techniques

To count Iba-1 positive microglia, free-floating sections were processed for immunohistochemistry using an anti-Iba-1 antibody (1:500) according to previously

described methods (Kajitani et al., 2006). After visualization of peroxidase reaction product using 3'3'-diaminobenzidine-tetrahydrochloride (DAB) (Vector Laboratories), sections were stained with Mayer's Hematoxylin (Muto Pure Chemicals, Tokyo, Japan) to detect cell nuclei and delineate the boundary of the hippocampal region. The sections were mounted on slides with Glycergel® Mounting Medium (Dako, Glostrup, Denmark). From each individual animal, six coronal sections, 160 µm apart (bregma -1.46 to -2.48 mm), were prepared following systematic random sampling. The number of Iba-1 positive cells in the hippocampal CA3 region, including the stratum lacunosum-moleculare, stratum radiatum, stratum lucidum, and pyramidal layer (Long et al., 1998) were counted blind, using the fractionator program of the Stereo Investigator system (MBF Bioscience, Williston, VT) (Yutsudo et al., 2013). For estimates of microglia density in the CA3 region, the area sampling fraction (asf) = 0.174 mm² [a(step) = 0.0144 mm²; a(frame) = 0.0025 mm²]. Five mice of each group were examined.

Statistical analysis

All results are expressed as the mean ± SEM. Statistical analysis for all data except microarray data was performed using JMP® Pro 9.0.2 software (SAS Institute, Cary, NC, USA). Each method of statistical analysis is shown in detail in each figure legend. Microarray data were compared using unpaired t-test using GeneSpring GX software. A value of $p < 0.05$ was considered statistically significant.

Results

Expression of the *Fosb* gene in microglia, astrocytes and neurons

Primary microglia and astrocytes from the cerebral cortex, and neurons from the hippocampus, were prepared from neonatal C57BL/6J mice to analyze the expression of the *Fosb* gene. RNA was extracted from the cells and relative levels of total *Fosb* mRNAs were compared using qPCR (Fig. 2A). Total *Fosb* mRNA levels were approximately the same in microglia and hippocampal neurons. In astrocytes, total *Fosb* mRNA levels were 40% lower than in microglia and neurons. Because expression of *Fosb* gene products in microglia, in contrast to neurons, has not previously been characterized, we investigated *Fosb* gene function in microglia. We isolated microglia from the cerebral cortex of wild-type and *Fosb*-null mice and examined expression levels of *Fosb* and *Fosb* mRNAs by RT-PCR using a primer set amplifying the alternatively spliced region in exon 4. In wild-type microglia, two RT-PCR products corresponding to the *Fosb* and *Fosb* mRNAs, respectively, were detected, but none were detected in *Fosb*-null microglia (Fig.2B).

Microarray analysis of wild-type and *Fosb*-null microglia

To identify genes whose expression is controlled by *Fosb* gene products in microglia, we performed microarray analysis using RNA prepared from wild-type and *Fosb*-null microglia. The expression levels of six genes were significantly altered with a fold change of 1.5 (ANOVA, $p < 0.05$; Table 2), and all six genes were significantly decreased in *Fosb*-null microglia, compared with wild-type (Table 2). Among these six genes, we focused on *C5ar1* and *C5ar2*. *C5ar1* showed the highest raw expression levels in wild-type microglia. Both *C5ar1* and *C5ar2* encode complement C5a receptors (Klos et al., 2009; Ward, 2009).

C5a is the final product of a complement pathway that is a part of the innate immune system. C5a has strong chemoattractant activity (Guo and Ward, 2005), and thus controls microglial motility (Nolte et al., 1996).

Expression of *C5ar1* and *C5ar2* genes and their functional significance in cultured microglia

To confirm the microarray data, we analyzed expression levels of *C5ar1* and *C5ar2* mRNAs using qPCR. Expression levels of *C5ar1* and *C5ar2* mRNAs in *Fosb*-null microglia were significantly decreased compared with those in wild-type (Fig. 3A & B). The *C5ar1* and *C5ar2* genes encode C5a anaphylatoxin chemotactic receptor 1 (C5aR1) and 2 (C5aR2), respectively. Strong C5aR1 immunoreactivity was found in the plasma membrane of cultured wild-type microglia, while no C5aR1 immunoreactivity was detectable in *Fosb*-null microglia (Fig. 3C).

Furthermore, we performed chemotaxis assays using the ChemoTx® Disposable Chemotaxis system with or without ATP, C5a and C5a^{desArg} (Fig. 3D). When cultured microglial cells were exposed to ATP, equivalent numbers of wild-type and *Fosb*-null cells moved towards the ATP, indicating that *Fosb* deficiency has no effect on the basic motility of cells. When exposed to C5a at concentration of 12 to 120 ng/ml, *Fosb*-null microglia exhibited similar motility to wild-type microglia. In contrast, microglia exposed to C5a^{desArg} exhibited dose-dependent motility, and *Fosb*-null microglia exhibited attenuated motility towards C5a^{desArg} compared with wild-type microglia (Fig. 3D), thus suggesting that *Fosb* may be involved in the regulation of microglial function. C5a^{desArg} is the desarginated form of C5a, and has higher affinity to C5aR2 than to C5aR1, while C5a binds to both C5aR1 and C5aR2 with similarly high affinity (Cain and Monk, 2002; Klos et al., 2009).

To compare the expression levels of *C5ar1* and *C5ar2* between the microglia, neurons and astrocytes, we examined levels of these two mRNAs in isolated microglia, astrocytes, and hippocampal neurons from wild-type mouse brains. Expression levels of both *C5ar1* and *C5ar2* mRNAs were highest in microglia, much lower in neurons and no expression was observed in astrocytes (Fig. 3E & F), indicating that *C5ar1* and *C5ar2* are mainly expressed in microglia in the mouse brain.

Fosb deficiency reduces basal and kainate-induced expression of *C5ar1* and *C5ar2* in the hippocampus

It has been reported that kainate can induce expression of both *Fosb* products and C5aR1 in the hippocampus (Mandelzys et al., 1997; Osaka et al., 1999; Yutsudo et al., 2013). Kainate is an agonist for kainate-class ionotropic glutamate receptors, and systemic administration of kainate to mice induces excitotoxicity, neurodegeneration and neuroinflammation (Wang et al., 2005).

To investigate the *in vivo* effect of *Fosb* deficiency in microglia, particularly in the neuroinflammatory state, kainate or saline was systemically administered to wild-type and *Fosb*-null mice. Western blot analysis of hippocampal extracts revealed that kainate treatment increases FOSB protein levels and to a lesser extent FOS protein levels in wild-

type but not *Fosb*-null hippocampus (Fig. 4A). Compared with wild-type, basal levels of *C5ar1* mRNA were significantly decreased in *Fosb*-null hippocampus, and in kainate-treated hippocampus the *C5ar1* mRNA level was less than 40% of the level detected in the wild-type condition (Fig. 4B). Basal levels of *C5ar2* mRNA in *Fosb*-null hippocampus were likewise significantly decreased compared with wild-type; however, *C5ar2* levels were not increased after kainate treatment (Fig. 4C).

Laser scanning confocal immunofluorescence microscopy revealed that C5aR1 expression was induced in the CA3 region of wild-type but not *Fosb*-null hippocampus after kainate treatment (Fig. 4D). The hippocampal CA3 region is the most sensitive region to excitotoxicity induced by kainate administration (Pollard et al., 1994; Rogove and Tsirka, 1998), and microglial cells are known to be activated 24 hours after kainate administration (Chen et al., 2005). In the CA3 region of kainate-treated wild-type hippocampus, most C5aR1 immunoreactivity was colocalized with Iba-1 immunoreactivity, indicating that activated microglia in wild-type but not *Fosb*-null hippocampus express C5aR1, as we observed in cultured microglia *in vitro* (Fig. 3A & C).

We then observed the seizure response of mice during the 90 minutes after kainate administration. Wild-type mice exhibited a severe seizure response within 35 min after kainate administration, and typically showed score 4 or 5 seizures (Fig. 4E). Forty percent of wild-type mice died within 90 min (Fig. 4F). In contrast, *Fosb*-null mice exhibited a relatively mild seizure response within 60 min after kainate administration, and showed seizure scores below 4 (Fig. 4E). No death of *Fosb*-null mice was observed within 90 min after kainate administration (Fig. 4F). We thus concluded that *Fosb*-null mice acquired resistance to kainate-induced excitotoxicity compared with wild-type mice.

Attenuated neuroinflammatory response in *Fosb*-null hippocampus after kainate treatment

It has been reported that C5a induces a proinflammatory response (Rittirsch et al., 2008; Ward, 2009), and that complement activation and neuroinflammation occur after brain injuries (Woodruff et al., 2010). To evaluate whether *Fosb* deficiency affects microglial activation, we examined CD68 immunoreactivity in the hippocampus as a marker for activated microglia. CD68 is an oxidized LDL-binding protein and is highly expressed in activated microglia performing phagocytosis (Streit and Xue, 2009). CD68 immunoreactivity was found in both kainate-treated wild-type and *Fosb*-null hippocampal CA3 regions but only low levels were observed in both saline-treated wild-type and *Fosb*-null hippocampal CA3 regions (Fig. 5A). This immunoreactivity was mostly colocalized with that of Iba-1 (Fig. 5B). We then compared the percentages of CD68-positive microglial cells in the hippocampal CA3 regions between wild-type and *Fosb*-null mice (Fig. 5C). More than 90% of microglia detected in untreated wild-type or *Fosb*-null CA3 regions exhibited no (Level 0) or weak (Level 1) CD68 immunoreactivity. After kainate administration, percentages of microglia with a strong CD68 immunoreactivity (Level 2) were significantly increased in wild-type but not *Fosb*-null CA3 regions.

It has been documented that activated microglia reduce the complexity of their shape by shortening (retracting) the branches of their processes so that they are resorbed into the cell body; the cell body thus becomes enlarged and the processes thicken (Kettenmann et al.,

2011). To compare the extent of morphological changes in microglia between wild-type and *Fosb*-null mice, we measured microglial cell body areas and the number and thickness of their processes in the hippocampal CA3 region (Fig. 5D, E and F). After kainate administration, microglial cell body areas were markedly increased in both wild-type and *Fosb*-null mice; however, the extent of this increase was significantly reduced in *Fosb*-null mice compared with wild-type mice (Fig. 5D). Accordingly, kainite-induced increases in the number and thickness of microglial processes in *Fosb*-null mice were significantly attenuated compared with that in wild-type mice (Fig. 5E and F). We thus concluded that morphological changes in microglial cells induced under excitotoxicity largely depend on *Fosb* gene products.

Proinflammatory cytokine mRNAs, for example, *Il1b*, *Il6* and *Tnf*, are known to be up-regulated during microglial activation (Zhang and Zhu, 2011). After kainate treatment, expression levels of *Il6* and *Tnf* mRNAs were increased in wild-type hippocampus, while those in *Fosb*-null hippocampus were significantly lower compared to those in wild-type (Fig. 5G). We thus concluded that *Fosb* deficiency diminishes the neuroinflammatory response induced by kainate administration.

We further compared the density of Iba-1 positive cells in the hippocampal CA3 region between wild-type and *Fosb*-null mice using unbiased stereological counting techniques (Fig. 6A). There was no difference in the basal density of Iba-1 positive cells in hippocampal CA3 regions between wild-type and *Fosb*-null mice. An approximately 1.5-fold increase in the density of Iba-1 positive cells was observed in both wild-type and *Fosb*-null CA3 regions 24 hours after kainate administration. There were few Iba-1 positive cells expressing a proliferative marker, Ki67 in both wild-type and *Fosb*-null CA3 regions 24 hours after kainite administration (Fig. 6B & C), suggesting that Iba-1 positive cells detected in the hippocampal CA3 region after kainate administration are likely to have infiltrated from surrounding tissues or via circulation and that *Fosb* gene products do not affect their infiltration.

Proteins encoded by both *Fosb* and *Fosb* mRNAs are required for expression of *C5ar1* and *C5ar2* genes in microglia

Our data demonstrated that *Fosb* gene products regulate the microglial expression of *C5ar1* and *C5ar2* genes both *in vitro* and *in vivo*. Because *Fosb*-null mice completely lack proteins encoded by both *Fosb* and *Fosb* mRNAs, we further investigated which proteins are required for expression of *C5ar1* and *C5ar2* genes in microglia. We have established two different mouse lines carrying a modified *Fosb* allele, *Fosb^F* or *Fosb^d*; the former encodes only FOSB and vFOSB proteins and the latter encodes FOSB and 2 FOSB proteins (Ohnishi et al., 2008; Ohnishi et al., 2011) (Fig. 7A).

Expression levels of *C5ar1* and *C5ar2* mRNAs were compared between microglia isolated from wild-type, *Fosb*-null, *Fosb^{F/F}* and *Fosb^{d/d}* mice using qPCR analysis (Fig. 7B). Expression levels of *C5ar1* and *C5ar2* mRNAs in *Fosb*-null, *Fosb^{F/F}* and *Fosb^{d/d}* microglial cells were significantly and similarly decreased compared with those in wild-type microglia. These results indicated that proteins encoded by both *Fosb* and *Fosb* mRNAs are required for normal expression of *C5ar1* and *C5ar2* genes in microglia.

Discussion

Our main conclusion is that in microglia *Fosb* gene products encoded by both *Fosb* and *Fosb* mRNAs are required for basal expression of both *C5ar1* and *C5ar2* genes, but only for kainate-induced expression of the *C5ar1* gene, and are necessary for microglial activation in the brain. Furthermore, we showed that *Fosb*-null mice exhibited significant resistance to kainate-induced seizures with attenuated activation of microglia in the hippocampus.

To date, functions of *Fosb* gene products in brain have been characterized almost exclusively in neurons, because neurons uniquely express high levels of *Fosb* products in response to several brain stimuli (Hope et al., 1994; Mandelzys et al., 1997; McClung et al., 2004; Kurushima et al., 2005; Nestler, 2008; Ohnishi et al., 2011). In the present study, we revealed for the first time that the *Fosb* gene is also expressed in microglia as well as neurons in the brain, and regulates different target genes in each type of brain cell. Although basal levels of *Fosb* gene products in hippocampal neurons are low compared to their levels in striatal medium spiny neurons (Nestler, 2008), we found that in primary cultured microglial cells, total *Fosb* mRNA levels were as high as in primary cultured hippocampal neurons (Fig.2). Given the appreciable expression of the *Fosb* gene in cortical microglia, we explored downstream target genes of *Fosb* products in microglia using microarray analysis. Expression levels of six genes including *C5ar1* and *C5ar2* were significantly decreased in *Fosb*-null microglia compared with wild-type. We focused on *C5ar1* and *C5ar2*, because of their function in the innate immune system.

The *C5ar1* and *C5ar2* genes encode C5aR1 and C5aR2, respectively; both are receptors for the anaphylatoxin C5a derived from complement C5. C5aR1 is present at the cell surface and mediates robust cell responses to C5a, while C5aR2 is predominantly intracellular and its function is still controversial (Bamberg et al., 2010; Li et al., 2013). Because microglia have higher expression levels of *C5ar1* and *C5ar2* mRNAs than neurons and astrocytes (Fig. 3), we further investigated properties of isolated microglia from wild-type and *Fosb*-null mice. Levels of both *C5ar1* and *C5ar2* mRNAs were significantly decreased in *Fosb*-null microglia, the former to less than 30% and the latter to less than 10% of wild-type microglial levels. C5aR1 immunoreactivity was detected mostly at the cell surface in wild-type microglia, and was not detected in *Fosb*-null microglia (Fig.3). Exposure of isolated microglia to C5a or C5a^{desArg}, a desarginated form of C5a, induced a chemotactic response. Interestingly, a reduced chemotactic response was observed in comparison with wild-type, only when *Fosb*-null microglia were exposed to C5a^{desArg}. The affinity of C5a for C5aR1 has been reported to be much higher than that of C5a^{desArg}, and about 30% of the wild-type level of *C5ar1* mRNA still remained in *Fosb*-null microglia, thus suggesting that the remaining C5aR1 in *Fosb*-null microglia might be enough to achieve a similar level of response to C5a as observed in wild-type microglia (Fig. 3D).

Two mature mRNAs, *Fosb* and *Fosb*, are formed by alternative splicing of the *Fosb* transcript in wild-type microglia (Fig. 1B). Because *Fosb*-null microglia express neither *Fosb* nor *Fosb* mRNAs, we used two different mouse lines carrying modified *Fosb* alleles, *Fosb*^F or *Fosb*^d, the former encoding only FOSB and vFOSB proteins and the latter

encoding only FOSB and 2 FOSB proteins (Fig. 7A). Our results indicate that products produced from both *Fosb* and *Fosb* mRNAs play a role in regulating expression of *C5ar1* and *C5ar2* genes in microglia (Fig. 7B), probably through their interaction with certain cis-elements in the genes. To understand the precise molecular mechanism of the regulation, it is essential in future work to identify which forms of *Fosb* gene products bind such cis-elements.

In mouse hippocampus, expression of *C5ar1* but not *C5ar2* was highly induced in after kainate administration, although basal levels of both mRNAs were significantly decreased in *Fosb*-null hippocampus compared with wild-type (Fig. 4). The cultured microglial cells showed the highest expression of *C5ar1* and *C5ar2* among the three types of brain cells examined (Fig. 3E & F), suggesting that hippocampal expression of *C5ar1* and *C5ar2* can be attributed to their expression mainly in microglia. Because expression levels of both *C5ar1* and *C5ar2* genes were significantly decreased in microglia isolated from *Fosb*-null, *Fosb^{F/F}* and *Fosb^{d/d}* mice compared with wild-type microglia (Fig. 7B), it is likely that other factor(s) are involved in kainate-induced expression of the *C5ar1* gene in hippocampus. Furthermore, we investigated various aspects of microglial activation after kainate administration (Fig. 5). The *Fosb*-null hippocampal CA3 region exhibited essentially the same density of microglial cells as seen in the wild-type with or without kainate administration (Fig. 6A). However, the *Fosb*-null microglia showed significant attenuation of morphological changes and expression of CD68, a marker of activated microglia with phagocytosis (Streit and Xue, 2009), upon kainate administration accompanied by decreased levels of *Il6* and *Tnf* mRNAs in the hippocampus. We thus concluded that *Fosb* promotes a neuroinflammatory response through the regulation of *C5ar1* and/or *C5ar2* gene expression in hippocampus.

It has been shown that microglial proliferation takes place 2 to 3 days after acute neuronal injury (Ladeby et al., 2005). It is therefore likely that the increase of microglial cell density in the hippocampal CA3 region 24 hours after kainate administration resulted from cell migration or infiltration rather than from in situ-proliferation. It was reported that kainate injection into the hippocampus causes microglial activation in osteopetrotic (op/op) mice in which injury-induced microglial proliferation does not take place, (Rogove et al., 2002). Excitotoxicity is thus likely to activate multiple pathways which differentially regulate microglial activation, proliferation and migration. *Fosb* gene products play a major role in this hippocampal microglial activation, including morphological and phagocytic changes accompanied by induction of *Il6* and *Tnf* mRNAs (Fig. 5).

It is noteworthy that *Fosb*-null mice acquired significant resistance to the kainate-induced seizure response (Fig. 4E & F). In a previous study, mice deficient in Toll-like receptor 4 (TLR4) were resistant to kainate-induced seizures in which high mobility group box 1 protein (HMGB1), a proconvulsant released from neurons and glia, interacts with TLR4 (Maroso et al., 2010). In addition, C5aR2 is required for the release of HMGB1 during sepsis (Rittirsch et al., 2008). Moreover, TLR4-induced expression of proinflammatory cytokines, such as IL-6 and TNF α , is synergistically augmented by C5a (Zhang et al., 2007). Taken together with these previous findings, our data suggest that the decreased expression

of C5aR1 and C5aR2 in *Fosb*-null microglia is responsible for the decreased acute seizure recurrence induced by kainate administration.

Neuroinflammation is a pivotal event in the CNS, especially in neurodegenerative disorders such as Alzheimer's disease (AD) (Wyss-Coray and Mucke, 2002). In AD, accumulating amyloid- β results in complement activation and a local, chronic inflammatory response that leads to attraction and activation of glial cells, thus producing proinflammatory cytokines (Veerhuis et al., 2011). It has been shown that C5aR1 antagonists reduce neuroinflammation and AD pathology in AD mouse models (Ager et al., 2010; Woodruff et al., 2010); however, involvement of C5aR2 remains to be established. In addition, over-activated microglia mediate neurotoxicity and worsen the progression of neurodegenerative disorders such as AD (Block et al., 2007). Therefore, restriction of *Fosb* gene expression may prevent over-activation of microglia and slow the progression of neurodegenerative disorders.

Moreover, it has been reported that complement components C1q and C3 play an important role in activity-dependent synaptic pruning by microglia especially in the postnatal retinogeniculate system (Stevens et al., 2007; Schafer et al., 2012). Both C1q and C3 are upstream of C5a production, and C5a signaling may be involved in this mechanism. Moreover, C1q knockout mice display enhanced seizure liability (Chu et al., 2010), and *Fosb*-null mice display spontaneous seizures in adulthood (Yutsudo et al., 2013) and a significant delay in the development of tolerance to electroconvulsive seizures (Hiroi et al., 1998), despite their reduced sensitivity to kainate shown in the present study. Further work is needed to investigate the possibility that abnormal synapse pruning in *Fosb*-null mice, because of a reduction of C5aR1 and C5aR2 in microglia, contributes to their abnormal seizure activity.

In summary, *Fosb* gene products regulate the expression of *C5ar1* and *C5ar2* genes encoding complement C5a receptors in microglia. The lack of the *Fosb* gene causes decreased microglial activation after kainate treatment. To regulate *C5ar1* and *C5ar2*, both FOSB and β -FOSB proteins appear to be required. Consequently, *Fosb* gene products contribute to microglial activation in the brain by regulating the expression of the complement C5a receptor genes *C5ar1* and *C5ar2* in microglia.

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Figure 1.

Genomic organization of the mouse *Fosb* gene, and its transcripts (*Fosb* and *Fosb* mRNAs) and translation products (FOSB, vFOSB, FOSB, and Δ 2 FOSB proteins) are shown. Yellow box; FH, N-terminal Fos homology domain; light blue box; BZIP, basic region and leucine zipper; blue box; C-TA, C-terminal transactivation domain; orange box; TBP-BD, TBP-binding domain. Each pair of dotted lines indicates the splicing of each intron and a red box indicates an "exonic intron" in exon 4, which is alternatively spliced out (red dotted lines). The black line shows primer position; primer set A (*Fosb* [A]) is for qPCR analysis of total *Fosb* mRNAs; primer set B (*Fosb* [B]) is for RT-PCR analysis of *Fosb* and *Fosb* mRNAs (Table 1).

Figure 2.

Quantitative detection of *Fosb* and *Fosb* mRNAs in isolated cortical microglia, astrocytes and hippocampal neurons. A. Relative expression levels of total *Fosb* mRNAs in wild-type microglia, neurons and astrocytes. Relative expression levels of total *Fosb* were estimated using microglial expression as a standard. Error bars show mean $\pm$ SEM. B. Agarose gel electrophoresis of *Fosb* PCR products in wild-type (WT) and *Fosb*-null (Null) microglia. PCR amplification results in different size fragments of *Fosb* (624 bp) and *Fosb* (484 bp). Control PCR reactions contain pBSKS(-)*Fosb* and pBSKS(-) *Fosb* plasmids (Nakabeppu and Nathans, 1991), either in combination (lane *Fosb*+ *Fosb*) or individually (lanes *Fosb* and *Fosb*), and were used to amplify *Fosb* and *Fosb*.

Figure 3.

Expression of *C5ar1* and *C5ar2* in isolated microglia, astrocytes and hippocampal neurons. A & B. Relative expression levels of *C5ar1* (A) and *C5ar2* (B) mRNAs in wild-type (WT) and *Fosb*-null (Null) microglia. Relative expression levels were estimated using the expression level in wild-type as a standard. * $p < 0.0001$ (Student's *t*-test). Error bars show mean $\pm$ SEM. C. C5aR1 expression in cultured microglia. Cultured microglia isolated from wild-type (WT) and *Fosb*-null (Null) mice were subject to laser scanning confocal immunofluorescence microscopy. Blue, DAPI; green, Iba-1; red, C5aR1. Scale bar = 20 μ m. D. Chemotaxis assay of wild-type (WT) and *Fosb*-null (Null) microglia. Number of cells moved means the number of cells traversing the porous membrane. Each experiment was independently repeated three times. Ctrl, without any chemoattractant; C5a and C5a^{desArg}, 120, 60, or 12 ng/ml of C5a or C5a^{desArg}, respectively; ATP, 100 μ M ATP. *Fosb*-null microglia exhibited significantly reduced chemotaxis towards 60 ng/ml C5a^{desArg} but not C5a or ATP, compared with wild-type * $p = 0.033$ (Student's *t*-test). Error bars show mean $\pm$ SEM. E & F Relative expression of *C5ar1* (E) and *C5ar2* (F) mRNAs in microglia, neurons and astrocytes. Relative expression levels were estimated using the expression level in astrocytes as a standard. E. One way ANOVA ($F_{2,6} = 165.13$, $p < 0.0001$, * $p < 0.0001$, Tukey-Kramer HSD *post hoc* comparison). F. One way ANOVA ($F_{2,6} = 68.64$, $p < 0.0001$, * $p = 0.0001$, Tukey-Kramer HSD *post hoc* comparison). Error bars show mean $\pm$ SEM. To quantify the expression level of each mRNA, total RNA was reverse-transcribed to cDNA and used for qPCR analysis using appropriate primer sets (Table 1).

Figure 4.

Fosb-dependent expression of *C5ar1* and *C5ar2* in mouse hippocampus. **A.** Western blotting of *Fosb* gene products. FOSB, 43 kDa; FOSB, 34-37 kDa; vFOSB, 31 kDa (see Fig. 7A); 2 FOSB, 25 kDa; GAPDH protein was detected as an internal control. **B & C.** Expression levels of *C5ar1* (**B**) and *C5ar2* (**C**) mRNAs in wild-type (WT) and *Fosb*-null (Null) hippocampus, 24 h after saline, or kainate (KA) injection. Relative expression levels were estimated using the expression level in wild-type hippocampus treated with saline as a control. * $p = 0.0059$, ** $p = 0.0089$, # $p = 0.0128$, ## $p < 0.0001$ (Student's *t*-test). Error bars show mean $\pm$ SEM. To quantify the expression level of each mRNA, total RNA was reverse-transcribed to cDNA and used for qPCR analysis using appropriate primer sets (Table 1). **D.** Inducible expression of C5aR1 in hippocampal CA3 region after kainate administration. Coronal sections prepared from wild-type (WT) and *Fosb*-null (Null) mouse

brains were subjected to laser scanning confocal immunofluorescence microscopy with anti-C5aR1 and anti-Iba-1 antibodies. Nuclei were counter-stained with DAPI. Top panels: Merged images of samples 24 h after saline injection. Scale bar = 100 μ m. Middle panels: Merged images of samples 24 h after kainate injection. Scale bar = 100 μ m. Bottom panels: High magnification images of samples 24 h after kainate injection. Blue, DAPI; green, C5aR1; red, Iba-1. Scale bar = 50 μ m. E. Seizure responses after kainate administration. *Fosb*-null mice exhibited significantly reduced seizure responses to kainate-induced excitotoxicity compared with wild-type mice (Generalized Linear Model, Difference; $p < 0.0001$, $\chi^2=821.7$, $df = 39$, Genotype; $p < 0.0001$, time; $p < 0.0001$, Interaction; $p < 0.0001$, WT N = 28, *Fosb*-null N = 10). Error bars show mean $\pm$ SEM. F. Survival rate after kainate administration. No *Fosb*-null mice died within 90 min after kainate administration (Kaplan-Meier method and log-rank test, $p = 0.012$, $\chi^2 = 6.14$, $df = 1$; WT, N = 32; *Fosb*-null, N = 12).

Figure 5.

Attenuated microglial activation in *Fosb*-null hippocampus 24 h after kainate administration.

A. Detection of activated microglia in hippocampal CA3 region 24 h after kainate injection. Coronal sections prepared from wild-type (WT) and *Fosb*-null (Null) mouse brains were subjected to laser scanning confocal immunofluorescence microscopy with anti-CD68 and anti-Iba-1 antibodies. Nuclei were counter-stained with DAPI. Top panels: Merged z-stacked images of samples 24 h after saline injection. Bottom panels: Merged z-stacked images of samples 24 h after kainate injection. Blue, DAPI; green, CD68; red, Iba-1. Scale bars = 100 μm. B. Higher magnification images of Figure 5A. Scale bars = 50 μm. C. The percentages of double CD68 and Iba-1 positive cells in *Fosb*-null (Null) CA3 regions 24 h after kainate administration compared with those in wild-type (WT). Level 0 (Lv. 0); completely CD68-negative cells, Level 1 (Lv. 1); exhibiting less than six CD68-positive dots in a single cell body, Level 2 (Lv. 2); carrying more than five CD68-positive dots or homogeneous

immunoreactivity in a cell body. One way ANOVA ($F_{3,8} = 26.35$, $p = 0.0002$ (Lv.2), $F_{3,8} = 25.01$, $p = 0.0002$ (Lv.1), * $p = 0.0002$, ** $p = 0.003$, # $p = 0.0003$, ## $p = 0.0021$, Tukey-Kramer HSD *post hoc* comparison). D. Microglial cell body areas in *Fosb*-null (Null) CA3 regions 24 h after kainate administration compared with those in wild-type (WT). One way ANOVA ($F_{3,716} = 274.38$, $p < 0.0001$, * $p < 0.0001$, Tukey-Kramer HSD *post hoc* comparison). E. Number of microglial processes in *Fosb*-null (Null) CA3 regions 24 h after kainate administration, compared with those in wild-type (WT). One way ANOVA ($F_{3,716} = 64.96$, $p < 0.0001$, * $p < 0.0001$, Tukey-Kramer HSD *post hoc* comparison). F. Thickness of microglial processes in *Fosb*-null CA3 regions 24 h after kainate administration compared with those in wild-type (WT). One way ANOVA ($F_{3,716} = 91.61$, $p < 0.0001$, * $p < 0.0001$, Tukey-Kramer HSD *post hoc* comparison). G. Reduced expression of *Il1b*, *Il6*, and *Tnf* mRNAs in *Fosb*-null (Null) hippocampus 24 h after kainate administration, compared with wild-type (WT). Saline, 24 h after saline injection; KA, 24 h after kainate injection. Relative expression levels were estimated using the expression level in wild-type treated with saline as a control. One way ANOVA [$F_{3,8} = 10.98$, $p = 0.0033$, * $p = 0.0426$, Hsu's multiple comparisons with best (MCB)]. One way ANOVA ($F_{3,8} = 31.50$, $p < 0.0001$, # $p = 0.0025$, Tukey-Kramer HSD *post hoc* comparison). Error bars show mean $\pm$ SEM. To quantify expression level of each mRNA, total RNA was reverse-transcribed to cDNA and used for qPCR analysis using appropriate primer sets (Table 1).

Figure 6.

Density of microglial cells in the hippocampal CA3 region was similarly increased in both wild-type and *Fosb*-null hippocampus 24 h after kainate administration. **A.** The density of Iba-1 positive cells in hippocampal CA3 region. One way ANOVA ($F_{3,16} = 7.36$, $p = 0.0026$, $*p = 0.0178$, $**p = 0.023$, Tukey-Kramer HSD *post hoc* comparison). **B & C.** Detection of proliferating microglia in hippocampal CA3 region 24 h after kainate injection. Coronal sections prepared from wild-type (WT) (**B**) and *Fosb*-null (Null) (**C**) mouse brains were subjected to laser scanning confocal immunofluorescence microscopy with anti-Ki67 and anti-Iba-1 antibodies. Nuclei were counter-stained with DAPI. Blue, DAPI; green, Ki67; red, Iba-1. Scale bars = 50 μm .

Figure 7.

Reduced expression of *C5ar1* and *C5ar2* mRNAs in microglia isolated from several lines of *Fosb* mutant mice. A. Western blotting of *Fosb* gene products in wild-type (WT), *Fosb*-null (Null), *Fosb^{F/F}* (F/F), and *Fosb^{d/d}* (d/d) hippocampus. FOSB, 43 kDa; ΔFOSB, 34-37 kDa;

2 FOSB, 25 kDa. vFOSB (31 kDa) was only detected in *Fosb^{F/F}* hippocampus. GAPDH protein was detected as an internal control. B. Expression levels of *C5ar1* and *C5ar2* mRNAs in wild-type (WT), *Fosb*-null (Null), *Fosb^{F/F}* (F/F) and *Fosb^{d/d}* (d/d) microglial cells. Relative expression levels were estimated using the expression level in wild-type as a control. One way ANOVA ($F_{3,8} = 14.60$, $p = 0.0013$ (*C5ar1*), $F_{3,8} = 98.10$, $p < 0.0001$ (*C5ar2*), * $p = 0.0026$, ** $p = 0.002$, *** $p = 0.0051$, # $p < 0.0001$, Tukey-Kramer HSD *post hoc* comparison). Error bars show mean ± SEM. To quantify the expression level of each mRNA, total RNA was reverse-transcribed to cDNA and used for qPCR analysis using appropriate primer sets (Table 1).

Table 1

Primers used for PCR analysis

Primer set	Upstream (5'-3')	Downstream (5'-3')
<i>qPCR</i>		
<i>Gapdh</i>	AAATGGTGAAGGTCGGTGTG	TGAAGGGGTCGTTGATGG
<i>Fosb</i> [A]	GCTCCCGGTGTAGTCATCA	CTGAAGATCCTGGCTGGTTGTG
<i>C5ar1</i>	GTCCTGTTACGACCGTTTT	ACGGTCGGCACTAATGGTAG
<i>C5ar2</i>	CTACCGTAGGCTGCTTCAGG	AGCCAAAGAGGAATCGAACA
<i>Il1b</i>	TGCACTACAGGCTCCGAGAT	AGGCCACAGGTATTTTGTCTG
<i>Il6</i>	ACTTCACAAGTCGGAGGCTT	GCAAGTGCATCATCGTTGTTC
<i>Tnf</i>	TGTCCCTTTCACTCACTGGC	CATCTTTTGGGGGAGTGCCT
<i>RT-PCR</i>		
<i>Fosb</i> [B]	GAAGAGACACTTACCCAGAAAG	CTCCTTGTTCCTTGCGGGTTTG

Table 2

List of genes for which expression was significantly altered between microglia derived from wild-type and *Fosb*-null mice.

Gene	Expression change		Raw expression level (Mean $\pm$ SD)	
	Null/WT	Fold decrease*	WT	<i>FosB</i> -null
<i>Apoc2</i>	down	4.45	996.1 $\pm$ 48.8	223.4 $\pm$ 5.1
<i>C5ar2</i>	down	3.39	885.2 $\pm$ 64.8	260.6 $\pm$ 11.7
<i>C5ar1</i>	down	1.83	7917.8 $\pm$ 326.3	4330.8 $\pm$ 126.7
<i>Zfp111</i>	down	1.73	524.4 $\pm$ 30.4	302.7 $\pm$ 11.1
<i>Vmn2r29</i>	down	1.70	301.3 $\pm$ 13.0	177.6 $\pm$ 13.3
<i>Ercc2</i>	down	1.51	777.3 $\pm$ 25.5	513.9 $\pm$ 5.3

* Fold change 1.5 with $p < 0.05$ (unpaired *t*-test).