

fosB-Null Mice Display Impaired Adult Hippocampal Neurogenesis and Spontaneous Epilepsy with Depressive Behavior

Noriko Yutsudo^{1,5}, Takashi Kamada^{2,4}, Kosuke Kajitani^{1,4}, Hiroko Nomaru^{1,4}, Atsuhisa Katogi¹, Yoko H Ohnishi^{1,6}, Yoshinori N Ohnishi^{1,7}, Kei-ichiro Takase², Kunihiko Sakumi^{1,3}, Hiroshi Shigeto² and Yusaku Nakabeppu^{*,1,3}

¹Division of Neurofunctional Genomics, Department of Immunobiology and Neuroscience, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan; ²Department of Neurology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan;

³Research Center for Nucleotide Pool, Kyushu University, Fukuoka, Japan

Patients with epilepsy are at high risk for major depression relative to the general population, and both disorders are associated with changes in adult hippocampal neurogenesis, although the mechanisms underlying disease onset remain unknown. The expression of *fosB*, an immediate early gene encoding FosB and Δ FosB/ Δ 2 Δ FosB by alternative splicing and translation initiation, is known to be induced in neural progenitor cells within the subventricular zone of the lateral ventricles and subgranular zone of the hippocampus, following transient forebrain ischemia in the rat brain. Moreover, adenovirus-mediated expression of *fosB* gene products can promote neural stem cell proliferation. We recently found that *fosB*-null mice show increased depressive behavior, suggesting impaired neurogenesis in *fosB*-null mice. In the current study, we analyzed neurogenesis in the hippocampal dentate gyrus of *fosB*-null and *fosB*^{dl/d} mice that express Δ FosB/ Δ 2 Δ FosB but not FosB, in comparison with wild-type mice, alongside neuropathology, behaviors, and gene expression profiles. *fosB*-null but not *fosB*^{dl/d} mice displayed impaired neurogenesis in the adult hippocampus and spontaneous epilepsy. Microarray analysis revealed that genes related to neurogenesis, depression, and epilepsy were altered in the hippocampus of *fosB*-null mice. Thus, we conclude that the *fosB*-null mouse is the first animal model to provide a genetic and molecular basis for the comorbidity between depression and epilepsy with abnormal neurogenesis, all of which are caused by loss of a single gene, *fosB*.

Neuropsychopharmacology (2013) **38**, 895–906; doi:10.1038/npp.2012.260; published online 16 January 2013

Keywords: immediate early gene; alternative splicing; adult neurogenesis; neural progenitor; proliferation; epilepsy

INTRODUCTION

Neurological and psychiatric disorders are highly prevalent in the general population. It has been shown that patients with epilepsy are at high risk for major depression relative

to the general population, with the incidence of depression being 5–20 times higher, but it is very often unrecognized and untreated (Danzer, 2012). Decreased neurogenesis in the adult hippocampus has been implicated in the pathogenesis of anxiety and depression (Snyder *et al*, 2011). In addition, adult hippocampal neurogenesis is known to be required for mediating the behavioral effects of antidepressants and electroconvulsive therapy (ECT) (Danzer, 2012).

In the adult rodent hippocampus, neurogenesis and expression of *fosB* (*Fosb*), an immediate early gene are increased by seizures induced by chemoconvulsants, such as kainic acid (KA), electroconvulsive seizures (ECS), an animal model of ECT, and forebrain ischemia. Among *fos* family genes, only the *fosB* gene produces multiple protein products, namely FosB, Δ FosB, and Δ 2 Δ FosB, by alternative splicing and translation initiation (Chen *et al*, 1997; Kurushima *et al*, 2005; Mandelzys *et al*, 1997; Morris *et al*, 2000; Nakabeppu and Nathans, 1991; Ohnishi *et al*, 2008; Ohnishi *et al*, 2011; Sabatakos *et al*, 2008). Δ FosB is a translation product of Δ *fosB* mRNA generated by

*Correspondence: Dr Y Nakabeppu, Division of Neurofunctional Genomics, Department of Immunobiology and Neuroscience, Medical Institute of Bioregulation, Kyushu University, 3-1-1 Maidashi Higashi-ku, Fukuoka 812-8582, Japan, Tel: +81 92 642 6800, Fax: +81 92 642 6791, E-mail: yusaku@bioreg.kyushu-u.ac.jp

⁴These authors contributed equally to this work.

⁵Current address: Laboratory for Cell Asymmetry, RIKEN Center for Developmental Biology, 2-2-3 Minatojima Minamimachi, Chuo-ku, Kobe 650-0047, Japan.

⁶Current address: Department of Medical Biophysics and Radiation Biology, Faculty of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan.

⁷Current address: Department of Pharmacology, Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan. Received 4 September 2012; revised 4 December 2012; accepted 9 December 2012; accepted article preview online 18 December 2012

alternative splicing, and lacks the C-terminal 101 amino acids containing the transactivation domain in the full-length FosB protein (Nakabeppu and Nathans, 1991). $\Delta 2\Delta$ FosB is an N-terminally truncated isoform of Δ FosB, which arises as a consequence of alternative initiation of translation from Met79 of the Δ fosB mRNA and lacks the N-terminal Fos homology domain as well as the C-terminal transactivation domain (Sabatakos *et al*, 2008). FosB and Δ FosB act in opposition to regulate Jun transactivity, and also regulate cell proliferation, differentiation, and cell death (Kurushima *et al*, 2005; Nakabeppu *et al*, 1993; Tahara *et al*, 2003). Δ FosB, but not $\Delta 2\Delta$ FosB, itself can act as a transcriptional activator or repressor (McClung and Nestler, 2003; Renthal *et al*, 2008; Sabatakos *et al*, 2008).

We recently reported that *fosB*-null mice exhibit depressive-like behaviors, including decreased locomotion, increased immobility during forced swimming, and increased anxiety-like responses (Ohnishi *et al*, 2011). Interestingly, *fosB*^{d/d} mice, expressing *fosB*^d alleles coding only the Δ FosB and $\Delta 2\Delta$ FosB proteins, did not show depressive-like behaviors, indicating Δ FosB/ $\Delta 2\Delta$ FosB can in part suppress the appearance of depressive-like behaviors. We have also observed that following transient forebrain ischemia in the rat brain, expression of Δ FosB, and to a lesser extent FosB, is induced in neural progenitor cells within the subventricular zone of the lateral ventricles and subgranular zone (SGZ) of the hippocampal dentate gyrus (DG). Furthermore, adenovirus-mediated expression of Δ FosB, and to a lesser extent FosB, promoted neural stem cell proliferation, whereas inhibition of FosB and Δ FosB expression caused neuronal differentiation in rat embryonic cortical cell cultures (Kurushima *et al*, 2005).

Based on these findings, we hypothesized that the *fosB* products, FosB, Δ FosB, and/or $\Delta 2\Delta$ FosB, promote basal and injury-induced neurogenesis in the adult hippocampus, and are involved in depression and epilepsy. To test our hypothesis, we analyzed neurogenesis in the hippocampal DG, as well as neuropathology and behaviors, of *fosB*-null and *fosB*^{d/d} mice. We show that *fosB*-null mice exhibit impaired neurogenesis and spontaneous epilepsy, in addition to depressive-like behaviors, while *fosB*^{d/d} mice do not.

MATERIALS AND METHODS

Animals

fosB^{+/-d} mice with a mutant *fosB*^d allele encoding Δ FosB and $\Delta 2\Delta$ FosB but not FosB, and *fosB*^{+/-G} mice with a null-mutant allele, *fosB*^G, were established. Heterozygous (*fosB*^{+/-d} and *fosB*^{+/-G}) mice were backcrossed onto C57BL/6J males (Clea Japan, Tokyo, Japan) and at least one time onto C57BL/6J females for maintenance of the strains (Ohnishi *et al*, 2011). To yield homozygous mutant mice, *fosB*^{d/d} and *fosB*-null (*fosB*^{G/G}), *fosB*^{+/-d} (N17 generation), or *fosB*^{+/-G} (N12 generation) mice were intercrossed. Homozygous *fosB*^{d/d} and *fosB*-null (*fosB*^{G/G}) mutant mice lines were maintained by inbreeding. Third and fourth generations were used in the present study. C57BL/6J mice were used as wild-type controls for all experiments. Genotyping of the

mutant alleles and animal housing conditions are provided in Supplementary Materials and Methods.

KA Treatment

Eight- to Ten-week-old male mice were used in this study. Mice were injected intraperitoneally with either saline (untreated control) or 25 mg/kg KA (Wako, Osaka, Japan). They were then injected twice daily for 3 days with 50 mg/kg BrdU (Sigma-Aldrich Japan, Tokyo, Japan). To determine the intensity of epileptiform seizures, we observed mice 2 h after KA administration and scored the seizure according to previously described criteria (Kajitani *et al*, 2006; Supplementary Materials and Methods). Animals were killed 1 day after the last BrdU injection, unless otherwise stated.

Antibodies

All antibodies used in the present study are described in Supplementary Materials and Methods.

Tissue Processing and Immunohistochemistry

Brain tissues were prepared and serial coronal sections (40 μ m) were cut on a cryostat and collected as free-floating sections. The floating sections were subjected to immunohistochemistry. Detailed methods for tissue processing and immunohistochemistry are in Supplementary Materials and Methods.

Western Blotting

Nuclear extracts were prepared and subjected to western blotting (Supplementary Materials and Methods).

Laser Scanning Confocal Immunofluorescence Microscopy

Free-floating sections were subjected to immunofluorescence microscopy as described in Supplementary Materials and Methods. Confocal images were acquired using an LSM510 META Confocal Microscope System (Carl Zeiss Microimaging Japan, Tokyo, Japan). All sections from each experimental animal, and groups to be compared, were processed in parallel.

Counting BrdU-Positive Cells

Free-floating sections were subjected to BrdU immunodetection, and the density of BrdU-positive cells in the granule cell layer (GCL) or hilus of the DG was determined using the fractionator program of the Stereo Investigator system (MBF Bioscience, Williston, VT) (Supplementary Materials and Methods).

Observation of Spontaneous Seizures

We observed behavioral seizure activity in animals during cage changing for ~20–30 min per week, and scored them as described previously (Kajitani *et al*, 2006). We only scored stage 3–5 seizures as seizure episodes, as

stage 1–2 seizures were hard to distinguish from normal behavior.

Recording of Electroencephalogram Discharges for Spontaneous Seizures

To investigate spontaneous electrical seizures using electroencephalogram (EEG) recordings, mice were grouped as follows: clinical seizures observed, *fosB*-null mice; clinical seizures not observed, *fosB*-null mice; *fosB*^{d/d} mice and wild-type mice. Recording electrodes for EEG monitoring were placed on the cerebral cortices and hippocampi of mice. The protocol was identical for all three groups (Supplementary Materials and Methods).

Microarray Hybridization and Data Analysis

Total RNA from the hippocampi of wild-type, *fosB*^{d/d}, and *fosB*-null mice was prepared using ISOGEN (Nippon Gene, Tokyo, Japan) and the RNeasy Mini Kit (Qiagen, Tokyo, Japan). For DNA microarray experiments, total RNA (100 ng) was added to poly-A control and treated using the WT Expression Kit (Ambion, Austin, TX, USA), according to manufacturer's instructions. cDNA was labeled using the WT terminal labeling kit (Affymetrix Japan, Tokyo, Japan) and hybridized against the GeneChip Mouse Gene 1.0 ST array (Affymetrix). The resulting list of expressed probes was imported into GeneSpring GX 10.0 (Agilent Technologies, Palo Alto, CA, USA) for data analysis. The microarray data discussed in this publication have been deposited in the NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number, GSE37056.

Real-Time RT-PCR Analysis

Total RNA was prepared from the hippocampus of mice using ISOGEN. First-strand cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Japan, Tokyo, Japan), according to manufacturer's instructions. For quantitative real-time RT-PCR of *Vgf*, *Smad3*, *Galanin*, *Trh*, and *Sema3e* mRNA, reactions were performed in triplicate as described in Supplementary Materials and Methods.

Statistical Analysis

Stereological counts were compared by analysis of variance (ANOVA), by fitting a linear model by least squares, followed by Tukey's honestly significant difference (HSD) test. Microarray data were compared using unpaired *t*-tests or two-way ANOVA. The *p*-values were adjusted for false discovery using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). The rest of the data were compared using the Kaplan-Meier method and log-rank tests, or Fisher's exact test as indicated. A value of *p* < 0.05 was considered as statistically significant. Except for microarray analysis data, all statistical analyses were carried out using JMP 9.02 software (SAS Institute, Cary, NC, USA).

RESULTS

Induced Expression of *fosB* Products in Activated Neural Progenitors, Immature-Newborn and Mature Neurons in the DG After KA Treatment

Western blotting analysis of hippocampal nuclear extracts using anti-FosB (5G4), which recognizes all *fosB* products (FosB, Δ FosB, and $\Delta\Delta$ FosB), detected a 43-kDa polypeptide, representing the full-length FosB protein, in wild-type mice only. While 32/36-kDa and 24-kDa polypeptides, representing Δ FosB and $\Delta\Delta$ FosB proteins respectively, were detected in wild-type and *fosB*^{d/d} mice (Figure 1a). No *fosB* products were detected in *fosB*-null mice.

Basal levels of Δ FosB and $\Delta\Delta$ FosB proteins were marginally higher in *fosB*^{d/d} compared with wild-type mice. Six hours after KA administration, there was a transient increase in FosB levels in wild-type mice. Levels of Δ FosB and $\Delta\Delta$ FosB were stably increased within 24 h after KA administration in both wild-type and *fosB*^{d/d} mice (Supplementary Figure S1a).

Immunohistochemistry using anti-FosB (N) or anti-FosB(5G4) in untreated wild-type hippocampus detected sparse expression of *fosB* products in most hippocampal neurons. Levels of *fosB* products were increased after KA administration (Figure 1b; Supplementary Figure S1b). In wild-type mice, immunoreactivity (IR) in DG granule neurons was highest among all subregions of the hippocampus, and maximal 6 h after KA administration (Figure 1b; Supplementary Figures S1b–d). A similar increase was observed in the hippocampus from *fosB*^{d/d} mice, 6–24 h after KA administration. No anti-FosB (N) IR was detected in *fosB*-null hippocampus (Figure 1b).

To distinguish expression of FosB and Δ FosB/ $\Delta\Delta$ FosB in wild-type hippocampus, we performed laser scanning confocal immunofluorescence microscopy (LSCIM), using three different antibodies against *fosB* products, anti-FosB (5G4), anti-FosB (C), and anti- Δ FosB. The latter two antibodies recognize FosB and Δ FosB/ $\Delta\Delta$ FosB, respectively (Figure 1c). Anti-FosB (C) IR was barely detectable in untreated hippocampus, and was transiently detected in the hippocampus 6 h after KA administration. Seventy-two hours after KA administration, anti-FosB (C) IR had returned to basal levels. Conversely, anti- Δ FosB IR was strongly detected in most neurons from untreated hippocampus, with significantly increased levels observed in hippocampal neurons from 6 to 72 h after KA administration. Moreover, we found that a number of anti-FosB (C) and anti- Δ FosB IR-positive cells, detected in the SGZ of DG 6 h after KA administration, were SOX2 positive, suggesting that expression of both FosB and Δ FosB/ $\Delta\Delta$ FosB was transiently induced in neural progenitor cells (Lugert *et al*, 2010).

Strong anti-FosB (5G4) IR was detected in the majority of NeuN-positive neurons throughout the hippocampus, 72 h after KA administration (Supplementary Figure S2), suggesting that increased expression of Δ FosB/ $\Delta\Delta$ FosB is chronically maintained in most hippocampal neurons after KA administration. After KA administration, anti-FosB (5G4) IR was detected in a few star-shaped glial fibrillary acidic protein (GFAP)-positive astrocytes in the DG hilus (arrowheads in Supplementary Figure S3, left panels), but not in radial glia-like GFAP-positive cells, that have a radial

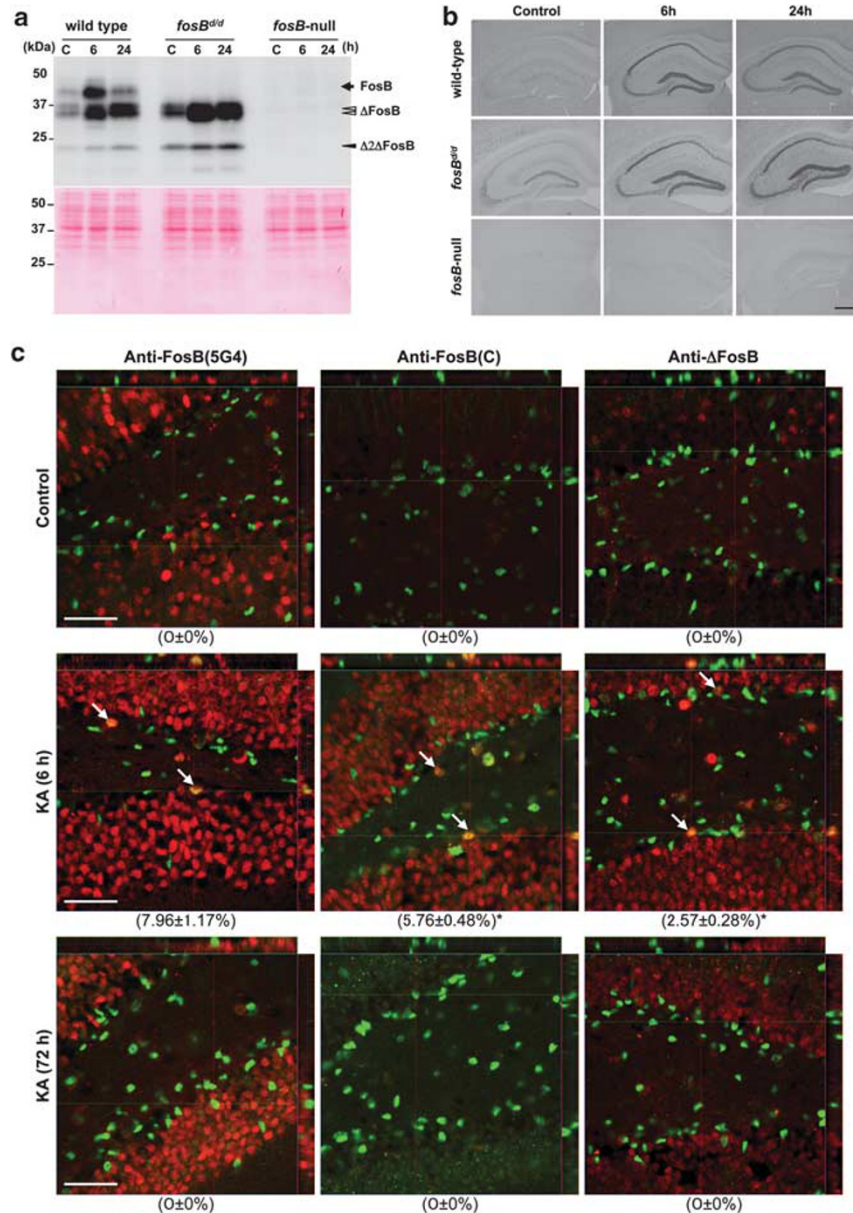


Figure 1 Wild-type, *fosB*^{Δf}, and *fosB*-null mice showed differential expression of FosB, ΔFosB, and Δ2ΔFosB in the hippocampus. (a) Western blotting of *fosB* products in hippocampal nuclear extracts. Nuclear extracts (12.5 μg protein per lane) were prepared from hippocampi at indicated times after saline (C) or KA (6 h, 24 h) administration, and were subjected to western blotting with anti-FosB (5G4) (upper panel). *fosB* products are highlighted: black arrow (FosB), open arrowheads (ΔFosB), and closed arrowhead (Δ2ΔFosB). Blots were post-stained with Ponceau S (lower panel). Quantified results are shown in Supplementary Figure S1a. (b) Immunohistochemistry of *fosB* products at 0 h (control), 6 h, or 24 h after KA administration in the adult hippocampus of wild-type, *fosB*^{Δf}, and *fosB*-null mice. Anti-FosB (N) was used to detect *fosB* products (FosB, ΔFosB, and Δ2ΔFosB). Scale bar: 500 μm. (c) Laser scanning confocal immunofluorescence microscopy of *fosB* products at 0 h (control), 6 h, or 72 h after KA administration in the DG of wild-type mice. Three different antibodies against *fosB* products, anti-FosB (5G4), anti-FosB (C), and anti-ΔFosB were used to detect *fosB* products (red). SOX2 protein (green), a marker for neural progenitor cells, was dominantly detected in SGZ of the DG. Arrows indicate cells expressing both *fosB* products and SOX2. Orthogonal projections throughout the cells are shown. Scale bars: 50 μm. Percentage of anti-FosB IR-positive cells in the SOX2-positive population is shown in parentheses with SEM. More than 100 SOX2-positive cells in each animal (*N* = 3) were observed. **p* = 0.0421 (anti-FosB (C) IR vs anti-ΔFosB IR-positive population 6 h after KA administration, Fisher's exact test).

process spanning the entire GCL and ramify in the inner molecular layer of the DG. Anti-FosB (5G4) IR was not observed in Ki67-positive proliferating cells detected in the SGZ, with or without KA administration (Supplementary Figure S3, middle). A proportion of doublecortin (DCX)-positive cells with axon-like processes in the GCL, representing immature newborn neurons (Lugert *et al*, 2010), exhibited relatively weak anti-FosB (5G4) IR 72 h

after KA administration (arrows in Supplementary Figure S3, right panels).

Overall, our results show that excitotoxicity transiently induces FosB expression in SOX2-positive/non-proliferating neural progenitors in the DG, as well as in mature neurons of the hippocampus. Moreover, expression of ΔFosB/Δ2ΔFosB is transiently induced in SOX2-positive neural progenitors, but chronically in mature hippocampal neurons, and to a

lesser extent in immature newborn neurons of the DG (Supplementary Figure S4).

***fosB*-Null Mice Show Decreased Proliferation and Increased Ectopic Migration of Neural Progenitor Cells in the Adult Hippocampus**

To determine if *fosB* products affect proliferation of neural progenitor cells in the adult hippocampus, we injected BrdU twice daily for 3 days in wild-type, *fosB^{d/d}*, and *fosB*-null mice, following KA or saline injection. Brain sections were prepared 1 day later (Figure 2a). BrdU-positive cells were predominantly distributed in the SGZ (Figure 2b). To confirm that BrdU-positive cells represented newborn neurons, LSCIM was performed using antibodies against BrdU, DCX, and NeuN (Figure 2c; Supplementary Figure S5). Irrespective of KA administration or genotype, >70% of BrdU-positive cells in the DG expressed both DCX and NeuN (Figure 2d and e). The remainder of BrdU-positive cells expressed either DCX or NeuN. Thus, the majority of BrdU-positive cells detected (Figure 2b) represented newborn neurons in the DG.

Next, we estimated the density of BrdU-positive cells in the DG using stereological counting methods (Figure 2f). The density of BrdU-positive cells in controls, without KA administration, was significantly decreased in *fosB*-null mice compared with wild-type mice ($p=0.0066$, Tukey–Kramer HSD *post hoc* comparison), while there was a slight, albeit non-significant, increase in *fosB^{d/d}* mice (Figure 2f, left). In all three genotypes, KA administration significantly increased the density of BrdU-positive cells in comparison with each untreated control (wild type, $p=0.0006$; *fosB^{d/d}*, $p=0.0006$; *fosB*-null, $p=0.0015$, *t*-test). The density of BrdU-positive cells in *fosB*-null mice, after KA administration, was significantly lower compared with wild-type mice ($p=0.002$, Tukey–Kramer HSD *post hoc* comparison) (Figure 2f, right).

LSCIM identified the majority of BrdU-positive cells in the hilus as newborn neurons, expressing DCX and NeuN, either individually or in combination (Figure 3a). We compared the density of BrdU-positive cells in the hilus, and found that without KA administration, *fosB*-null mice had a slightly higher density of BrdU-positive cells compared with wild-type and *fosB^{d/d}* mice. Following KA administration, the density of BrdU-positive cells in both *fosB^{d/d}* and *fosB*-null mice was increased slightly, but to a lesser extent compared with wild-type mice (Figure 3b). The percentage of BrdU-positive cells in the hilus (calculated from the total number of BrdU-positive cells in the whole DG) was highest in *fosB*-null and lowest in *fosB^{d/d}* mice, regardless of KA administration, suggesting that newborn neurons in *fosB*-null, but not *fosB^{d/d}* mice, show increased migration into the hilus in comparison with wild type (Figure 3c).

We next examined survival of newborn neurons in the DG. BrdU was injected into wild-type and *fosB*-null mice twice daily for 3 days, following saline administration (Supplementary Figure S6a). The density of BrdU-positive cells in the DG was determined 1 day and 70 days after the last BrdU injection. There was no significant difference in the survival of BrdU-positive cells in the DG after 70 days (wild type: $19.47 \pm 3.50\%$; *fosB*-null: $14.85 \pm 0.66\%$) (Supplementary Figure S6b). In both mouse lines, all BrdU-positive cells were NeuN positive and were detected

in the GCL/SGZ and hilus (Supplementary Figure S6c), suggesting that *fosB* products do not affect survival and maturation of newborn neurons.

We compared seizure scores and hippocampal damage in wild-type, *fosB^{d/d}*, and *fosB*-null mice after KA administration. We found no significant difference in both seizure response 2 h after KA administration and hippocampal damage 6 days after KA administration, among the three mouse lines (Supplementary Figure S7).

***fosB*-Null Mice Display Spontaneous Epilepsy**

fosB-null mice occasionally exhibited spontaneous seizures, often associated with behavioral arrest and forelimb clonus (Supplementary video S1). Seizures were observed from 13 weeks of age and the non-incidence rate of seizures decreased as age increased. Approximately, 80% of *fosB*-null mice exhibited spontaneous seizures by 80 weeks (Figure 4a). The seizures were similar to KA-induced seizures that are widely accepted as a model of temporal lobe epilepsy (Ben-Ari and Cossart, 2000). Neither wild-type nor *fosB^{d/d}* mice displayed seizures.

To confirm electrophysiological changes of epilepsy in *fosB*-null mice, EEG recordings were performed using 50- to 70-week-old wild-type, *fosB^{d/d}*, and *fosB*-null mice (Supplementary Figure S8; Figure 4b). Wild-type and *fosB^{d/d}* mice displayed low baseline amplitude throughout the observation period (upper and middle panels in Figure 4b). However, in *fosB*-null mice, typical spontaneous electrographic seizures were observed, with the onset of epileptiform discharge from the hippocampus and secondary generalization to the cortex (bottom panel in Figure 4b). Spontaneous epileptiform EEG activity was verified in 27.3% of *fosB*-null mice examined. The spontaneous seizures had durations of up to 47 s. The majority of recorded seizures were strong, with generalized tonic-clonic episodes that occurred in the hippocampus and spread to the cortex. We concluded that *fosB*-null mice display hippocampal-onset epileptic seizures with secondary generalization to tonic-clonic convulsions.

An abnormal hippocampal cell arrangement including thinning of the GCL of the DG was characteristic of *fosB*-null mice regardless of behavioral seizures (Figure 4c). Neither wild-type nor *fosB^{d/d}* mice showed any histological abnormalities. We also examined the density of parvalbumin-positive interneurons in the DG, and found that the density was slightly decreased in both *fosB^{d/d}* and *fosB*-null mice compared with wild-type mice, albeit non-significantly (Supplementary Figure S9). Thus, our results indicate that expression of Δ FosB and/or $\Delta 2\Delta$ FosB is sufficient to suppress spontaneous epilepsy, as determined by an absence of spontaneous seizures, epileptic EEGs, and abnormal DG structures in the adult brain of wild-type and *fosB^{d/d}* mice.

***fosB* Products Regulate Expression of Various Genes Involved in Neurogenesis, Depression, Migration, and Epilepsy**

To examine *fosB* regulation of neurogenesis, depression, and epilepsy, we compared the gene expression profiles of wild-type, *fosB^{d/d}*, and *fosB*-null mice. Total RNA was

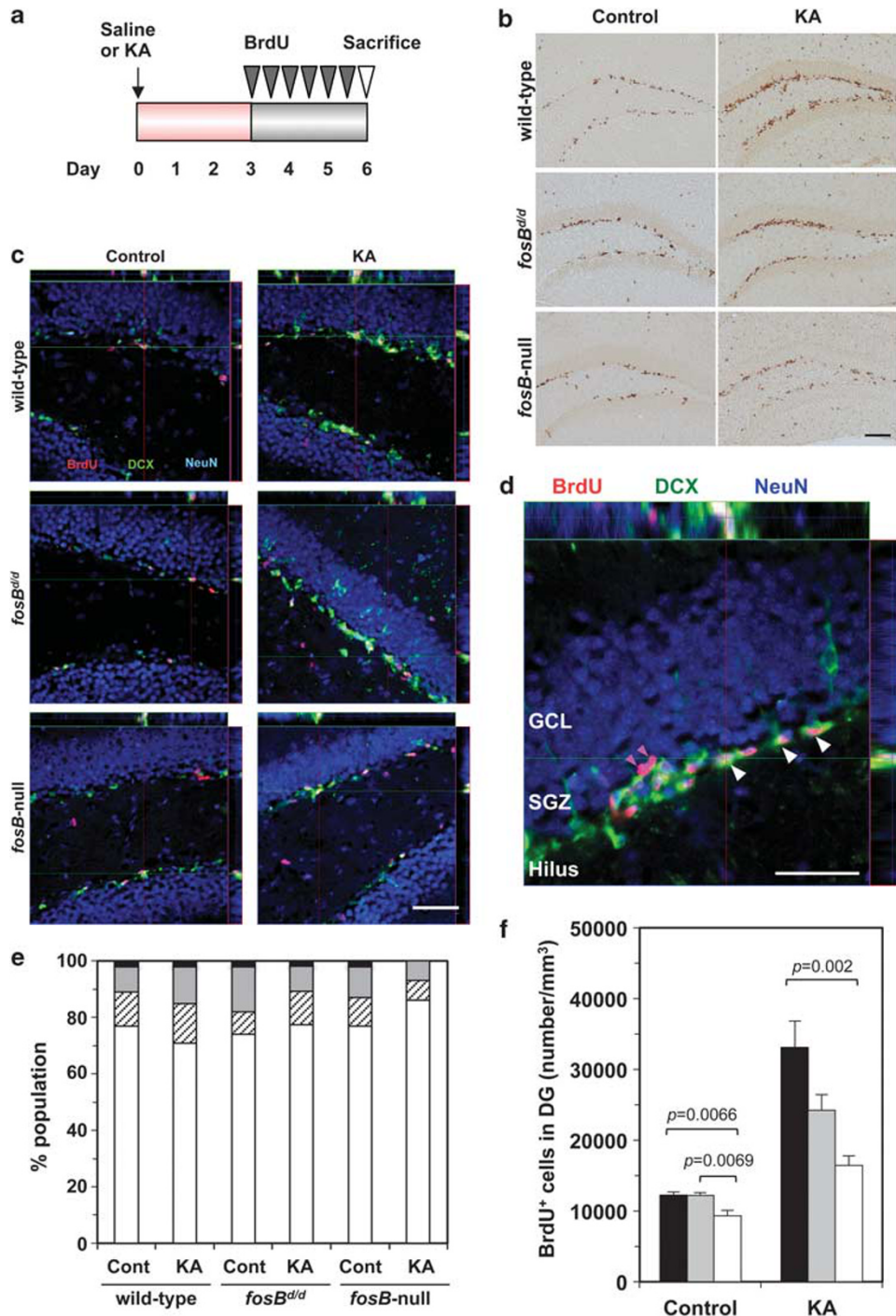


Figure 2 Adult *fosB*-null mice show reduced basal and KA-induced proliferation of neural progenitor cells in the hippocampus. (a) Experimental design. For 3 days following saline (control) or KA injection, mice were injected with BrdU twice daily. Brain sections were prepared on the 6th day, and BrdU-positive cells were detected. (b) Immunohistochemistry for BrdU in adult DG of wild-type, *fosB^{Δ/Δ}*, and *fosB*-null mice, following saline (control) or KA injection. Scale bar: 200 μ m. (c) Laser scanning confocal immunofluorescence microscopy for DCX (green) and NeuN (blue), both neuronal lineage markers, and BrdU (red). Scale bar: 50 μ m. (d) Phenotype of BrdU-positive cells in the DG. Orthogonal projections throughout the cells are shown to demonstrate triple (BrdU⁺/DCX⁺/NeuN⁺ shown with white arrowheads) and double-labeled (BrdU⁺/NeuN⁺ shown with magenta arrowheads) cells. BrdU⁺ single-labeled or BrdU⁺/DCX⁺ double-labeled cells are not seen in this panel. Scale bar: 50 μ m. (e) *fosB* products do not affect differentiation of newborn neurons. One hundred BrdU-positive cells, within immunofluorescence-stained sections from each animal, and two animals for each group, were examined by confocal microscopy to determine the phenotype of BrdU-positive cells. In all three genotypes, the majority of BrdU-positive cells express DCX and/or NeuN. Bar graphs show percentages of BrdU⁺/DCX⁻/NeuN⁻ (black), BrdU⁺/DCX⁻/NeuN⁺ (gray), BrdU⁺/DCX⁺/NeuN⁻ (hatched), and BrdU⁺/DCX⁺/NeuN⁺ (open) cells. (f) Density of BrdU-positive cells in the DG was determined. $N=5$ in each group; error bars, mean \pm SEM; black bars, wild type; gray bars, *fosB^{Δ/Δ}*; open bars, *fosB*-null. One-way ANOVA (control, $F_{2,12}=9.56$, $p=0.0033$; KA, $F_{2,12}=10.08$, $p=0.0027$). The p -values (Tukey–Kramer HSD *post hoc* comparison) are shown.

isolated from hippocampi of wild-type, *fosB^{d/d}*, and *fosB*-null mice 24 h after administration of saline or KA, and subjected to microarray analysis.

Expression levels of 63 genes were significantly altered in *fosB*-null mice compared with wild-type and/or *fosB^{d/d}* mice, with or without KA administration (Supplementary Table S1). In young saline controls, 23 genes were significantly downregulated and one gene significantly upregulated in *fosB*-null mice compared with *fosB^{d/d}* or wild-type mice. Twenty-four hours after KA administration in young mice, 29 genes were significantly downregulated

and 12 genes significantly upregulated in *fosB*-null mice compared with *fosB^{d/d}* or wild-type mice. In aged untreated mice, expression of two genes was significantly higher in *fosB*-null mice compared with wild-type mice. Expression levels of *Vgf*, *Smad3*, *Gal*, *Trh*, and *Sema3e* were confirmed by quantitative RT-PCR analysis (Supplementary Table S2; Supplementary Figure S10).

Our results indicate that *fosB* differentially regulates expression of a number of genes, particularly following KA administration. Among them, 10 genes (*Vgf*, *Dlk1*, *Smad3*, *Gal*, *Trh*, *Islr2*, *Penk*, *Srxn1*, *Serinc2*, and *Car12*; Table 1) are likely to be involved in neurogenesis, depression, migration, or epilepsy, as discussed later. Among the 10 genes, *Vgf*, *Gal*, *Trh*, *Penk*, and *Srxn1* have AP-1 sites in their promoter regions, whereas SMAD3 interacts with AP-1 complexes (FOS, JUN, and JUNB), to regulate TGF- β signaling (Zhang *et al*, 1998; Table 1; Supplementary Figure S11).

DISCUSSION

***fosB*-Null Mice Display Impaired Neurogenesis in the Adult Hippocampus, Increased Depressive-like Behaviors and Spontaneous Epilepsy**

In the current study, we have shown that *fosB*-null mice exhibit decreased proliferation of neuronal progenitor cells, increased ectopic migration of newborn neurons during basal and KA-induced neurogenesis in the adult hippocampus, and adult-onset spontaneous epileptic seizures, epileptic EEGs, and abnormal hippocampal structures. Conversely, compared with wild-type mice, *fosB^{d/d}* mice exhibited a similar level of basal proliferation of neuronal progenitor cells, with decreased KA-induced proliferation and decreased ectopic migration of newborn neurons in the hippocampus, but importantly, *fosB^{d/d}* mice did not exhibit spontaneous seizures, epileptic EEGs, or abnormal hippocampal structures.

In the present study, we compared various phenotypes of *fosB^{d/d}* and *fosB*-null mutant mice with those of C57BL/6J mice as a wild-type strain, onto which the mutant mouse lines were backcrossed >12 generations. However, a

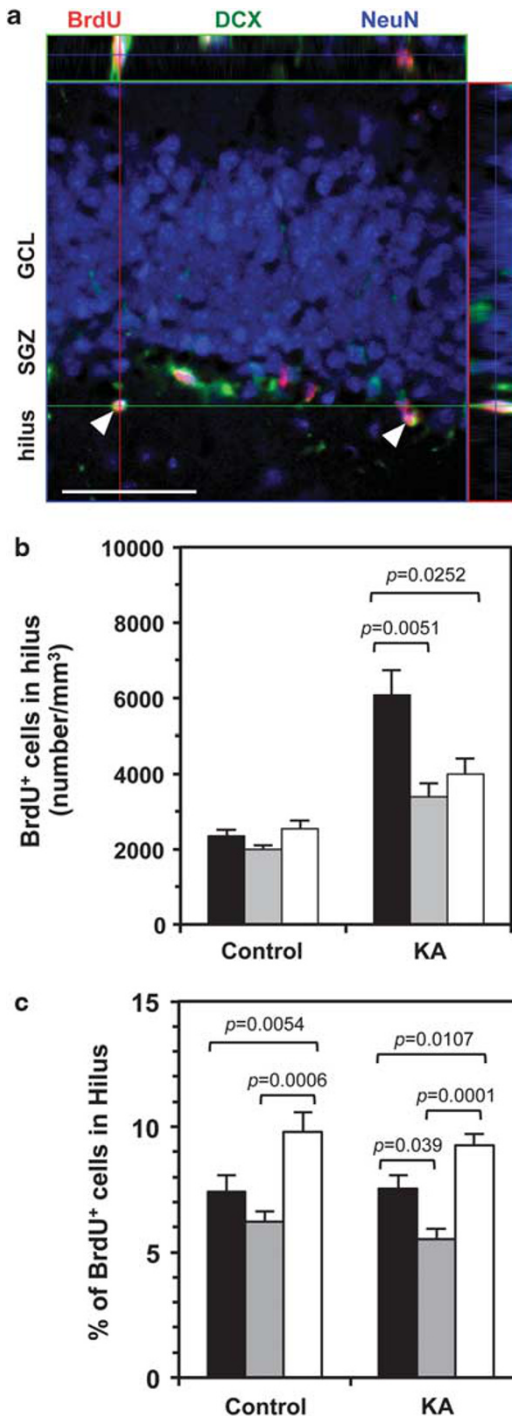


Figure 3 Adult *fosB*-null mice show increased ectopic migration of neural progenitor cells in the hippocampus. As shown in Figure 2a, for 3 days following saline (control) or KA injection, mice were injected with BrdU twice daily. Brain sections were prepared on the sixth day, and BrdU-positive cells were detected. (a) Laser scanning confocal immunofluorescence microscopy for BrdU (red), DCX (green), and NeuN (blue) in the hilus of *fosB*-null mice. The arrowheads indicate ectopic neuroblasts or immature neurons (BrdU + /DCX + /NeuN +). Orthogonal projections throughout the cells are shown. Scale bar: 50 μ m. (b) The density of BrdU-positive cells in the hilus. $N = 5$ in each group; error bars, mean \pm SEM; black bars, wild type; gray bars, *fosB^{d/d}*; open bars, *fosB*-null. One-way ANOVA (control: $F_{2,12} = 2.53$, $p = 0.121$; KA: $F_{2,12} = 8.58$, $p = 0.0049$). The p -values (Tukey–Kramer HSD *post hoc* comparison) are shown. (c) The percentage of BrdU-positive cells in the hilus, calculated from the total number of BrdU-positive cells in the entire DG. $N = 5$ in each group; error bars, mean \pm SEM; black bars, wild-type; gray bars, *fosB^{d/d}*; open bars, *fosB*-null. One-way ANOVA (control: $F_{2,12} = 14.53$, $p = 0.0006$; KA: $F_{2,12} = 20.17$, $p = 0.0001$). The p -values (Tukey–Kramer HSD *post hoc* comparison) are shown.

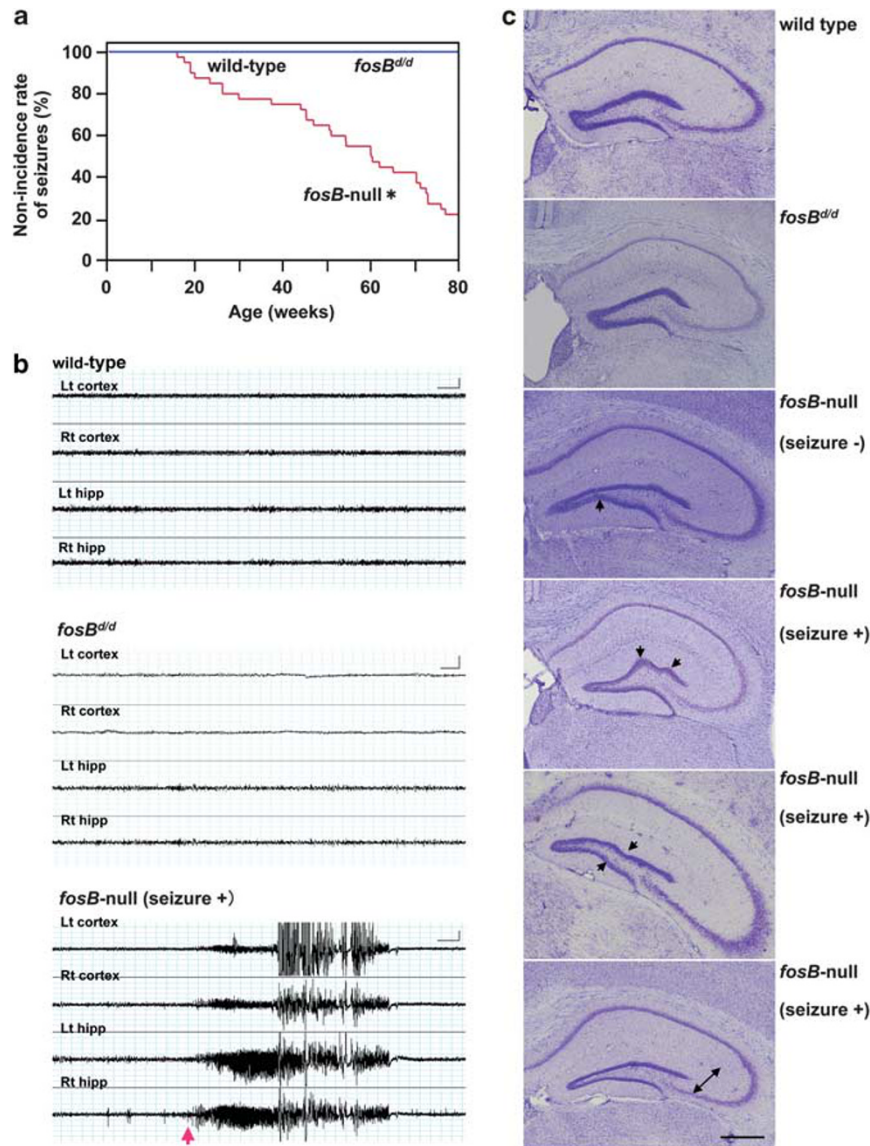


Figure 4 Aged *fosB*-null mice exhibit spontaneous seizures with epileptiform electroencephalograph discharges that originate in the hippocampus, and an abnormal DG structure. (a) The non-incidence rate of spontaneous seizures. Wild-type and *fosB^{d/d}* mice, $N = 10$; *fosB*-null mice, $N = 40$. $*p < 0.0001$, Kaplan–Meier method and log-rank test ($\chi^2 = 40.94$, $df = 2$). (b) Concurrent hippocampal and cortical EEGs in 50- to 70-week wild-type (upper panel), *fosB^{d/d}* (middle panel), and *fosB*-null mice (lower panel). Lt cortex, left cortex; Rt cortex, right cortex; Lt hipp, left hippocampus; Rt hipp, right hippocampus. $N = 5$ in each group. Scale bars: X axis, 4 s; Y axis, 0.5 mV. (c) Nissl stained coronal hippocampal sections from wild-type, *fosB^{d/d}*, and *fosB*-null mice. Characteristic abnormal arrangements (arrows) and thinning (bidirectional arrow) of the GCL are observed in *fosB*-null mice regardless of seizures. Seizure –, without any seizures; Seizure +, with seizures. Scale bar: 500 μ m.

considerable amount of allelic differences are still expected near the mutated *fosB* locus on chromosome 7 between C57BL/6J and mutant mice (Flaherty and Bolivar, 2007). *fosB*-null mice exhibited largely different phenotypes in comparison with C57BL/6J and *fosB^{d/d}* mice, the latter two exhibited rather similar phenotypes regarding spontaneous epilepsy, depressive-like behaviors, and extent of adult neurogenesis in the hippocampus, and these phenotypes were maintained through the generations of backcross ($N = 4$ to $N = 12$ or 17) (unpublished observation). Although it remains possible that allelic differences in the chromosome 7 modify the phenotypes, our results indicate that the phenotypes reported here are determined by the *fosB* locus, at least in part.

We previously found that *fosB*-null mice exhibit increased depressive-like behaviors, whereas *fosB^{+/-d}* mice, with reduced expression of FosB and enhanced expression of Δ FosB and $\Delta 2\Delta$ FosB, exhibit antidepressive-like behaviors (Ohnishi *et al*, 2011). These data indicate that *fosB* products, especially Δ FosB and/or $\Delta 2\Delta$ FosB, promote and maintain intact neurogenesis in the adult hippocampus, and ensure suppression of depression and spontaneous epilepsy. A previous study demonstrated that repeated ECS induces expression of *fosB* products in the prefrontal cortex and tolerance to repeated ECS is significantly attenuated in *fosB*-null mice (Hiroi *et al*, 1998). Taken together, the results suggest that *fosB* products have multiple effects on various aspects of seizures in multiple regions of the brain.

Table 1 Candidate Genes for Neurogenesis, Depression, and Epilepsy Regulated by *fosB* Products

Symbol	Gene name	Fold change						Literature ^a				
		Untreated			24 h after KA			AP-I	ECS	Neurogenesis	Depression	Epilepsy or seizures
		null/wt	dd/wt	null/dd	null/wt	dd/wt	null/dd					
<i>Young</i>												
<i>Vgf</i>	VGF nerve growth factor inducible			- 1.29	- 1.42		- 1.23	Canu et al (1997)	↑ Wang et al (2010)	Thakker-Varia et al (2007)	Thakker-Varia et al (2007)	Newton et al (2003)
<i>Dlk1</i>	Delta-like 1 homolog (<i>Drosophila</i>)	- 1.22								Sumacz et al (2011)		
<i>Smad3</i>	MAD homolog 3 (<i>Drosophila</i>)				- 1.67			Verrecchia et al (2001)		Wang and Symes (2010)		
<i>Gal</i>	Galanin prepropeptide			- 2.30*	- 1.86	- 1.24		Anouar et al (1999)	↑ Wang et al (2010)	Abbosh et al (2011)	Abbosh et al (2011)	Mazarati et al (2000)
<i>Trh</i>	Thyrotropin-releasing hormone			- 3.26*	- 2.50*	- 1.30		Cote-Velez et al (2005)	↑ Wang et al (2010)		Sattin et al (1994)	Deng et al (2006)
<i>Islr2</i>	Immunoglobulin superfamily containing leucine-rich repeat 2				- 1.33							Pramparo et al (2011)
<i>Penk</i>	Preproenkephalin 1				- 2.17*	- 1.51	- 1.44	Hyman et al (1988)			Ogden et al (2004)	Lason et al (1994)
<i>Srxn1</i>	Sulfiredoxin 1 homolog (<i>S. cerevisiae</i>)			- 1.21				Soriano et al (2008)				Soriano et al (2008)
<i>Serinc2</i>	Serine incorporator 2						- 1.38					Inuzuka et al (2005)
<i>Aged</i>												
<i>Ca12</i>	Carbonic anhydrase XII	2.21			ND	ND	ND					Halmi et al (2006); Winum et al (2009)

Among 63 genes altered in the hippocampus of *fosB*-null mice, 10 genes are related to neurogenesis, depression, or epilepsy. Fold changes (null/wt, *fosB*-null vs wt; dd/wt, *fosB^{dd}* vs wt; null/dd, *fosB*-null vs *fosB^{dd}*). Numbers in bold indicate fold change > 1.2 or < - 1.2, and $p < 0.05$ by unpaired *t*-test. Numbers with an asterisk indicate fold change > 2.0 or < - 2.0, and $p < 0.05$ by ANOVA. *p*-value computation; Asymptotic Multiple Testing Correction; Benjamini Hochberg FDR (Benjamini and Hochberg, 1995).

^aLiterature columns indicate previous evidence for the following categories. AP-I: existence of AP-I sites in the promoter regions or reported interactions with AP-I transcription factors (+); ECS: evidence of upregulation by ECS (↑); NG: involvement in neurogenesis; DP: involvement in depression; E/S: seizure- or epilepsy-associated regulation. ND, not done.

***fosB* Regulates Specific Genes to Maintain Intact Neurogenesis and Suppress Spontaneous Epilepsy**

Gene expression profiling suggests that expression of *Vgf*, *Gal*, *Trh*, *Penk*, and *Srxn1* is likely to be regulated by FosB and to a lesser extent Δ FosB/ Δ 2 Δ FosB. Among them, *Vgf* and *Gal* are reported to be involved in both neurogenesis and seizures or epilepsy (Abbosh *et al*, 2011; Mazarati *et al*, 2000; Newton *et al*, 2003; Thakker-Varia *et al*, 2007), and whose expression is likely to be upregulated by both FosB and Δ FosB/ Δ 2 Δ FosB (Supplementary Figure S10). These results may explain why only *fosB*-null, but not *fosB*^{d/d}, mice exhibit spontaneous epilepsy together with a severe decline in neurogenesis (Figures 2f and 4).

It is well known that GABAergic interneurons play an important role in suppressing epilepsy (Perucca and Mula, 2012). In this study, we found a slight decrease in the density of parvalbumin-positive interneurons in both *fosB*^{d/d} and *fosB*-null DG, the latter only exhibiting spontaneous epilepsy (Supplementary Figure S9). However, *fosB*-null mice exhibited the lowest expression of mRNA for TRH, which promotes GABA release (Deng *et al*, 2006), after KA administration, while *fosB*^{d/d} mice exhibited the highest TRH mRNA level in untreated conditions (Supplementary Figure S10). This may also contribute to the spontaneous epilepsy seen in *fosB*-null mice. It is noteworthy that the level of *Car12* mRNA encoding carbonic anhydrase 12 was increased >2-fold only in aged *fosB*-null mice (Table 1). Because topiramate 1, an inhibitor of carbonic anhydrases, is a broad-spectrum anti-epileptic drug (Winum *et al*, 2009), it is likely that Δ FosB and/or Δ 2 Δ FosB as well as FosB repress *Car12* expression, thereby resulting in epilepsy suppression.

Δ FosB and/or Δ 2 Δ FosB Maintain the Intact Structure of Adult Hippocampus

fosB-null mice exhibited abnormalities in hippocampal structures, which were similar to those observed in brains of rodent models and human patients with epilepsy (Fahrner *et al*, 2007). Undulation or thinning of the GCL of the DG was observed in *fosB*-null mice, and may be due to either a neuronal migration defect or decreased hippocampal neurogenesis. Tyrosine kinase *fyn*-deficient mice have previously been reported to have a similar undulation of the DG (Grant *et al*, 1992), and also show increased susceptibility to audiogenic seizures (Miyakawa *et al*, 1995). *fosB*^{d/d} mice did not show these structural abnormalities, indicating that the presence of Δ FosB and/or Δ 2 Δ FosB was sufficient to suppress them.

Among the 10 *fosB*-regulated genes, *Islr2*, which encodes the immunoglobulin superfamily containing leucine-rich repeat 2, is downregulated in mouse models of human neuronal migration defects, and is also mutated in seizure patients (Pramparo *et al*, 2011). SMAD3 and DLK1 are also reported to be involved in cell migration (Wang and Symes, 2010; Yin *et al*, 2006). We previously reported that Δ FosB/ Δ 2 Δ FosB positively, whereas FosB negatively, regulates cell-matrix adhesion and the TGF- β 1 signaling pathway in mouse embryonic stem cells (Ohnishi *et al*, 2008). Moreover, E-cadherin, an important cell-cell adhesion molecule, is an indirect target of *fosB* gene products in the

adult mouse striatum and hippocampus (Ohnishi *et al*, 2011), suggesting that *fosB* products indirectly regulate cell migration and structural maintenance of the hippocampus.

***fosB* May Suppress Depressive-Like Behaviors Through Regulation of Downstream Genes That Enhance Neurogenesis**

Recently, it was reported that adult-born hippocampal granule neurons dynamically regulate stress reactivity at both the endocrine and behavioral levels (Snyder *et al*, 2011). VGF and GAL exert antidepressant action by enhancing neurogenesis in the hippocampus and/or through other effects. *Vgf* is downregulated in animal models of depression and human bipolar postmortem brain (Thakker-Varia *et al*, 2010, 2007). GAL exhibits antidepressant-like effects in human patients with depression (Murck *et al*, 2004), which may be partly mediated by a direct effect of GAL on hippocampal neurogenesis (Abbosh *et al*, 2011). Moreover, hippocampal TRH induction has also been proposed to have antidepressant effects (Sattin *et al*, 1994). Taken together, our present findings suggest that *fosB* suppresses depressive-like behavior through upregulation of these genes (Supplementary Figure S11).

***fosB* Targets May Act in an Autocrine/Paracrine Manner**

Expression of *fosB* products was significantly increased in mature hippocampal neurons, SOX2-positive/GFAP-negative neural progenitor cells, and immature newborn neurons in the DG after exposure to KA. Ki67-positive proliferating cells did not express *fosB* products. Since exogenous expression of FosB or Δ FosB in quiescent fibroblasts and embryonic cortical cells induces their proliferation (Kurushima *et al*, 2005; Nakabeppu *et al*, 1993), it is likely that brain stimuli such as KA treatment or ischemic insults immediately and transiently induce *fosB* expression in neural stem-like cells, thus stimulating their own proliferation.

Of the genes listed in Table 1, 10 are expressed in hippocampal neurons, as determined by *in situ* hybridization (ISH) images (<http://mouse.brain-map.org/>) provided by the Allen Institute for Brain Science. As VGF, TRH, GAL, and PENK are secretory molecules, it is feasible that these proteins are expressed and function within *fosB*-expressing hippocampal neurons, but also act in an autocrine/paracrine manner to regulate neighboring progenitor cells.

Conclusion

The present study demonstrates that the *fosB*-null mouse is the first animal model to provide a genetic and molecular basis for the comorbidity of epilepsy and depression with abnormal neurogenesis, all of which are caused by loss of a single gene, *fosB*. Since depression and epilepsy are known to share a genetic basis (Hesdorffer *et al*, 2012), it will be important in the future to determine if alterations in the *fosB* gene, or its expression, are associated with these disorders in humans. Identifying alterations will provide insight into the diagnosis and treatment of these complicated disorders.

ACKNOWLEDGEMENTS

We thank Dr Z Sheng (Kyushu University, Fukuoka, Japan), Dr N Osumi (Tohoku University, Sendai, Japan) and Dr M Matsumata (Brain Science of Institute, RIKEN, Wako, Japan) for technical advice, and Ms S Kitamura, Ms A Matsuyama, and Ms K Nakabeppu for technical help. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Japan Society for the Promotion of Science (18300124, 22221004, and 23657116 to Y Nakabeppu), and the Kyushu University Global COE Program (Cell-rate Decision Function and Dysfunction in Homeostasis).

DISCLOSURE

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (<http://www.nature.com/npp>)