available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

Neurobehavioral basis of the impaired nurturing in mice lacking the immediate early gene *FosB*

Kumi O. Kuroda[a,b,*], *Michael J. Meaney*[c], *Noriko Uetani*[d], *Tadafumi Kato*[b]

[a]*Kuroda Research Unit for Affiliative Social Behavior, RIKEN Brain Science Institute, Saitama 351-0198, Japan.*
[b]*Laboratory for Molecular Dynamics of Mental Disorder, RIKEN Brain Science Institute, Saitama 351-0198, Japan*
[c]*Developmental Neuroendocrinology Laboratory, Douglas Hospital Research Centre, McGill University, Montreal, Quebec, Canada H4H 1R3*
[d]*McGill Cancer Centre, Department of Biochemistry, McGill University, Montreal, Quebec, Canada H3G 1Y6*

ARTICLE INFO

Article history:
Accepted 26 February 2008
Available online 18 March 2008

Keywords:
FosB
Maternal behavior
Glial fibrillary acidic protein (GFAP)
Medial preoptic area (MPOA)
Oligonucleotide microarray
Emotional behavior

ABSTRACT

The transcription factor FosB is induced in neurons of the medial preoptic area (MPOA) during parenting, through activation of the extracellular signal-regulated kinase (ERK). *FosB* mutant (–/–) postpartum mice and virgin mice that are exposed to pups show defective nurturing behavior. The *FosB* (–/–) MPOA fails to fully up-regulate *SPRY1* and *Rad*, the feedback regulators of ERK and calcium signaling, respectively. Here we studied FosB function by examining the gene expression profiles and the behavioral characteristics of *FosB* (–/–) mice. We found that *FosB* (–/|–) mice exhibited not only decreased parenting but also decreased infanticide compared with (+/) littermates. We then performed gene expression analysis in the MPOA of *FosB* (–/–) mice compared with the wild-type littermates. We found up-regulation of glial fibrillary acidic protein *(GFAP)*, *C4*, and *Ela1* mRNA in the MPOA of *FosB* (–/–) mice; all of these gene products were implicated in general neuropathological conditions. Immunohistochemical analysis showed that up-regulation of GFAP was not restricted to MPOA but extended throughout the forebrain, including the cerebral cortex and striatum. Such pervasive GFAP up-regulation suggested that *FosB* (–/–) mice might have other behavioral abnormalities than nurturing. Indeed, these mice showed a clear alteration in emotionality, detected by the acoustic startle, elevated plus maze, and passive avoidance tests. These results suggest that *FosB* (–/–) mice have broader neurobehavioral dysfunctions, with which the nurturing defect might share the common mechanism.

* *Corresponding author.*
E-mail address: oyako@brain.riken.jp (K.O. Kuroda).

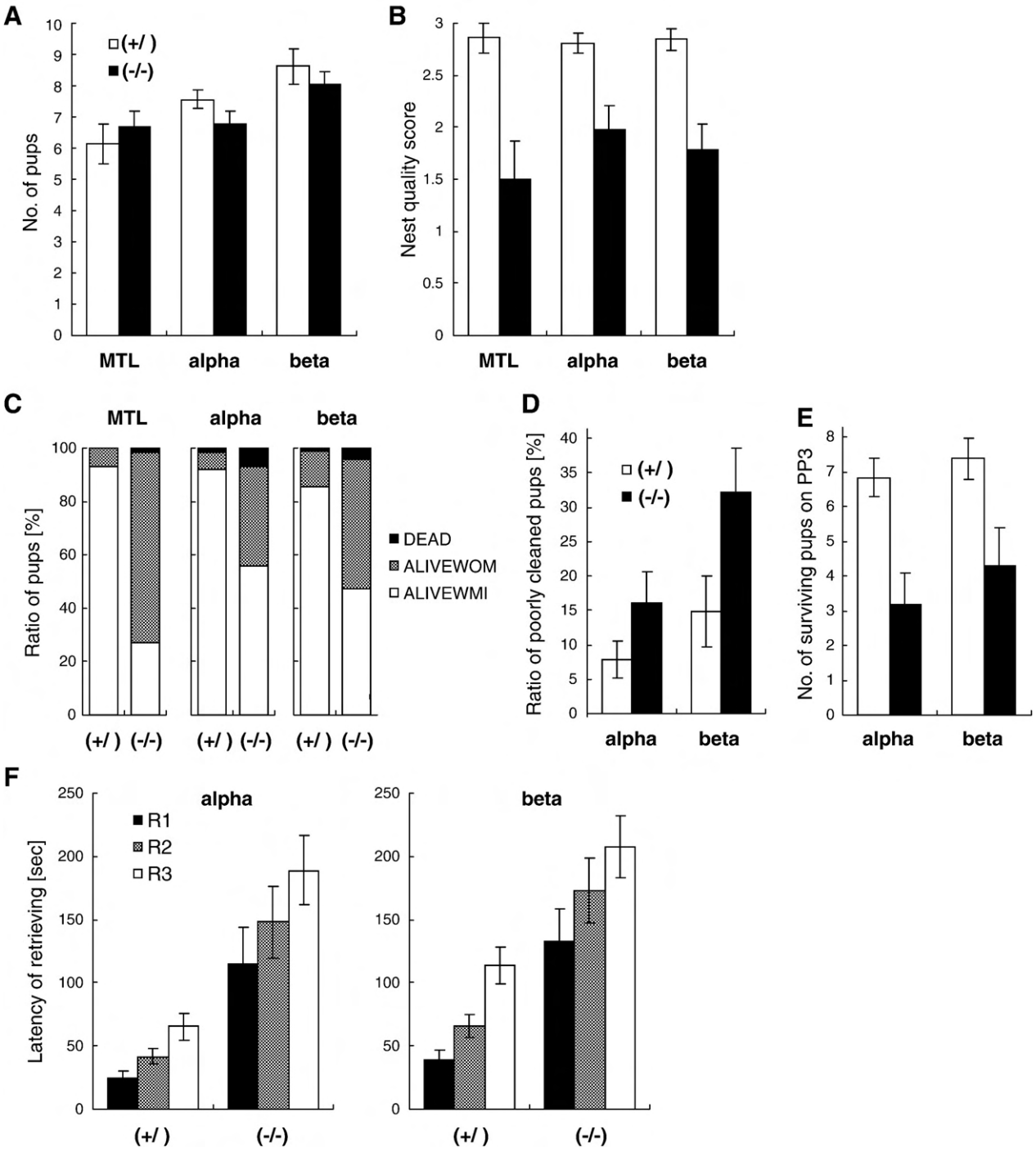
Abbreviations: ANOVA, analysis of variance; AOR, adjusted odds ratio; PPI, prepulse inhibition; CI, confidence interval; CNS, central nervous system; CORT, corticosterone; df, degree of freedom; ERK, extracellular signal-regulated kinase; FC, fold change; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; IHC, immunohistochemical; GLM, generalized linear model; MPOA, medial preoptic area; MPOAdl, dorsolateral part of the MPOA; MTL, Montreal; PBS, sodium phosphate-buffered saline; qRT-PCR, quantitative real-time polymerase chain reaction; RIKEN, Rikagaku Kenkyuu Sho (The institute of Chemical and Physical Research)

0006-8993/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2008.02.100

1. Introduction

Childhood abuse and neglect become risk factors for a wide range of mental disorders such as depression, anxiety disorders, and personality disorders (Heim and Nemeroff, 2001). Understanding the molecular, cellular, and neurobiological basis of parental behavior would be helpful for the prevention of child maltreatment. Because parental care, such as nursing, is essential for all mammalian infants to grow, the basic brain mechanism of parenting should be conserved among mammals. Therefore, we can expect to gain our knowledge about human parental behavior from basic research using other mammalian models.

The neural mechanism of parental behavior has been studied most extensively in rodents (Krasnegor and Bridges, 1990; Numan and Insel, 2003). Accumulating evidence supports the idea that the medial preoptic area (MPOA) of the hypothalamus plays a key role in the expression of parental retrieving behavior (i.e., gathering scattered pups into the nest) (Morgan et al., 1999; Numan, 1994). When a rat or mouse takes care of pups, c-Fos, which is an important molecule for AP-1 transcription activity (Herdegen and Leah, 1998), is induced in MPOA



(Calamandrei and Keverne, 1994; Li et al., 1999; Numan and Numan, 1994). MPOA lesions, especially in the dorsolateral MPOA (MPOAdl), specifically inhibit retrieving, without affecting feeding, general locomotion, female reproductive functions, or sexual behaviors (Jacobson et al., 1980; Kalinichev et al., 2000a,b; Lee and Brown, 2002; Numan et al., 1990; Rosenblatt et al., 1996; Terkel et al., 1979).

Brown et al. (1996) generated genetically engineered mice lacking FosB, another AP-1 transcription factor homologous to c-Fos, and found that these mutant mice had profound defects in nurturing behavior without any cognitive and sensorimotor impairment . Together with c-Fos, FosB is induced in MPOA neurons during performance of parental behavior in mice (Brown et al., 1996) and in rats (Kalinichev et al., 2000a,b; Numan et al., 1998). These studies suggest that c-Fos and FosB are induced in MPOA neurons and then, in turn, induce the expression of downstream genes required to facilitate parental behavior. To identify such downstream genes, we recently investigated the gene expression profiles of the MPOA in parental and nonparental mice (Kuroda et al., 2007). We identified up-regulation of *NGFI-B* (also designated as *Nr4a1/Nur77/TR3/NAK1NP10/GFRP1*), *SPRY1*, and *Rad* in parental mice, together with *c-Fos* and *FosB*. A common inducer of these genes, the extracellular signal-regulated kinase (ERK) was transiently phosphorylated in MPOA neurons on pup exposure. Pharmacologic blockade of ERK phosphorylation inhibited the *FosB* up-regulation in MPOA and the initiation of pup retrieving in virgin female mice but did not affect the established parenting in parous females. Furthermore, induction of *SPRY1* and *Rad* was impaired in MPOA of nonparental *FosB* (–/–) mice. Therefore, the ERK-FosB-SPRY1/Rad signaling seemed to play a crucial role in the initiation of parental care.

In this study, to extend our knowledge of the molecular mechanism of mammalian parental behavior, we first re-examined the behavioral characteristics of *FosB* (–/–) mice and performed the gene expression analysis in the MPOA. We found that: (1) *FosB* (–/–) mice were defective not only in parenting but also in infanticide; (2) *FosB* (–/–) mice showed marked up-regulation of glial fibrillary acidic protein (GFAP) throughout the forebrain including the MPOA; and (3) *FosB* (–/–) mice displayed abnormal emotionality in behavioral test batteries. These results suggest that *FosB* (–/–) mice have broader neurobehavioral dysfunctions, with which the nurturing defect might share the common mechanism.

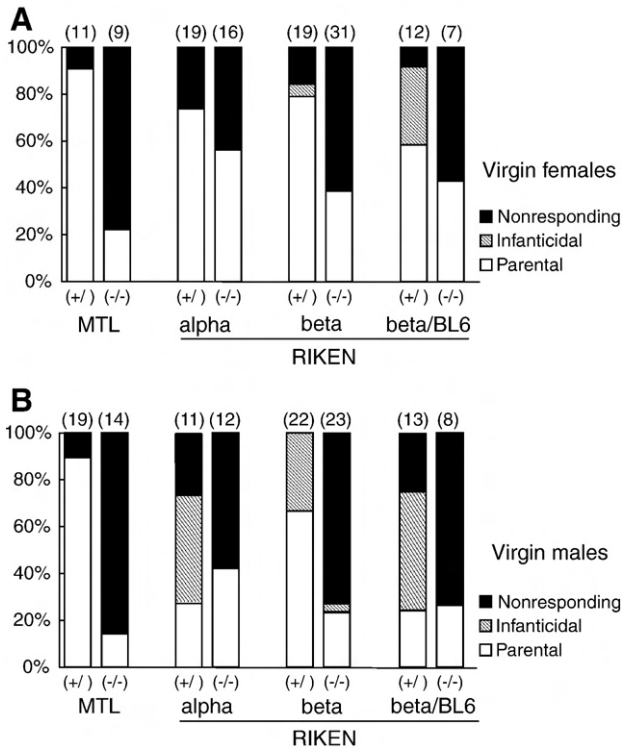


Fig. 2 – Results of pup retrieval assays in *FosB* (–/–) virgin female (A) and male (B) mice. Numbers in parentheses indicate the number of mice studied. Retrieved all 3 pups within 30 min (parental, open area), committed infanticide within 30 min (infanticidal, shaded area), or neither of them (nonresponding, solid area). The results of the second day are shown for male mice. MTL, Montreal; alpha, Alpha-Dri for bedding; beta, Beta-Chip for bedding, and beta/BL6, Beta-Chip for bedding and used the mice backcrossed to C57BL6/J for at least 5 generations. (+/) means wild-type (+/+) or heterozygous (+/–), whereas (–/–) means homozygous *FosB* mutant mice.

Fig. 1 – Maternal behavior of *FosB* (–/–) postpartum mothers. The aspects of maternal behavior were assessed on the day of delivery (A–D, F) or on postpartum day (PPD) 3 (E). (A) Total number of pups found in the cage. (B) The nest quality. (C) The ratio of dead pups (DEAD, solid area), live pups with milk in the stomach (ALIVEWMI, open area), and live pups without milk in the stomach (ALIVEWOM, shaded area). (D) The ratio of poorly cleaned pups (i.e., with placenta, amniotic membrane, or umbilical cords). (E) The number of surviving pups on PPD 3. (F) The latency to retrieve the first (R1, solid area), second (R2, shaded area), and the last (R3, open area) pups in the retrieving assay. (+/) means wild-type (+/+) or heterozygous (+/–), whereas (–/–) means homozygous *FosB* mutant mice. The numbers of *FosB* (+/) and (–/–) mothers observed in each condition were 7 and 10 (Montreal [MTL]), 37 and 23 (alpha at RIKEN), and 13 and 21 (beta at RIKEN), respectively. By the two-way ANOVA with the intersubject factors of genotype and experimental condition for panels A–E, no significant effect of genotype (df = 1) was found in the total number of pups ([A], $F = 0.43$, $p = 0.51$) or in the percentage of dead pups ([C], $F = 1.69$, $p = 0.20$). On the other hand, significant effects of genotype were found in the next quality ([B], $F = 32.2$, $p < 0.000$), the percentage of live pups with milk ([C], $F = 40.2$, $p < 0.001$), the percentage of live pups without milk ([C], $F = 35.3$, $p < 0.000$), the ratio of poorly cleaned pups ([D], $F = 13.0$, $p = 0.001$), and the number of surviving pups on PPD3 ([E], $F = 18.3$, $p < 0.001$). No significant effect of the experimental condition was found except in the total number of pups ([A], $F = 6.94$, $p = 0.001$). This effect might be an artifact derived from the different degrees of ease in searching pups buried in bedding, especially dead ones, in different conditions. No significant interaction was found between genotype and the experimental condition. Panel F shows the three-way repeated measures ANOVA with the intersubject factors of genotype and experimental condition, and the within-subject factor of R1, R2, and R3. A significant effect of genotype was found ($F = 28.7$, $p < 0.001$) and in R1-3 ($F = 71.5$, $p < 0.001$), whereas no significant effect of the experimental condition was found ($F = 1.69$, $p = 0.20$).

2. Results

2.1. *Nurturing behavior of FosB (–/–) mice*

To clarify the molecular mechanism of nurturing defects caused by the lack of *FosB* gene, we bred the *FosB* (–/–) mouse strain by *FosB* (+/–) intercross; that is, all of the subject mice used in this study were reared by *FosB* (+/–) mothers. And since Brown et al, as well as ourselves (data not shown), did not detect any nurturing defects in *FosB* (+/–) mice in comparison with (+/+) littermates, we combined the data of (+/–) with those of (+/+) throughout the parental behavior analyses and denoted them as (+/), according to the previous report.

This study was started at McGill University in Montreal, Canada (MTL). Postpartum female *FosB* (–/–) mice showed impairment of maternal behavior such as incomplete nest building (Fig. 1, MTL), increased ratio of pups without milk in the stomach (Fig. 1C, MTL), higher ratio of poorly cleaned pups and low survival rate of pups (Fig. 1E, MTL), consistent with the previous report (Brown et al., 1996). We also performed pup retrieval assays in virgin females and males. *FosB* (–/–) mice showed less parental retrieving compared with (+/) mice, both in females Fig. 2A MTL) and males (Fig. 2B MTL). These results are well in accordance with the previous report (Brown et al., 1996).

The rest of the experiments were performed at RIKEN (Wako, Japan). We confirmed nurturing defects in postpartum (–/–) mothers compared with (+/) littermates, although the effect was somewhat milder than in MTL (Fig. 1A–C, alpha). For example, for the (–/–) mothers on the day of delivery, the percentage of the pups without milk in the stomach was 54% in alpha, RIKEN, whereas it was 25% in MTL (Fig. 1C). The proportion of parenting virgin (–/–) females did not differ significantly from that of (+/) littermates in the initial experiments at RIKEN (Fig. 2A, alpha, $p=0.311$ by a Fisher's exact test). The same applied to virgin males (Fig. 2B, alpha, $p=0.667$ by a Fisher's exact test). In addition, unlike in MTL, we observed the high rate of infanticide by *FosB* (+/) virgin males at RIKEN (Fig. 2B, alpha), whereas (–/–) virgin males did not commit infanticide in either condition. Because the genetic background, cage size, and the ventilated caging system were identical in both experiments, we speculated that the difference of the bedding, wood chips (Beta Chip) in MTL and paper chips (Alpha-dri) at RIKEN might have affected the results. When wood chips were used at RIKEN, we could clearly observe the nurturing defect of *FosB* (–/–) mice both in females (Fig. 2A, beta) and males (Fig. 2B, beta). The response pattern of (+/) virgin males was still clearly distinct in MTL and in RIKEN beta ($p=0.001$ by a Fisher's exact test), suggesting that other unspecified environmental factors might also be different in these institutions. *FosB* (–/–) virgin female and male mice exhibited different response patterns from their (+/) littermates also in the background crossed at least five times to C57BL/6 (Fig. 2A and B, beta/BL6).

To extract the effects of gender, genotype, and the experimental condition on the pup-directed behavior, the data shown in Fig. 2 were further analyzed by a general linear model (GLM) approach (Agresti, 1996) (see Experimental procedures section 4.7 for details). The results were summarized in Table 2. Most importantly, it turned out that *FosB* (–/–) mice were more nonresponding, less parental and less infanticidal than their (+/) littermates (adjusted odds ratios (AOR) are 23.33, 0.19, and 0.02, respectively). Other major findings included that males were significantly more infanticidal (AOR 8.02) and less parental (AOR 0.39) than females and, in MTL, mice were rarely infanticidal (AOR 0.00) and more parental (AOR 4.75) than in RIKEN, with the same bedding (Beta-Chip). Genotype-condition interaction was found as *FosB* (–/–) mice were more parental in RIKEN, alpha (AOR 4.16), and were less parental (AOR 0.10) in MTL, compared with (–/–) mice in RIKEN, beta.

Table 1 – Genes up-regulated in mice lacking *FosB*

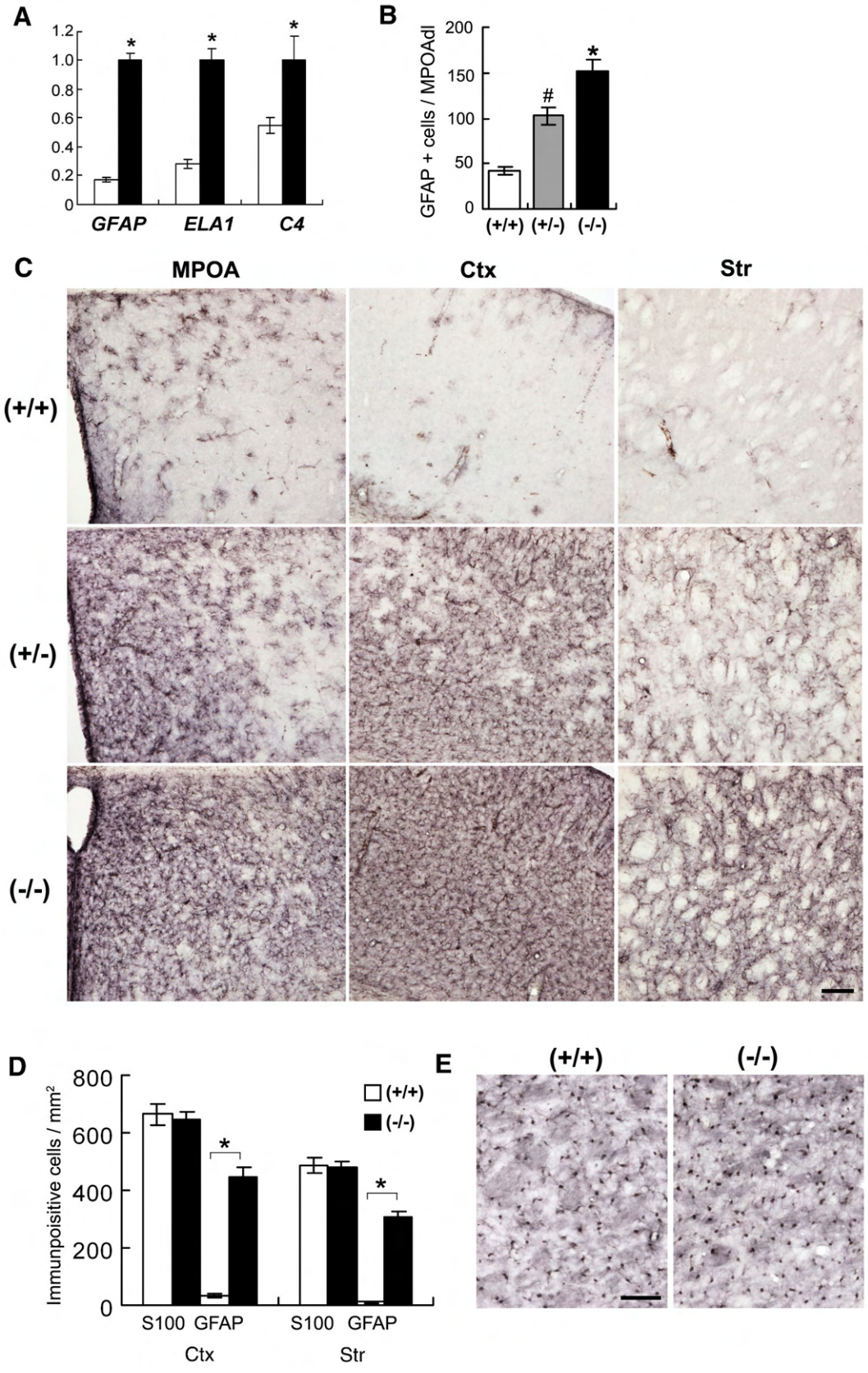
Gene	Probe set ID	FC^{-1} Female	FC^{-1} Male	Description
GFAP	1426509_s_at	5.35	3.60	Glial fibrillary acidic protein
Ela1	1423693_at	3.65	1.61	Elastase 1
C4	1418021_at	3.46	2.03	Complement component 4
GFAP	1426508_at	2.93	1.53	Glial fibrillary acidic protein

FC (fold change)$^{-1}$=(expression in (–/–) group)/(expression in (+/) group).

Fig. 3 – Glial fibrillary acidic protein (GFAP) up-regulation in the forebrain of *FosB* (–/–) mice. (A) Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of expression levels of the genes identified by DNA microarray analysis. *FosB* (+/+) (open bars) and (–/–) (solid bars) postpartum females ($N=5$ each). *Significantly different from (+/+) ($p<0.05$). Data are expressed as arbitrary units normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), with highest values of the (–/–) group set to 1 for appearance's sake. (B) Mean ± SEM number of GFAP-immunopositive cells in the unilateral dorsomedial preoptic area (MPOAdl) of *FosB* (+/+), (+/–), and (–/–) postpartum mothers. One-way ANOVA detected a significant effect of genotype ($F=33.3$, $df=2$, $p<0.001$). #Significantly different from (+/+) ($p<0.001$, Tukey HSD) and from (–/–) ($p=0.004$). *Significantly different from (+/+) ($p<0.001$) and from (+/–) ($p=0.004$). (C) GFAP immunohistochemical (IHC) analysis of *FosB* (+/+), (+/–), and (–/–) in brains of postpartum mothers. Coronal sections depicting the dorsal part of MPOA (MPOA, the third ventricle at the left, and the anterior commissure at the top), somatosensory cortex (middle, the corpus callosum at the left-bottom corner, and the surface at the right-top corner), and striatum (right). (D) Mean ± SEM number of GFAP and S100 immunopositive cells of *FosB* (+/+) (open bars) and (–/–) (solid bars) males in the somatosensory cortex and the striatum. *significantly different between (+/+) and (–/–) ($p<0.05$). (E) S100 IHC analysis in the striatum of *FosB* (+/+) and in (–/–) male mice. Scale bars, 100 μm. We did not find any gender difference for these data (data not shown).

2.2. *Gene expression analysis*

To identify the molecular basis of impaired nurturing behavior in *FosB* (–/–) mice, we performed gene expression analysis in the MPOA. Two comparisons were made. The first was the comparison between wild-type mother mice caring for their pups on the day of parturition and *FosB* (–/–) mothers who delivered pups but did not show nurturing behavior. The



second was the comparison between the virgin *FosB* (+/) males who showed retrieving behavior to the unfamiliar pups and the virgin male (–/–) mice that were poorly responsive to the unfamiliar pups. The reasons we used virgin males instead of females were threefold: first, the behavioral difference depending on genotype was clearer in males than in females at RIKEN. Second, the adult virgin *FosB* (+/) males spontaneously showed either of two contrasting behaviors, parenting or infanticide. This fact enabled us to compare the MPOA of these parental and infanticidal virgin males by performing another set of DNA microarray analyses, as described previously (Kuroda et al., 2007). Lastly, we observed similar patterns of gene expression profiles at the MPOA during parenting in both male and female mice (Kuroda et al., 2007).

The genes whose expressions were significantly altered in both of the experiments were selected with the criteria including the fold change (FC, Table 1) or $FC^{-1}>1.5$, and $p<0.05$ (for details, see Experimental procedures). Four transcripts were found to be up-regulated in the MPOA of *FosB* (–/–) mice in comparison with the *FosB* (+/) mice (Table 1). Two of them were for GFAP, the main constituent of intermediate filaments in mature astrocytes (Eng et al., 2000). This microarray result was confirmed by the quantitative real-time polymerase chain reaction (qRT-PCR) analysis (Fig. 3A). When the cut-off FC or FC^{-1} was set to 1.2, *SPRY1* was up-regulated in FosB (+/) parental mice compared to nonparental (–/–) mice in both mothers and in virgin males, as previously reported (Kuroda et al., 2007).

2.3. *GFAP up-regulation throughout the FosB (–/–) forebrain gray matter*

GFAP up-regulation in *FosB* (–/–) brain was further verified by immunohistochemical (IHC) analysis. Under normal conditions, the astrocytes in the gray matter contain few GFAP-containing fibrils. As shown in Fig. 3B and C, GFAP staining in *FosB* (–/–) mice was markedly elevated in astrocytes in the MPOA compared with their wild-type littermates. Unexpectedly, this finding was not limited to MPOA but seen throughout the forebrain gray matter, especially prominent in cerebral cortex and striatum (Fig. 3C and D), where very few GFAP-expressing astrocytes were found in the wild-type brain. In contrast, GFAP expression was not altered in astrocytes in the white matter such as corpus callosum. qRT-PCR showed that *GFAP* mRNA as well as the other two transcripts, *Ela1* and *C4*, were also up-regulated in *FosB* (–/–) frontal cortex (data not shown).

Repeated measures analysis of variance (ANOVA) revealed a significant effect of genotype on GFAP-immunopositive cells (Fig. 3D) ($F(1, 30)=387.547$, $p<0.001$). IHC analysis using anti-S100 antibody, which stains all kinds of astrocytes, did not show an increased number of astrocytes in *FosB* (–/–) compared to those in (+/+) ($F(1, 30)=0.30$, $p=0.864$; Fig. 3D and E). These findings indicate that up-regulation of GFAP does not reflect an increased number of astrocytes but is caused by the increased amount of GFAP per astrocyte in gray matter. Extensive GFAP up-regulation was observed in all *FosB* (–/–) mice tested ($N>20$) ranging from 3 weeks to 1.5 years of age, without discernible progression with age or gender difference (data not shown). In brains of mice younger than 3 weeks of age, this phenomenon could not be assessed clearly by GFAP IHC analysis because *FosB* (+/+) astrocytes in gray matter and also radial glia expressed GFAP strongly during the central nervous system (CNS) development (data not shown).

Up to this point, we regarded the heterozygous *FosB* (+/–) mice as normal with regard to the defect of maternal behavior, based on the previous study and also our own experience. However, it turned out that *FosB* (+/–) mice showed intermediate up-regulation of GFAP in MPOAdl as well as cerebral cortex and striatum by IHC analysis (Fig. 3B and C). The moderate up-regulation of GFAP was not uniform in the (+/–) forebrain; for example, astrocytes in the MPOAdl were less GFAP immunopositive than the astrocytes in the dorsomedial part of MPOA in (+/–) mothers (Fig. 3C). Similarly, the fifth layer of the somatosensory cortex of (+/–) animals contained fewer GFAP-immunopositive astrocytes than the sixth layer (Fig. 3C, Ctx). Taking this observation into account, we analyzed the *FosB* (+/–) mice separately in the following behavioral analysis.

2.4. *Behavioral characteristics and neuroendocrine response to stress*

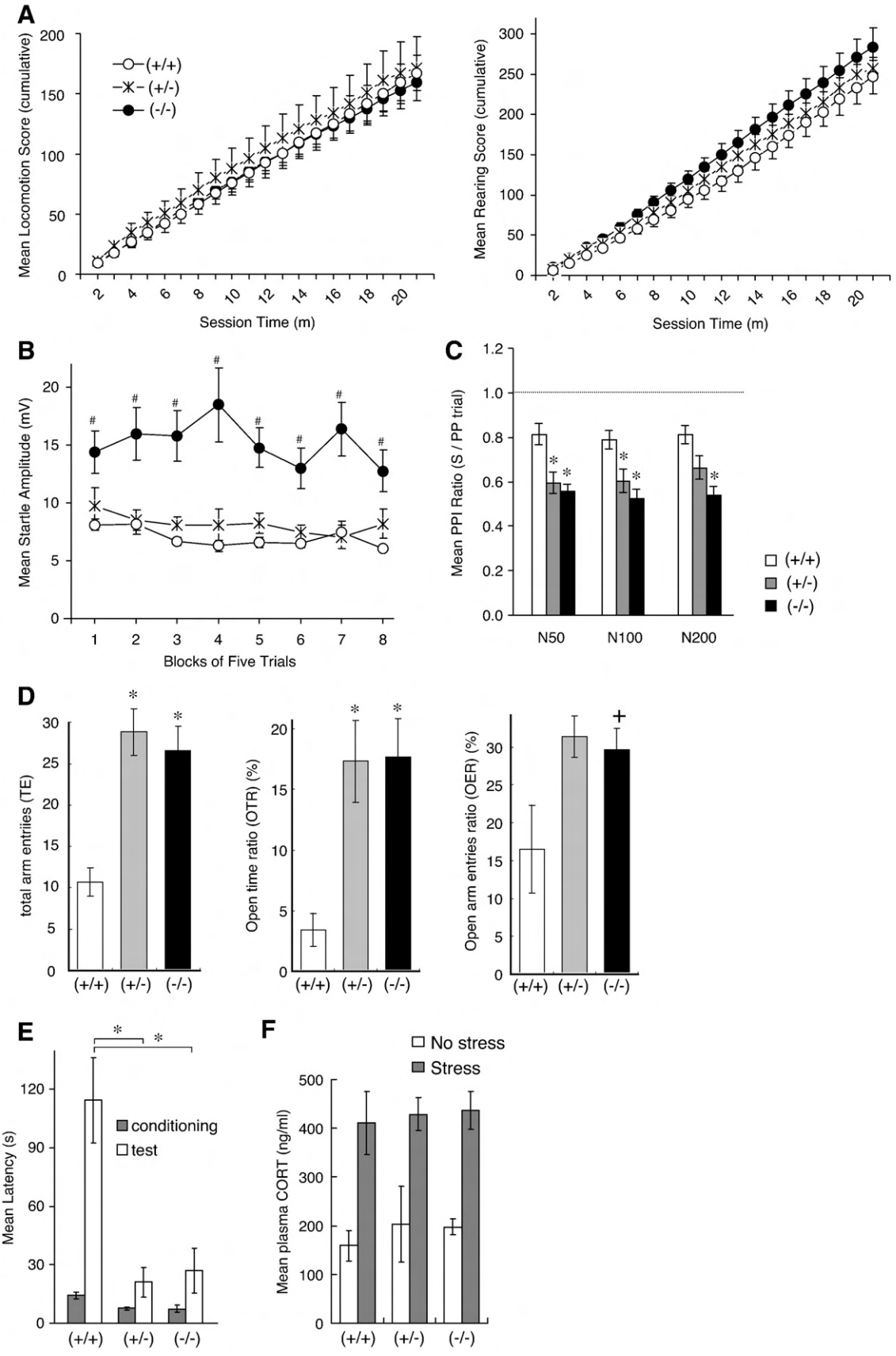
Because the marked overexpression of GFAP was observed not only in the MPOA but also throughout the forebrain, we postulated that lack of FosB caused wide-ranging behavioral abnormalities rather than the specific defect in nurturing behavior. We anecdotally noticed that *FosB* (–/–) mice appeared to be nervous, uneasy, and overreactive to daily handling. *FosB* (–/–) mothers often responded in a panic-like manner when their nests were disturbed. They often broke their nest by themselves, ran around the cage, picking up and dropping the pups. These crude observations prompted us to examine their emotionality, anxiety, and stress reactivity. Phenotypic behavioral differences between *FosB* (–/–), (+/–), and (+/+) mice were assessed with a specific behavioral test battery consisting of open-field activity, passive avoidance learning, elevated plus maze, and prepulse inhibition (PPI). At the end of the test sequence, mice were subjected to restraint stress and the plasma corticosterone (CORT) level was measured.

2.4.1. *Open-field test*

No significant effect of genotype (df=2) was found for the latency to onset of locomotion ($F=0.32$, $p=0.72$), the latency to onset of rearing ($F=0.12$, $p=0.88$), total locomotion during the 20-min session ($F=0.11$, $p=0.90$), and total scores of rearing ($F=0.76$, $p=0.47$) by one-way ANOVA (Fig. 4A). Possible interaction of time after the initiation of the test and the genotype

Fig. 4 – Behavioral characteristics of *FosB* (–/–) mice. (A) Open-field test. Mean locomotion (left) and rearing (right) score. (B) Acoustic startle response. The symbols are the same as those used in Fig. 1. #Significantly different from (+/+) and from (+/–) ($p<0.05$). (C) PPI. *Significantly different from (+/+) ($p<0.05$). (D) Elevated plus maze. Time spent in open arms (left), open time ratio (middle), open arm entries ratio (right). *Significantly different from (+/+) ($p<0.05$). †Difference from (+/+) is close to significance ($p=0.05$). (E) Passive avoidance test. *$p<0.05$. (F) Plasma corticosterone (CORT) levels after immobilization stress.

on the locomotor activity was tested by the two-way repeated measures ANOVA with the intersubject factor of genotype (df=2) and within-subject factor of time (df=19). Although a significant effect of time was found ($F=2.58$, df=6.09, $p=0.019$), neither significant interaction between time and genotype ($F=1.0$, df=36.3, $p=0.36$) nor significant effect of genotype



($F=0.10$, df=2, $p=0.90$) was found. Therefore, (–/–) animals did not show significant differences in the general locomotor and exploratory activity in this open-field test.

2.4.2. *Acoustic startle response and PPI*

The startle response to acoustic stimuli was assessed by two-way repeated measures ANOVA with the intersubject factor of genotype and the within-subject factor of block (Fig. 4B). No significant effects of block ($F=1.4$, df=7, $p=0.18$) and the interaction of block and genotype ($F=1.4$, df=14, $p=0.11$) were found. On the other hand, a significant effect of genotype was found ($F=7.6$, df=2, $p=0.002$). *FosB* (–/–) mice showed significantly higher levels of startle response compared with *FosB* (+/–) ($p=0.042$) and *FosB* (+/+) mice ($p=0.003$, Tukey's honest significant difference [HSD]).

PPI is a reduction of the startle response to an intense stimulus seen when this stimulus is preceded by a weaker stimulus, which itself does not cause the startle response (Crawley, 2000). It is used as an operational measure of sensorimotor gating. Two-way ANOVA revealed a significant effect of genotype ($F=31.7$, df=2, $p<0.001$), whereas no significant effect of amplitude of prepulse sound ($F=0.31$, df=2, $p=0.72$) and no interaction between genotype and amplitude ($F=0.17$, df=4, $p=0.95$) were found (Fig. 4C). Both *FosB* (–/–) ($p<0.001$) and *FosB* (+/–) ($p<0.001$) mice showed increased PPI.

2.4.3. *Elevated plus maze*

The total arm entry (TE), which is a supposed index of locomotor activity in the apparatus, was significantly different among the genotypes (Fig. 4D) ($F=10.0$, df=2, $p<0.001$, one-way ANOVA). Both *FosB* (–/–) and (+/–) mice entered the open and closed arms more frequently than *FosB* (+/+) mice ((–/–), $p<0.001$, (+/–), $p=0.004$, Tukey's HSD). The open time ratio (OTR) ($F=6.5$, df=2, $p=0.004$) and the open arm entries ratio (OER) ($F=3.6$, df=2, $p=0.039$) were also significantly different among the genotypes. Both *FosB* (–/–) and (+/–) mice stayed in the open arm longer than (+/+) mice ((–/–), $p=0.004$, (+/–), $p=0.037$, Tukey's HSD). *FosB* (–/–) mice showed a tendency to enter the open arm more frequently than (+/+) mice ($p=0.050$, Tukey's HSD).

2.4.4. *Passive avoidance test*

When two-way repeated measures ANOVA with the intrasubject factor of day (day 1, conditioning; day 2, test) and the intersubject factor of genotype were applied, significant effects of day ($F=21.6$, df=1, $p<0.001$), day-genotype interaction ($F=9.7$, df=2, $p=0.001$), and genotype ($F=10.4$, df=2, $p<0.001$) were found for the latency to move (Fig. 4E). Both *FosB* (–/–) and (+/–) mice showed shorter latency at the test session than (+/+) mice.

2.4.5. *Plasma CORT levels after the restraint stress*

At the end of the behavioral test battery, 15-min restraint stress was given to half the mice and the effect of stress on the CORT level was examined (Fig. 4F). Although stress significantly increased the CORT levels ($F=47.0$, df=1, $p<0.001$, two-way ANOVA with the intersubject factors of genotype and stress), no effects of genotype ($F=0.43$, df=2, $p=0.65$) and genotype-stress interaction ($F=0.01$, df=2, $p=0.98$) were found.

2.4.6. *Summary of behavioral test battery*

In summary, the *FosB* (–/–) adult male mice showed: (1) enhanced acoustic startle response and increased PPI, (2) increased total arm entries and stay for open arms by elevated plus maze, and (3) diminished passive avoidance, compared with the wild-type littermates.

3. Discussion

In this study, we confirmed and extended the previous finding that *FosB* (–/–) mice showed impairment of nurturing behavior (Brown et al., 1996). We noticed that the pup-directed behavior of not only *FosB* (–/–) mice but also of (+/) mice is affected by genetic background and the experimental conditions, such as the cage bedding used during retrieval assay (Table 2). Infanticide was strongly inhibited in the MTL condition, even if the case size, the cage bedding, and the genetic background were identical with the RIKEN beta condition. Therefore, some unspecified factors should have contributed to dictate the behavior of the males. In any case, it should be emphasized that the both parenting and infanticide can be sensitive to experimental conditions used, as are many other behaviors (Crabbe et al., 1999). A different line of *FosB* (–/–) mice was created, but these mice did not seem to show marked impairment of reproductive functions, since they could be maintained in a *FosB* (–/–) colony (Gruda et al., 1996). Together with differences in the targeting constructs or the genetic backgrounds, differences in the experimental conditions might be involved in this apparent discrepancy.

The effect of genotype on postpartum nurturing behaviors was confirmed (Fig. 1) as previously reported (Brown et al., 1996). Moreover, even though the pup-directed behavior of virgin animals was greatly dependent on the experimental conditions, *FosB* (–/–) virgin animals were consistently more nonresponding and less infanticidal toward donor pups than (+/) littermates in all conditions (Fig. 2 and Table 2). It should be noted that both infanticide and parenting are adaptive reproductive strategies at least in certain circumstances (Hrdy, 1977; vom Saal and Howard, 1982). The observation that *FosB* (–/–) males are even more defective in infanticide rather than in parenting (e.g., Fig. 2B, alpha and beta/BL6, see also infanticidal and parental AOR for the effect of genotype in Table 2) recalls the previous reports that prenatal stress and lower social rank inhibit infanticide of virgin males (vom Saal, 1983; vom Saal and Howard, 1982). It has also been reported that virgin male mice with a targeted mutation of progesterone receptor show no infanticide and enhanced parental behaviors (Schneider et al., 2003). Moreover, juvenile males are spontaneously parental, and only during postweaning development do they start to avoid or attack pups in rats (Bridges et al., 1974) and in house mice (McCarthy and vom Saal, 1986). Therefore, infanticide might be added later in ontogeny as a regulatory system for parenting. *FosB* (–/–) brain may have deficits in both systems or have a problem in decision-making between two behavioral repertoires. For maternal behaviors of postpartum mothers, the effects of experimental conditions did not reach statistical significance. This observation may reflect the fact that the maternal behavior of postpartum mothers is more robust and resistant in various conditions than that of virgin animals.

Table 2 – Summary of GLM for pup retrieval assays in virgins

		Parental			Infanticidal			Nonresponding		
		AOR	95%CI	*p* value	AOR	95%CI	*p* value	AOR	95%CI	*p* value
Main effect										
Gender	Female	1[a]			1[a]			NA		
	Male	0.39	(0.22–0.69)	0.001**	8.02	(2.63–24.44)	<0.000***	NA		
Genotype	(+/)	1[a]			1[a]			1[a]		
	(–/–)	0.19	(0.08–0.48)	<0.000***	0.02	(0.00–0.19)	<0.000***	23.33	(6.35–85.73)	<0.000***
Condition	MTL	4.75	(1.19–18.88)	0.027*	0.00	(0.00–0[b])	0[b],***	1.41	(0.26–7.51)	0.689
	RIKEN,alpha	0.50	(0.18–1.38)	0.180	0.73	(0.21–2.57)	0.621	4.61	(1.11–19.19)	0.036*
	RIKEN,beta	1[a]						1[a]		
	RIKEN,beta/BL6	0.29	(0.10–0.83)	0.021*	2.62	(0.83–8.24)	0.100	2.41	(0.49–11.82)	0.277
Interaction										
(–/–) ×	MTL	0.10	(0.02–0.65)	0.016*	NA			1.83	(0.23–14.50)	0.566
	RIKEN,alpha NA	4.16	(1.03–16.80)	0.046*	NA			0.12	(0.02–0.65)	0.014*
	RIKEN,beta NA	1[a]			NA			1[a]		
	RIKEN,beta/BL6	3.88	(0.75–20.07)	0.106	NA			0.45	(0.06–3.32)	0.433

AOR, adjusted odds ratio; 95%CI, 95% confidence interval.
NA: excluded from the model by stepwise backward elimination procedure (i.e. the effect on the behavior was significantly small.).
[a] Baseline category.
[b] Theoretical value.*$p<0.05$, **$p<0.01$, ***$p<0.001$.

There was a tendency that the retrieving behavior and pup-cleaning behavior of postpartum mothers were slightly perturbed in RIKEN beta compared with RIKEN alpha, although this effect did not reach statistical significance (Fig. 1D and F). In the case of Fyn knock-out mice, the substance responsible for the bedding-dependent impairment of nurturing behavior was identified as hexanal (Hamaguchi-Hamada et al., 2004), a volatile substance contained in plants, causing the grassy odor. However, in our preliminary study, hexanal did not seem to alter the maternal behaviors on the day of delivery in either *FosB* (+/) or (–/–) mothers. In addition, hexanal is contained in the hard-wood chips, and beta-chip is made of soft wood. This crude observation does not exclude the possibility that some other ingredients contained in wood chips or the different bedding texture might affect the parental behavior.

In this study, we identified GFAP up-regulation per astrocyte in gray matter in *FosB* (–/–) brain. However, the cause of GFAP up-regulation remains unclear. One possibility is the astrocyte activation by neurotoxic process. In response to essentially any CNS pathology, such as stroke, brain trauma, tumor, and neurodegeneration, astrocytes exhibit hypertrophy and increase GFAP synthesis massively (Pekny and Nilsson, 2005). Such processes are often accompanied by inflammatory reactions; for example, elastase secreted by activated neutrophils is involved in the development of cerebral damage induced by ischemia (Shimakura et al., 2000). The presenilin conditional knock-out mice exhibited age-dependent neurodegeneration and showed complement component C1q up-regulation together with GFAP in the cerebral cortex (Beglopoulos et al., 2004). Another possibility of GFAP up-regulation in *FosB* (–/–) forebrain is the altered differentiation of astrocytes, as seen in *Olig2* mutant mice (Cai et al., 2007). The former possibility is favorable to explain concomitant up-regulation of C4 and Ela1, the fact suggesting lymphocyte infiltration, in *FosB* (–/–) mice. However, neither we nor other researchers (Brown et al., 1996; Hiroi et al., 1997; Hiroi et al., 1998) could detect any gross neuronal loss or other signs of neuropathology by routine histology, IHC analysis for various neurotransmitters, or by fluoro-jade B staining detecting degenerated neurons (our unpublished observation). Moreover, GFAP up-regulation was already obvious on postnatal day 21 and did not seem to progress with age. Therefore, astrocyte activation may occur during CNS development and be kept as a chronic state in *FosB* (–/–) mice. ERK signaling plays a vital role during CNS development by mediating functions of growth factors such as the brain-derived neurotrophic factor (Moriguchi et al., 1996). FosB protein has a role in inducing feedback regulators of the ERK signaling during pup exposure (Kuroda et al., 2007). Therefore, it is not surprising that *FosB* (–/–) neurons may suffer from ERK dysregulation during CNS development. This issue should be addressed further in future studies.

It takes at least an hour for FosB protein to be up-regulated in MPOA neurons after pup exposure, whereas most of the virgin females of laboratory mice start parenting toward offspring within 30 min. Thus, it is hard to assume that immediate induction of FosB protein in response to pup exposure is required for ongoing parenting, as pointed previously (Jin et al., 2005). It would be more reasonable to postulate that FosB expression in the MPOA during development might be needed to modify or strengthen the neural circuit for improved parental behavior in future. Indeed, during everyday pup exposure, the retrieving latency tends to be shortened both in rats (Bridges and Scanlan, 2005) and in mice (our unpublished observation).

Widespread GFAP up-regulation in *FosB* (–/–) mice suggested that these (–/–) mice not only have a specific abnormality in nurturing behavior, but may also have other behavioral phenotypes. Although no abnormalities in cognitive and sensorimotor functions were found in the *FosB* (–/–) mice used in this study (Brown et al., 1996), Hiroi et al detected mild hyperactivity under novel or stressful environment (Hiroi et al., 1997; Zhu et al., 2007). *FosB* (–/–) mice also showed exaggerated locomotor activation in response to initial cocaine

exposures, a robust conditioned preference to a lower dose of cocaine, as well as attenuated behavioral tolerance to repeated electroconvulsive shock, compared with wild-type littermates (Hiroi et al., 1997, 1998). In the present study, the *FosB* (–/–) mice showed altered behavioral characteristics, such as the enhanced acoustic startle response, exaggerated PPI, increased TE and OTR by elevated plus maze, and diminished passive avoidance (Fig. 4). On the other hand, they were not distinguishable in spontaneous activity in the open field and in endocrine response to restraint stress.

While increased stay and entry in the open arm in elevated plus maze are normally interpreted as the reduction of anxiety, this measure has been reported to be significantly influenced by locomotor activity (Weiss et al., 1998). Colnsistent with previous studies (Hiroi et al., 1997; Zhu et al., 2007) and our assumption, increased TE on the EPM apparatus suggested that *FosB* (–/–) mice are hyperactive under a stressful environment. Similarly, the decreased latency in the passive avoidance task is implicated not only in the deficient learning and memory, but also in the impulsivity and abnormal behavioral inhibition (Patterson and Newman, 1993). Since the previous study reported the normal learning of *FosB* (–/–) mice in the Morris water maze (Brown et al., 1996), the diminished passive avoidance may be due to their impulsivity, rather than simply to their cognitive deficits. The psychological mechanism of human syndromes of disinhibition, such as psychopathy and childhood hyperactivity, has been linked to impulsivity, poor passive avoidance (i.e., failures to learn from aversive feedback), and fearlessness by some accounts (Patterson and Newman, 1993). The abnormal parental behavior and our anecdotal observation during daily handling seem to fit into this viewpoint. On the other hand, the enhanced PPI is rare even in the literature and difficult to understand. This finding might have been caused by the enhanced acoustic startle response rather than by better sensorimotor gating in (–/–) mice. At least we could say that *FosB* (–/–) mice are clearly different from (+/+) mice in some behavioral tasks, possibly related to the altered emotionality.

Interestingly, (+/–) mice behave similarly to (+/+) in parental behaviors and in acoustic startle response, but rather similar to (–/–) in PPI, elevated plus maze, and passive avoidance tests. Therefore, it seemed that some traits follow Mendel's law of dominance but others do not. GFAP upregulation of (+/–) also depends on the brain area. In the medial part of MPOA and in the sixth layer of the somatosensory cortex, (+/–) is more similar to (–/–) than to (+/+) (Fig. 3C), whereas in the MPOAdl and in the striatum GFAP upregulation of (+/|–) is the midpoint of that of (+/+) and (–/–). In terms of parental care, *FosB* (+/–) animals are indistinguishable from (+/+) animals, in both postpartum mothers and virgins (Brown et al., 1996) (data not shown). Therefore, the requirement of FosB protein seems to be different in each brain area and the relevant behavior.

We have recently shown that the ERK-FosB signaling has an important role in the initiation of parental behavior (Kuroda et al., 2007). Although *FosB* (–/–) mice exhibit abnormalities in many other behaviors as shown in the previous reports (Zhu et al., 2007) and in this study, maternal nurturing has been the most prominent and readily discernible defect. Maternal nurturing may be the most complex behavioral task for mice living in laboratory conditions and may require the finest tuning of the ERK-FosB signaling, which could act for morphologic plasticity of neurons and resultant behavioral adaptation. Further investigation in molecular neuropathology caused by lack of FosB is necessary to fully explain diverse behavioral alterations of *FosB* (–/–) mice, including both infanticide and parenting.

4. Experimental procedures

4.1. *Animals*

All animal experiments were conducted in accordance with the National Institutes of Health guide "Principles of Laboratory Animal Care" (NIH publication no. 85–23, revised 1985) and were approved by the Animal Experiment Judging Committee of McGill University and RIKEN. Wild-type C57BL/6J mice were purchased from Japan SLC (Hamamatsu, Japan). A *FosB* (–/–) mouse strain was obtained from the Jackson Laboratory (strain C;129S-*Fosb*^{tm1Meg}/J; stock number 3077). All of the subject mice used in this study were reared by *FosB* (+/–) mothers, so that the phenotype observed between (+/+), (+/–), and (–/–) offspring were derived from altered genotype but not from the parental care. It has been shown that the maternal behavior reflected in pup survival of *FosB* (–/–) and (+/) mothers is not affected by the genotype of the pups (Brown et al., 1996). For initial characterization, we used the F2 generation of this original strain and C57BL/6J. The results shown in Figs. 2 and 3 were confirmed using the *FosB* mutant mice backcrossed to wild-type C57BL/6J at least five times. The mice were housed in ventilated shoebox cages (267×483×152 mm) and were maintained under a 12:12-h light/dark cycle with lights on from 08:00 to 20:00. The wood chips (Beta Chip, NEPCO, Warrensburg, NY) were used as cage bedding at McGill University for both breeding and for behavioral testing. At RIKEN, the TEK-Fresh Standard bedding was used for normal animal housing. This bedding was flaky, bulky, and nice for daily housing and breeding, but sometimes made it hard to observe the animal's behavior. Therefore, for parental behavior observations, two types of cage bedding, purified paper chips (Alpha-Dri, Shepherd Specialty Papers, Watertown, TN) and wood chips (Beta Chip), were used at RIKEN.

4.2. *Pup retrieval assay and evaluations of postpartum maternal behavior*

We used separate animals in each parental behavior analysis so that their behavior was not compromised by the previous experience with pups; that is, all the subject animals were exposed to pups for the first time in their life except for their own littermates. Since Brown et al, (1996) as well as ourselves, did not detect any nurturing defects in *FosB* (+/–) mice in comparison with (+/+) littermates, we combined the data of (+/–) with those of (+/+) throughout the parental behavior analyses and denoted them as (+/), according the previous report. To quantify parental responsiveness, pup retrieval assays were performed essentially as described previously (Brown et al., 1996; Rosenblatt, 1967). Briefly, *FosB* (+/) and (–/–) adult virgin females, 10–16 wk of age were individually housed for at least 1 d prior to the experiment. Together with the bedding, they were

provided with a cotton square (Nestlet, Ancare, Bellmore, NY) as nest material. On the test day, each animal was exposed to three 1- to 6-day-old pups. One pup was placed in each corner of the cage distant from the nest. The cages were continually observed for the next 30 min and the following measures were recorded: latency to sniff a pup for the first time, to retrieve each pup into the nest, and to crouch over the pups continuously for >1 min. If any of the pups was attacked during the test, all the pups were immediately removed and the wounded pup was euthanized as described previously (Perrigo et al., 1993). This subject was deemed as infanticidal. Otherwise, pups were left in the cage for 30 min. If the subject mouse retrieved all three pups to the nest, they were labeled as parental. If the subject mouse was neither parental nor infanticidal, it was labeled as nonresponding. Unlike the case in rats, most of the virgin female mice were parental at the first retrieval assay as previously reported (Mathieson et al., 2002). For virgin males, the retrieval assay was repeated on two sequential days, and the score of the second day was used for their behavioral classification because sometimes little nurturing occurred on the first day in all genotypes (Brown et al., 1996; Rosenblatt, 1967). Both infanticide and parenting are valid reproductive strategies in rodents, depending on their mating experience, social status, and developmental environment such as intrauterine positioning (vom Saal and Howard, 1982) (see also Hrdy, 1977).

For the postpartum mothers in normal housing condition, the nest quality was evaluated before the pup retrieval assay. The nest was graded as 3 (excellent) when all the pups and almost all of nest materials were gathered tightly at one nest place and the nest was shaped like a hollow surrounded by a bank. The nest was 2 (good) when only one or two pups were separated from the other pups (out of the nest or buried under the bank of the nest), when the nest was rather flat but still discrete, or when less than one third of the nest materials were out of nest place. The nest was 0 (poor) when the pups and the nest materials were distributed randomly in the cage. The nest was 1 (OK) when the conditions were in between 0 and 2. Then, three of the pups were taken out of the nest and placed in each corner. They were observed for only 10 min because the retrieving behavior of normal postpartum mothers is very quick. Finally, all the pups were taken out of the nest and were investigated and classified either as "alive with milk in the stomach," "alive without milk," or "dead". In addition, it was recorded if there were any remaining amniotic membrane, umbilical cord, or placenta material attached to the body.

Data from BL6 background mothers were not included because there was a significant discrepancy between maternal behavior and pup survival on the BL6 background, and this made the interpretation of the results difficult. For example, almost all of the (+/–) females made nice nests and retrieved of all cleaned pups in the nest, whole litters of 60% (63 cases of 105 deliveries) of the (+/–) primiparous mothers died within a few days. These dying pups were all in the nest and their mother's nipples showed sign of vigorous suckling, but pups' stomach were empty. The same tendency was also seen in (–/–) mothers; some (–/–) mothers did not care for pups, but others actually did parenting, and still the pups could not survive. Therefore, we speculated that the milk production or ejection was compromised in the postpartum female mice of FosB (+/–) and (–/–) females on the BL6 background, but not on the original 129-based background. This issue needs further investigation and is tangential to the present study. Therefore, we did not include these data of BL6 background mothers in this study.

4.3. *Behavioral assays for gene expression analysis*

To compare gene expression patterns between parental and nonparental groups of mice both in females and males, two sets of experiments were conducted. For the postpartum female experiments, *FosB* (+/+) or (–/–) virgin females were mated with male mice and singly housed at late gestation. *FosB* (+/+) females were mated with *FosB* (+/–) males, and *FosB* (–/–) females were mated with *FosB* (+/+) males, respectively, to minimize the difference of pups' genotype. Again, it has been shown that the maternal behavior reflected in pup survival of *FosB* (+/) mothers is not affected by the genotype of the pups (Brown et al., 1996). On visible pregnancy, females were housed individually in a new cage and provided with a cotton square as nest material. Between 12:00 and 15:00 on the day of parturition, the mothers were subjected to the nest evaluation and the pup retrieval assay using their own pups and sacrificed. As reported previously (Brown et al., 1996), pregnancy and parturition of *FosB* (–/–) females were normal, but their nurturing behavior was defective in our experimental condition (Fig. 1). Mothers who had not delivered within 5 week after mating, who had not finished delivery by 9:00, and who had four pups or fewer were excluded from this study because the small amount of sensory stimuli from small litters interferes with the initiation of maternal behavior (Stern and Johnson, 1990). Three *FosB* (+/+) mice showing normal parental behavior and three *FosB* (–/–) mice showing defective nurturing were used for the DNA microarray analysis.

For the male experiments, *FosB* (+/–) and (–/–) adult virgin males were singly housed using wood chips, and on the next day, they were subjected to the pup retrieval assay by exposing them to foreign pups for 30 min. The following day they were subjected to the pup retrieval assay again, but the pups were left in the cage for 2 h until the subject males were sacrificed to maximally induce transcriptional activation. Behavior at the second retrieval assay was used for behavioral classification. The three *FosB* (+/–) mice showing parental behavior and the four *FosB* (–/–) mice, which were neither parental nor infanticidal toward the pups, were used for DNA microarray analysis.

4.4. *DNA microarray analyses*

Subject mice were humanely killed by cervical dislocation. The brains were immediately removed, soaked in RNAlater (Ambion, Austin, TX) at 4°C overnight, and stored at −20 °C. Coronal sections (200 μm) containing the posterior half of the anterior commissure were micropunched (Palkovits micropunch, 0.35 mm; Fine Science Tools, Foster City, CA) to dissect the dorsal parts of the MPOA. The bilateral tissues from each animal were collected and subjected to total RNA isolation (Qiagen RNeasy Micro Kit, Qiagen, Hilden, Germany). The quantity and quality of RNA were measured using NanoDrop ND-1000 (NanoDrop, Wilmington, DE) and Agilent 2100 Bioanalyzer with a RNA 6000 Pico LabChip kit (Agilent, Santa Clara, CA). Total RNA (10 ng) was then processed to make biotin-labeled cRNA using the Two Cycle cDNA Synthesis and IVT labeling kit

(Affymetrix, Santa Clara, CA). The resultant cRNA samples were verified using Test2chip (Affymetrix). Each biotin-labeled cRNA sample from one animal was hybridized to a single Affymetrix GeneChip Mouse Genome 430A 2.0 Array (Affymetrix) that contained >22,600 probe sets representing transcripts from 14,000 mouse genes.

The hybridization signal on the chip was scanned using an HP GeneArray scanner (Hewlett-Packard, Palo Alto, CA), processed by GeneSuite software, and analyzed using a GeneSpring software (Silicon Genetics, Redwood, CA). The normalized values of gene expression were subjected to the Student *t* test and transcripts satisfying the following three criteria were regarded as significantly changed: (1) Flag, an index of signal fidelity, was "present" or "marginal" in at least three samples in each experiment. (The Affymetrix system actually measures each gene with 11 independent perfectly matched probes and 11 corresponding mismatched probes. It subtracts the values of the mismatched from the matched probes, then does the one-sided Wilcoxon signed rank test to see whether, based on 11 independent measurements, the difference between the signals of the matched and mismatched probes are statistically significant. It uses these measures to label each probe signal as "present" when the signal was deemed significant, "absent" when it was not significant, or "marginal".) Using this cut-off criterion, 15,227 (67%) or 15,601 (69%) probes among 22,600 probes analyzed were deemed to be reliably measured in the experiment of females or males, respectively. (2) The fold change (FC) = average expression in *FosB* (+/) group/average expression in *FosB* (–/–) group, and FC>1.5 or FC<0.67. (3) Expression was significantly different between *FosB* (–/–) and (+/) by Student *t* test ($p<0.05$). The purpose of our microarray screens was to pick up candidate genes differentially regulated by the presence of FosB, for the following validation by qRT-PCR. Therefore, we simply applied the independent *t* test to the expression values of each gene here without further calibration for multiple comparisons such as Bonferroni correction. We found seven probe sets down-regulated in *FosB* (–/–) mice compared with the (+/) littermates in both of the female and male experiments. Loci of these seven probes, however, resided inside of or very close to the *FosB* locus (chromosome 7, A1–B3). These changes might have been caused by the insertion of FosB targeting allele or by the flanking gene problem (Wolfer et al., 2002) and were not investigated further in this study. All of the up-regulated genes in Table 1 were not in chromosome 7, so that they should not be caused by such construction artifacts.

4.5. *qRT-PCR detection of mRNA*

Total RNA from the MPOA micropunches was linearly amplified through cDNA synthesis and in vitro transcription, the same as in DNA microarray sample preparation. The second-round first-strand cDNA was synthesized with SuperScript II reverse transcriptase using oligo(dT) primers (Invitrogen Japan, Tokyo, Japan). qRT-PCRs were performed with an ABI PRISM 7000 Sequence Detector Systems and *Taq*Man or SYBR Green Universal PCR Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The relative amount of specific mRNA was calculated as $2^{(Ct\ (each\ gene)-Ct\ (GAPDH))}$ in triplicate. The average Ct (*GAPDH*) did not differ between groups (data not shown). *Taq*Man probe IDs used in this study are Mm99999915_g1 (*GAPDH*), Mm00546086_m1 (*GFAP*), Mm00712898_m1 (*Ela1*), and Mm00550309_m1 (*C4*).

4.6. *ICH analyses*

IHC analyses of free-floating brain sections were performed essentially as described (Berghorn et al., 1994). Briefly, mice were anesthetized and perfused with 4% paraformaldehyde in sodium phosphate-buffered saline (PBS) at pH 7.4. The brains were removed, immersed in the same fixative at 4 °C overnight, and then in PBS containing 30% (wt/vol) sucrose, and cryosectioned at 40 μm. Every third section from the serial sections was washed with PBS, bleached with 0.3% H_2O_2 in methanol, incubated overnight with a primary antibody (1:1000 dilution), washed, and then incubated with an appropriate biotin-conjugated secondary antibody. The signal was intensified and visualized using a Vectastain Elite ABC kit and a DAB substrate kit (Vector, Burlingame, CA). Primary antibodies used in this study were an anti-GFAP mouse monoclonal antibody ASTRO6 (Lab Vision, Fremont, CA; Fig. 3B–D) and an anti-S100 rabbit polyclonal antibody (Dako, Glostrup, Denmark; Fig. 3D and E).

Following IHC, these sections were mounted on glass slides, dehydrated, and cover-slipped with Entellan New (Merck Japan, Tokyo). Neuroanatomical areas were determined (Paxinos and Franklin, 2001), and bright-field images were acquired using a digital camera DXM1200C and an Eclipse 80i microscope (Nikon, Kawasaki, Japan). Using the printed photographs, GFAP or S100 immunopositive cells within the frontal cortex or the striatum were counted manually by the experimenter blind to the experimental groups.

4.7. *Statistical analyses*

For the statistical analysis of the data, Fisher's exact probability test, Student's *t* tests, and various kinds of ANOVA were used. When sphericity was rejected by the Mauchly test, the Greenhouse-Geisser estimate was used. When a significant effect was found by ANOVA, Tukey post-hoc comparisons or the Fisher least-squares difference (LSD) test were applied. For the statistical analysis of comprehensive behavioral test battery, repeated measures ANOVA with genotype (–/–, +/–, +/+) as the between-subject factor and repeated measures such as period or day as the within-subject factor, were conducted on the data in each test. Significance levels were set at $p<0.05$ (two-tailed). These statistical analyses were performed using SPSS 16.0 for Windows (SPSS Japan, Tokyo, Japan); *df* indicates degree of freedom.

For further analysis of the multivariate categorical data shown in Fig. 2, two approaches could be used (Agresti, 1996), one was a generalized linear model (GLM), and the other was a logit model for multinomial responses (multinomial logistic regression). Although many statistical software have different function to perform these analyses, the latter has been shown to be a special case of the former (Nelder and Wedderburn, 1972). We performed both analyses using R (R Development Core Team, 2007) and libraries (nnet, MASS, and epicalc), and obtained consistent results (see online supplementary material). Here we presented the procedure of the GLM analysis, because the results of multinomial logistic regression were

shown as a ratio of two behavioral probabilities and slightly more complicated than the GLM results. First, we converted the 3-level nominal data consisting of "Parental", "Infanticide", and "Nonresponding" into the set of parameters (*P* score, *I* score, *N* score). If one animal showed any of these behaviors within 30 min of pup exposure, it was given the corresponding score of "1", and "0" for the other two behavioral scores. Because these measures were mutually exclusive, each subject animal showed behavioral parameter either (1,0,0), (0,1,0) or (0,0,1). Then *glm* function was applied to explain each of these behavioral scores of P, I and N, with three independent variables of genotype (+/ or –/–), gender (female or male) and the experimental conditions. The model was selected by stepwise backward elimination procedure using Akaike's information criterion (Akaike, 1969) using *stepAIC* function in R, and examined by a likelihood-ratio test. Then adjusted odds ratios, 95% Confidence Intervals and p values were calculated, double-checked using SPSS, and were shown in Table 2.

4.8. *Behavioral test battery*

Phenotypic behavioral differences between *FosB* (–/–), (+/–), and (+/+) mice were assessed with a specific behavioral test battery consisting of open-field activity, passive avoidance learning, elevated plus maze, and prepulse inhibition. These tests were run in sequence to one set of 11-week-old male mice (*FosB* [–/–] mice, $n=16$; [+/–], $n=6$, and [+/+], $n=10$). The order of the tests was the same as the order of presentation in Fig. 4. This order was arranged from noninvasive tests to invasive ones to avoid the possible effect of the previous tests. All of these animals have been exposed to pups for two successive days beforehand. These experiments were carried out in cooperation with the Behavioral and Medical Sciences Research Consortium (BMRC, Hyogo, Japan) and animal handling was done in accordance with the "Guidelines for Animal Experimentation at BMRC".

4.8.1. *Open-field activity*

A transparent acrylic box (30×30×30 cm) housed in a ventilated sound-attenuating shell was used. In the light condition, an overhead incandescent bulb provided room lighting that measured approximately 110 lx inside the open-field arena. In addition, a fan attached in the upper part of the wall at one end of the shell presented a masking noise of 45 dB. On each X and Y bank of the open field, two infrared beams were attached 2 cm above the floor in 10-cm intervals making a flip-flop circuit between two beams. Total number of circuit breaks was counted as the locomotor activity. On the X bank, 12 infrared beams were attached 4.5 cm above the floor in 2.6-cm intervals, and the total number of beam crossings was counted as the rearing activity. Animals were allowed to explore freely in the open-field arena for 20 min.

4.8.2. *Acoustic startle response and PPI*

Each mouse was enclosed in a transparent acrylic box (7×7×10 cm) mounted on a wooden frame on four rubber balls, which absorb vibration quickly, within a ventilated enclosure. Whole-body startle responses of the mouse caused vibrations of the box. These vibrations were converted to analog signals by a GH-313A piezoelectric accelerometer (Keyence, Osaka, Japan) attached to the underside of the box and then digitized and recorded by a computer. The acoustic startle pulse of broadband burst (115 dB, 50 ms) and tone prepulse (1000 Hz, 85 dB, 30 ms) were presented via a speaker located in front of the box. Throughout the session, a background noise level of 70 dB was maintained. The experimental session consisted of two periods. During the first period, 40 startle pulses were presented to test for basal startle responsiveness. The second period included three different trial types: a startle pulse alone, a tone prepulse followed by a startle pulse, and a light prepulse followed by a startle pulse. Prepulses preceded the startle pulse by either 50, 100, or 200 ms from a prepulse onset to the onset of the startle pulse. The mean intertrial interval was 20 s (range: 10–30 s) throughout the session. The startle response was recorded for 200 ms (measuring the response every 1 ms) starting with the onset of the startle pulse. PPIs were indexed in terms of the PPI ratio. The ratio was calculated using the formula: PPI ratio = PP/S, where PP is designated the mean response of trial with prepulse, and S is designated the mean response of trial without prepulse. Thus, a smaller ratio indicates a stronger PPI. Because light pulse did not significantly inhibit startle response, the data of light pulse were not presented.

4.8.3. *Elevated plus maze*

The gray acrylic floor of the maze consisted of four arms 5 cm wide and 30 cm long that met at a 5×5-cm center zone, and the apparatus was mounted on a pedestal that placed the arms 50 cm above the floor. The two closed arms had clear plastic walls 15 cm high, whereas the open arms had a low rim 3 mm high. The room lighting created a light intensity of approximately 20 lx on the maze. A fan attached in the upper part of the wall at one end of the shell presented a masking noise of 45 dB. The animal was placed gently onto the center of the maze and was allowed to explore the maze freely for 10 min. The number of entries into each arm and time spent in each arm were recorded from videotapes.

The time spent in open arms was extremely long in one of the *FosB* (+/+) mice (437 s during 10 min). This mouse was regarded as an outlier (Smirnov–Grubbs test, $p<0.01$, $n=34$) and excluded from further statistical analysis.

4.8.4. *Passive avoidance learning*

Animals were trained in a step–through-type passive avoidance apparatus consisting of two compartments, one light (10×10×20 cm) and one dark (10×10×20 cm), with a grid floor. A guillotine door separated the two compartments. In the acquisition trial, mice were individually placed in the light compartment. Five seconds later, the door to the dark compartment was opened. When the mouse moved into the dark compartment, the guillotine door was closed, and 10 and 18 s later, a scrambled electrical shock (160 V, 3 s) was delivered through the grid floor by a shock generator. Twenty-four hours later, the retention test without shock was conducted. Each mouse was placed in the light compartment and the latency to enter the dark compartment was recorded up to a maximum of 180 s.

4.9. *Restraint stress and plasma CORT measurements*

At the end of the behavioral test battery described above, the 15-min acute restraint stress was given to half the subject mice using a mouse holder (Ishizawa Coop., Tsukuba, Japan). The rest

of mice were left without stress for 15 min. Then the mice were humanely killed by cervical dislocation. Trunk blood was immediately collected and the blood plasma was subjected to CORT measurements by radioimmunoassay, as described (Nabors et al., 1974). This experiment was performed within 10–12 a.m.

Acknowledgments

We thank Michel L. Tremblay for kindly providing working space and laboratory equipment; Michael E. Greenberg for the discussions and for the protocol of PCR genotyping of the *FosB* (–/–) strain; Chikuma Hamada, Hirotaka Onishi and Masahiro Kaneseki for the statistical method selection; the R Core Team for the software and packages, Fukiko Isono, Tetsuaki Ara, and Taeko Nemoto for their technical assistance; the RIKEN Research Resource Center for the hybridization and scanning of GeneChips and maintenance of animals; and Doe Nobutaka for the behavioral testing at BMRC. This research was supported by grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (2004–2007 to K.K. and K.T.), a Long-Term Fellowship of the Human Frontier Science Program (2002–2004 to K.K.), and DRI Research Grants from RIKEN (2005–2006 to K.K.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.brainres.2008.02.100.

REFERENCES

Agresti, A., 1996. An introduction to categorical data analysis. John Wiley & Sons.

Akaike, H., 1969. Fitting autoregressive models for prediction. Ann. Inst. Statist. Math. 21, 243–247.

Beglopoulos, V., Sun, X., Saura, C.A., Lemere, C.A., Kim, R.D., Shen, J., 2004. Reduced beta-amyloid production and increased inflammatory responses in presenilin conditional knock-out mice. J. Biol. Chem. 279, 46907–46914.

Berghorn, K.A., Bonnett, J.H., Hoffman, G.E., 1994. cFos immunoreactivity is enhanced with biotin amplification. J. Histochem. Cytochem. 42, 1635–1642.

Bridges, R.S., Scanlan, V.F., 2005. Maternal memory in adult, nulliparous rats: effects of testing interval on the retention of maternal behavior. Dev. Psychobiol. 46, 13–18.

Bridges, R.S., Zarrow, M.X., Goldman, B.D., Denenberg, V.H., 1974. A developmental study of maternal responsiveness in the rat. Physiol. Behav. 12, 149–151.

Brown, J.R., Ye, H., Bronson, R.T., Dikkes, P., Greenberg, M.E., 1996. A defect in nurturing in mice lacking the immediate early gene fosB. Cell 86, 297–309.

Cai, J., Chen, Y., Cai, W.H., Hurlock, E.C., Wu, H., Kernie, S.G., Parada, L.F., Lu, Q.R., 2007. A crucial role for Olig2 in white matter astrocyte development. Development 134, 1887–1899.

Calamandrei, G., Keverne, E.B., 1994. Differential expression of Fos protein in the brain of female mice dependent on pup sensory cues and maternal experience. Behav. Neurosci. 108, 113–120.

Crabbe, J.C., Wahlsten, D., Dudek, B.C., 1999. Genetics of mouse behavior: interactions with laboratory environment. Science 284, 1670–1672.

Crawley, J.N., 2000. Whats wrong with my mouse? Wiley-Liss, New York. 329 pp.

Eng, L.F., Ghirnikar, R.S., Lee, Y.L., 2000. Glial fibrillary acidic protein: GFAP-thirty-one years (1969–2000). Neurochem. Res. 25, 1439–1451.

Gruda, M.C., van Amsterdam, J., Rizzo, C.A., Durham, S.K., Lira, S., Bravo, R., 1996. Expression of FosB during mouse development: normal development of FosB knockout mice. Oncogene 12, 2177–2185.

Hamaguchi-Hamada, K., Sanbo, C., Hamada, S., Yagi, T., 2004. Exposure to hexanal odor influences maternal behavior and induces neonatal death in Fyn tyrosine kinase-deficient mice. Neurosci. Res. 48, 259–267.

Heim, C., Nemeroff, C.B., 2001. The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. Biol. Psychiatry. 49, 1023–1039.

Herdegen, T., Leah, J.D., 1998. Inducible and constitutive transcription factors in the mammalian nervous system: control of gene expression by Jun, Fos and Krox, and CREB/ATF proteins. Brain Res. Brain Res. Rev. 28, 370–490.

Hiroi, N., Brown, J.R., Haile, C.N., Ye, H., Greenberg, M.E., Nestler, E.J., 1997. FosB mutant mice: loss of chronic cocaine induction of Fos-related proteins and heightened sensitivity to cocaine's psychomotor and rewarding effects. Proc. Natl. Acad. Sci. U. S. A. 94, 10397–10402.

Hiroi, N., Marek, G.J., Brown, J.R., Ye, H., Saudou, F., Vaidya, V.A., Duman, R.S., Greenberg, M.E., Nestler, E.J., 1998. Essential role of the FosB gene in molecular, cellular, and behavioral actions of chronic electroconvulsive seizures. J. Neurosci. 18, 6952–6962.

Hrdy, S.B., 1977. Infanticide as a primate reproductive strategy. Am. Sci. 65, 40–49.

Jacobson, C.D., Terkel, J., Gorski, R.A., Sawyer, C.H., 1980. Effects of small medial preoptic area lesions on maternal behavior: retrieving and nest building in the rat. Brain Res. 194, 471–478.

Jin, S.H., Blendy, J.A., Thomas, S.A., 2005. Cyclic AMP response element-binding protein is required for normal maternal nurturing behavior. Neuroscience 133, 647–655.

Kalinichev, M., Rosenblatt, J.S., Morrell, J.I., 2000a. The medial preoptic area, necessary for adult maternal behavior in rats, is only partially established as a component of the neural circuit that supports maternal behavior in juvenile rats. Behav. Neurosci. 114, 196–210.

Kalinichev, M., Rosenblatt, J.S., Nakabeppu, Y., Morrell, J.I., 2000b. Induction of c-fos-like and fosB-like immunoreactivity reveals forebrain neuronal populations involved differentially in pup-mediated maternal behavior in juvenile and adult rats. J. Comp. Neurol. 416, 45–78.

Krasnegor, N.A., Bridges, R.S., 1990. Mammalian parenting: biochemical, neurobiological, and behavioral determinants. Oxford UP, New York. 502 pp.

Kuroda, K.O., Meaney, M.J., Uetani, N., Fortin, Y., Ponton, A., Kato, T., 2007. ERK-FosB signaling in dorsal MPOA neurons plays a major role in the initiation of parental behavior in mice. Mol. Cel. Neurosci. 36, 121–131.

Lee, A.W., Brown, R.E., 2002. Medial preoptic lesions disrupt parental behavior in both male and female California mice (*Peromyscus californicus*). Behav. Neurosci. 116, 968–975.

Li, C., Chen, P., Smith, M.S., 1999. Neural populations in the rat forebrain and brainstem activated by the suckling stimulus as demonstrated by cFos expression. Neuroscience 94, 117–129.

Mathieson, W.B., Wilkinson, M., Brown, R.E., Bond, T.L., Taylor, S.W., Neumann, P.E., 2002. FOS and FOSB expression in the medial

preoptic nucleus pars compacta of maternally active C57BL/6J and DBA/2J mice. Brain Res. 952, 170–175.
McCarthy, M.M., vom Saal, F.S., 1986. Infanticide by virgin CF-1 and wild male house mice (*Mus musculus*): effects of age, prolonged isolation, and testing procedure. Dev. Psychobiol. 19, 279–290.
Morgan, H.D., Watchus, J.A., Milgram, N.W., Fleming, A.S., 1999. The long lasting effects of electrical simulation of the medial preoptic area and medial amygdala on maternal behavior in female rats. Behav. Brain Res. 99, 61–73.
Moriguchi, T., Gotoh, Y., Nishida, E., 1996. Roles of the MAP kinase cascade in vertebrates. Adv. Pharmacol. 36, 121–137.
Nabors Jr., C.J., West, C.D., Mahajan, D.K., Tyler, F.H., 1974. Radioimmunoassay of human plasma corticosterone: method, measurement of episodic secretion and adrenal suppression and stimulation. Steroids 23, 363–378.
Nelder, J.A., Wedderburn, R.W.M., 1972. Generalized linear models. J. R. Statist. Soc. A. 135, 370–384.
Numan, M., 1994. Maternal behavior. In: Knobili, E., Neill, J.D. (Eds.), The physiology of reproduction, vol. 2. Raven, New York, pp. 221–302.
Numan, M., Numan, M.J., 1994. Expression of Fos-like immunoreactivity in the preoptic area of maternally behaving virgin and postpartum rats. Behav. Neurosci. 108, 379–394.
Numan, M., Insel, T.R., 2003. The neurobiology of parental behavior. Springer-Verlag, New York, p. 418.
Numan, M., McSparren, J., Numan, M.J., 1990. Dorsolateral connections of the medial preoptic area and maternal behavior in rats. Behav. Neurosci. 104, 964–979.
Numan, M., Numan, M.J., Marzella, S.R., Palumbo, A., 1998. Expression of *c-fos*, fos B, and egr-1 in the medial preoptic area and bed nucleus of the stria terminalis during maternal behavior in rats. Brain Res. 792, 348–352.
Patterson, C.M., Newman, J.P., 1993. Reflectivity and learning from aversive events: toward a psychological mechanism for the syndromes of disinhibition. Psychol. Rev. 100, 716–736.
Paxinos, G., Franklin, K.B.J., 2001. The mouse brain in stereotaxic coordinates. Academic Press, San Diego.
Pekny, M., Nilsson, M., 2005. Astrocyte activation and reactive gliosis. Glia 50, 427–434.
Perrigo, G., Belvin, L., Quindry, P., Kadir, T., Becker, J., van Look, C., Niewoehner, J., vom Saal, F.S., 1993. Genetic mediation of infanticide and parental behavior in male and female domestic and wild stock house mice. Behav. Genet. 23, 525–531.
R Development Core Team, 2007. R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna.
Rosenblatt, J.S., 1967. Nonhormonal basis of maternal behavior in the rat. Science 156, 1512–1514.
Rosenblatt, J.S., Hazelwood, S., Poole, J., 1996. Maternal behavior in male rats: effects of medial preoptic area lesions and presence of maternal aggression. Horm. Behav. 30, 201–215.
Schneider, J.S., Stone, M.K., Wynne-Edwards, K.E., Horton, T.H., Lydon, J., O'Malley, B., Levine, J.E., 2003. Progesterone receptors mediate male aggression toward infants. Proc. Natl. Acad. Sci. U. S. A. 100, 2951–2956.
Shimakura, A., Kamanaka, Y., Ikeda, Y., Kondo, K., Suzuki, Y., Umemura, K., 2000. Neutrophil elastase inhibition reduces cerebral ischemic damage in the middle cerebral artery occlusion. Brain Res. 858, 55–60.
Stern, J.M., Johnson, S.K., 1990. Ventral somatosensory determinants of nursing behavior in Norway rats. I. Effects of variations in the quality and quantity of pup stimuli. Physiol. Behav. 47, 993–1011.
Terkel, J., Bridges, R.S., Sawyer, C.H., 1979. Effects of transecting lateral neural connections of the medial preoptic area on maternal behavior in the rat: nest building, pup retrieval and prolactin secretion. Brain. Res. 169, 369–380.
vom Saal, F.S., 1983. Variation in infanticide and parental behavior in male mice due to prior intrauterine proximity to female fetuses: elimination by prenatal stress. Physiol. Behav. 30, 675–681.
vom Saal, F.S., Howard, L.S., 1982. The regulation of infanticide and parental behavior: implications for reproductive success in male mice. Science 215, 1270–1272.
Weiss, S.M., Wadsworth, G., Fletcher, A., Dourish, C.T., 1998. Utility of ethological analysis to overcome locomotor confounds in elevated maze models of anxiety. Neurosci. Biobehav. Rev. 23, 265–271.
Wolfer, D.P., Crusio, W.E., Lipp, H.P., 2002. Knockout mice: simple solutions to the problems of genetic background and flanking genes. Trends Neurosci. 25, 336–340.
Zhu, H., Lee, M., Agatsuma, S., Hiroi, N., 2007. Pleiotropic impact of constitutive fosB inactivation on nicotine-induced behavioral alterations and stress-related traits in mice. Hum. Mol. Genet. 16, 820–836.