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ERK-FosB signaling in dorsal MPOA neurons plays a major role in the initiation of parental behavior in mice

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During mouse parental behavior, neurons in the dorsal medial preoptic area (MPOAd) are activated and express transcription factors such as c-Fos and FosB. *FosB*-knockout mice are reported to be defective in parental care. To clarify molecular signaling responsible for parental behavior, we investigated gene expression profiles in the MPOAd of parental versus nonparental mice. We identified upregulation of *NGFI-B*, *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 phosphorylated in MPOAd neurons upon pup exposure. Pharmacological blockade of ERK phosphorylation inhibited the FosB upregulation in MPOAd, 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 MPOAd of nonparental *FosB*-knockout mice. These results suggest the pivotal role of ERK-FosB signaling in the initiation of parental care.

Introduction

Parental behavior is defined as the collection of behaviors (e.g., nursing, nesting, retrieving) that can increase offspring survival (Krasnegor and Bridges, 1990; Numan and Insel, 2003). For all mammalian infants, maternal care including nursing is essential for survival. In some rodent species such as laboratory mice, fathers also take care for their offspring (Lonstein and De Vries, 2000). Virgin animals, especially males, often avoid or attack unfamiliar pups at first, but after some period of cohabitation they can be sensitized and care for donor pups (pup sensitization) (Rosenblatt, 1967). Once they become fully parental, such "parental memory" can last for at least several days in virgins and for many months in postpartum mothers without further pup exposure (Bridges, 1996; Bridges and Scanlan, 2005). Therefore, nurturing experience can modify parental responsiveness in a long-lasting manner, but the molecular mechanism of this phenomenon is not known.

The medial preoptic area (MPOA) is a critical brain region for the expression of parental retrieving behavior (i.e., gathering scattered pups into the nest) (Numan, 1994; Morgan et al., 1999). 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; Numan and Numan, 1994; Li et al., 1999). MPOA lesions, especially in the dorsal MPOA (MPOAd), specifically inhibit retrieving, without affecting feeding, general locomotion, female reproductive functions, or sexual behaviors (Terkel et al., 1979; Jacobson et al., 1980; Numan et al., 1990; Rosenblatt et al., 1996; Kalinichev et al., 2000a; Lee and Brown, 2002). No other brain area has been reported to consistently and specifically fulfill these both conditions as MPOAd does.

A growing number of gene-targeted mouse strains have revealed defective parental behavior (Leckman and Herman, 2002) (Gammie, 2005). *FosB* mutant mice were reported to be profoundly deficient in parenting behavior, in spite of showing no abnormalities in general health or cognitive functions, such as olfactory discrimination and spatial learning (Brown et al., 1996). *FosB*−/− females mate normally and deliver no less healthy pups than those of the wild-type females, but they often leave the pups scattered in the cage and nurture them little, so that the majority of the pups die before weaning. The genotype of pups is not the cause of this nurturing defect. Virgin *FosB*−/− females and males also show a deficit in retrieving behavior, suggesting that this defect is independent from the peripartum hormonal milieu. *FosB* is an immediate early gene (IEG) homologous to *c-Fos* and is induced in a pattern similar to that of c-Fos in MPOAd neurons during parenting (Brown et al., 1996; Kalinichev et al., 2000b).

These previous reports suggest that c-Fos and FosB are induced in MPOAd neurons and these transcription factors then induce the expression of other genes required to actuate parental behavior. It

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Table 1
Genes upregulated with parental behavior in both experiments 1 and 2

Common name	Probe set ID	Experiment 1				Experiment 2		Description
		2 vs. 0 h FC	*t**	6 vs. 0 h FC	*t**	PAR vs. INF FC	*t***	
c-Fos	1423100_at	**3.50**	3.69	**2.29**	3.82	**2.22**	2.63	FBJ osteosarcoma oncogene
NGFI-B	1416505_at	1.30	0.88	**1.76**	2.89	**1.98**	4.96	NGF-inducible factor-B
Rad	1422565_at	2.23	2.09	**1.74**	3.06	**1.85**	2.71	RGK-family small G protein
Spry1	1415874_at	**1.22**	4.72	1.06	2.08	**1.29**	5.06	Sprouty homologue 1

The fold change (FC)=average expression in the parental group divided by the average expression in the nonparental or infanticidal group. Bold numbers indicate significant changes ($P<0.05$). *df=4, **df=5.

remains unclear, however, which signal transduction pathway is activated by sensory stimuli from pups to upregulate c-Fos and FosB in the MPOAd and what c-Fos- and FosB-mediated cellular events occur in these neurons. To address these questions, we investigated molecular mechanism essential for parenting in the MPOAd.

Results

DNA microarray analyses and quantitative real-time PCR

We examined gene expression profiles in the MPOAd of parental vs. nonparental mice upon pup exposure by means of DNA microarray analyses to identify molecular events relevant to parental behavior. In experiment 1, lactating females were separated from their litters for 2 days and then exposed to donor pups for 0 h, 2 h, or 6 h. In the DNA microarray analysis, transcripts showing significantly higher expression in either of the parental groups (2 h or 6 h) than those in the nonparental groups were selected using the criteria described in the Experimental methods. The numbers of transcripts selected by the criteria was 310. This included *c-Fos*, a positive control of this gene expression analysis (Table 1).

Some of these expression changes, however, might have been caused by sensory stimuli from pups' suckling or different activity levels and might not be directly implicated in the expression of parental behavior. To eliminate such indirect effects on gene expression, we performed another experiment comparable to experiment 1 using virgin male mice. We used male mice since most virgin female mice are spontaneously maternal (Numan, 1985; Calamandrei and Keverne, 1994). In contrast, in our experimental condition, half of adult virgin males were spontaneously parental and the reminders were infanticidal and bit pups within a few minutes (Fig. 1), consistent with the previous reports (Lonstein and De Vries, 2000). This fact enabled us to compare the MPOAd of these parental and infanticidal virgin males by performing another set of DNA microarray analyses (experiment 2). The number of transcripts selected by our criteria for experiment 2 was 265.

We then compared the results of experiments 1 and 2 focusing on transcripts that showed significant upregulation related to parental care in both studies. Only six transcripts showed changes in both studies and increases in four of these transcripts (*c-Fos*, *NGFI-B*, *Rad*, and *SPRY1*) (Table 1) were successfully confirmed in the parental group using qRT-PCR in both experiments 1 and 2 (Figs. 2A and B, respectively). *FosB* does not appear in Table 1, mainly because the GeneChip data of *FosB* mRNA did not fulfill the selection criterion 3 (see the Experimental methods), suggesting that the expression level of *FosB* was lower than the detection limit by this GeneChip analysis. Since FosB induction during parental care has already been shown by IHC (Brown et al., 1996; Numan et al., 1998; Kalinichev et al., 2000b), we tested the expression of *FosB* mRNA by qRT-PCR. In all experiments, *FosB* mRNA was significantly higher in the parental group than in the nonparental group (Figs. 2A and B). This finding was also confirmed by IHC using two antibodies for FosB protein (data not shown). Therefore, we concluded that *FosB* should be actually upregulated in the parental group of both experiments 1 and 2.

Consistent with previous studies (Calamandrei and Keverne, 1994; Numan and Numan, 1994; Li et al., 1999), c-Fos immunopositive cells in MPOAd of parental postpartum mothers significantly increased following exposure to pups (see Fig. 4C). We also found that c-Fos was induced in the MPOAd of parental males. *c-Fos* expression measured as mRNA and protein were significantly higher in parental males than those in infanticidal males (Table 1, Figs. 2B–D).

ERK activation is involved in parental behavior

SPRY1 is a murine homologue of *Drosophila Sprouty* (Hacohen et al., 1998; Minowada et al., 1999; Impagnatiello et al., 2001). Sprouty proteins represent a major class of ligand-inducible inhibitors of the receptor tyrosine kinase (RTK)-dependent Ras-ERK MAP kinase signaling pathway (Mason et al., 2006). *SPRY1* induction

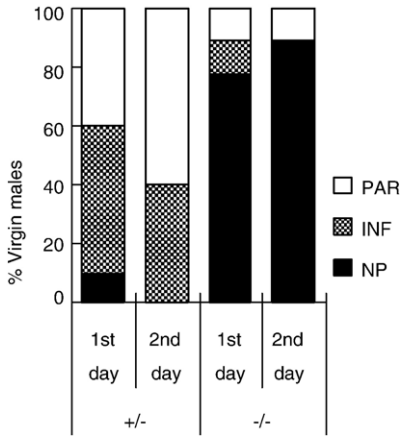

Fig. 1. Pup retrieval assays of *FosB*+/− and −/− virgin male mice. Results of two consecutive pup retrieval assays of *FosB*+/− (n=10) and −/− (n=9) virgin males. PAR (parental): Retrieved all three pups within 30 min (open area). INF (infanticidal): Bit one or more pups within 30 min (shaded area). NP (nonparental): Neither of them (solid area). The results from the second day were used for behavioral classification.

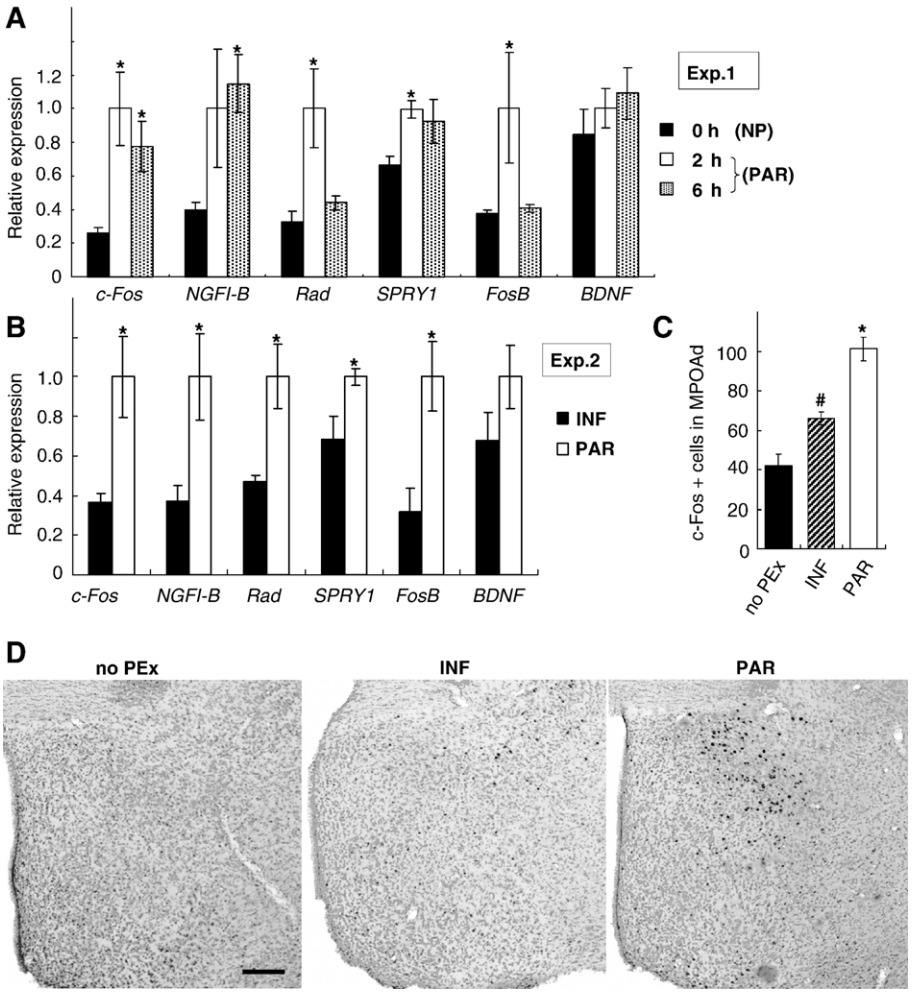

Fig. 2. Transcripts upregulated in parental than in nonparental animals in both experiments 1 and 2. (A, B) Mean±SEM relative expression levels of genes listed in Table 1 plus *FosB* and *BDNF* examined by qRT-PCR. Data are expressed as arbitrary units normalized to *GAPDH*, with values of the 2-h (A) or parental (PAR) (B) group set to 1. *Significant difference ($P<0.05$) from the nonparental (NP) (A) or from parental (PAR) (B) group. (A) Experiment 1: postpartum mothers without (0 h, solid bars, $n=6$) or with pup exposure for 2 h (open bars) or for 6 h (shaded bars; $n=3$ each); one-way ANOVA with a post hoc Tukey's test: $F(2,9)=12.60$, 6.45, 8.94, 5.49, 5.49, 1.12, respectively, from the left. (B) Experiment 2: infanticidal (solid bars, $n=4$) or parental (open bars, $n=4$) virgin males; Student's t tests: $t_6=3.00$, 2.74, 3.18, 2.50, 3.28, 1.50, respectively, from the left. (C) Mean±SEM number of c-Fos immunopositive neurons per unilateral MPOAd of male mice. Wild-type virgin males without pup exposure (no PEx, solid bar, $n=9$), exposed to pups for 2 h and bit one or more pups (INF, shaded bar, $n=11$), or retrieved all the pups within 30 min (PAR, open bar, $n=12$). $F(2,27)=33.54$; $^{\#}$significantly different from no PEx ($P<0.05$); *significantly different from no PEx ($P<0.001$) and from INF ($P<0.001$). (D) Representative c-Fos IHC in coronal sections of MPOA from the experiment in (C). The sections were counterstained with hematoxylin. The anterior commissure at the top, the third ventricle to the left. Scale bar, 150 μm.

during parental behavior suggests that the RTK-Ras-ERK pathway should be activated in MPOAd neurons. *c-Fos*, *FosB*, and *NGFI-B* are also known targets of the MAP kinase signaling pathway (Watson and Milbrandt, 1989; Chaudhuri, 1997). Rad is a member of the RGK- (Rad, Gem, and Kir) family small GTP-binding proteins that function as potent inhibitors of voltage-dependent calcium channels (Kelly, 2005). Both of Rad's close homologues, Gem (Maguire et al., 1994) and Kir (Cohen et al., 1994), are induced by RTK-ERK signaling, although this has not been shown for Rad directly. These previous reports together with the present findings suggest that, in MPOAd neurons, RTK-ERK signaling is activated during the performance of parental behavior, which induces transcription factors such as c-Fos, NGFI-B, and FosB and consequently stimulates expression of actuators such as SPRY1 and Rad (see Fig. 5). If so, the activated form of ERK (dual-phosphorylated, pERK) should also be detectable. IHC with a pERK-specific antibody confirmed elevated pERK-like immunoreactivity in MPOAd neurons of parental mothers (Fig. 3A). The number of pERK-positive neurons was significantly increased at 15 min after pup exposure, but their numbers rapidly declined and were not significantly different from baseline after 30 min (Fig. 3B).

Blocking ERK phosphorylation inhibits the initiation of retrieving but does not disturb the consolidated retrieving behavior

To clarify the role of ERK activation for performance of parental behavior, we blocked the specific upstream activator of ERK, MEK1/2. Administration of 100 mg kg^{-1} SL327, a MEK inhibitor, at 1 h before contextual fear conditioning diminishes ERK phosphorylation in the hippocampus and blocks fear conditioning (Atkins et al., 1998). Diminished ERK phosphoryla-

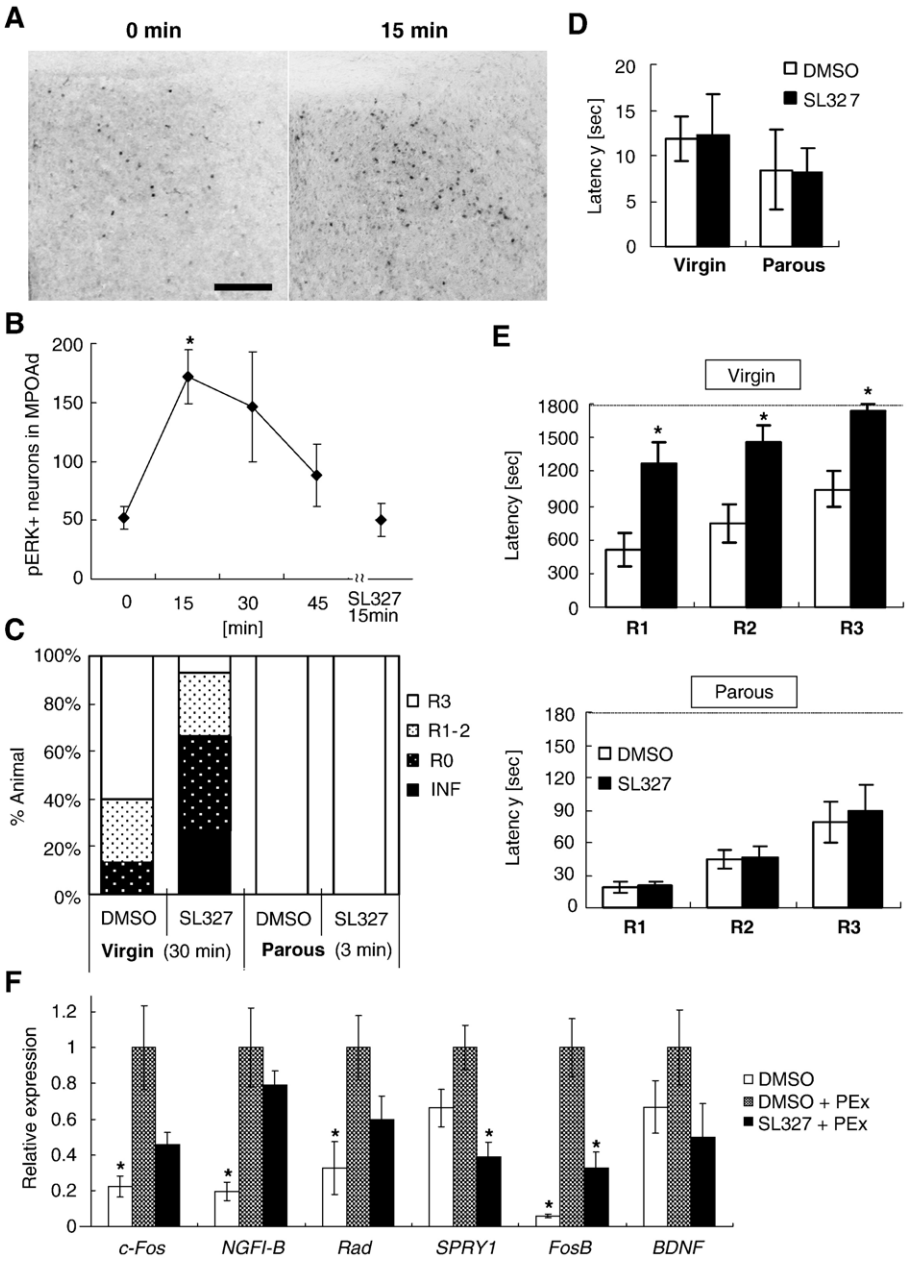

Fig. 3. ERK phosphorylation in MPOAd neurons and its role for the initiation of retrieving behavior and transcriptional activation. (A) pERK IHC in MPOAd. Postpartum mothers deprived of pups at the postpartum day (PPD) 5 and no further pup exposure (0 min) or pup re-exposure for 15 min on PPD7. All photographs are representative of results from at least five samples in each group. The anterior commissure at the top, the third ventricle to the left. Scale bar, 150 μm. (B) Time course of mean±SEM number of pERK positive neurons per unilateral MPOAd of PPD7 mothers ($n=5$ for each time point), induced by pup exposure from 0 min. $F(4, 16)=4.13$; *significantly different from 0 min and from SL327, 15 min ($P<0.05$). (C) Results of pup retrieval assays of virgin ($n=15$ per group) or parous ($n=5$ per group) female mice treated with 50% DMSO (vehicle) or 100 mg kg^{-1} SL327. Duration of the assay was set to 30 min for virgin females and to 3 min for parous females. Retrieved all three pups within this assay period (R3, open area), retrieved only one or two pups (R1-2, lightly shaded area), retrieved no pup (R0, darkly shaded area), or bit one or more pups (INF, solid area). Four of 15 virgins injected SL327 bit donor pups instead of parenting, with an average latency of 6 min (D) Mean±SEM latency to sniff the pup for the first time in the experiment described in panel C; $t_{28}=0.09$ for virgin, $t_8=0.04$ for parous females. (E) Mean±SEM latency to retrieval of each pup (R1, R2, R3) in the experiment described in panel C; $t_{25.4}=3.06$, $t_{28}=3.32$, $t_{17.5}=3.87$ from the left for virgins, respectively; $t_8=0.06$, 0.15, 0.32 from the left for parous females, respectively. *Significantly different from DMSO ($P<0.05$). (F) Mean±SEM relative expression levels of genes listed in Table 1 plus *FosB* and *BDNF* examined by qRT-PCR. Virgin female mice treated with 50% DMSO and without pup exposure (DMSO, open bars), treated with vehicle and exposed to pups for 90 min (DMSO+PEx, shaded bars), treated with SL327 and exposed to pups for 90 min (SL327+PEx, solid bars) ($n=5$ per group). Data are expressed as arbitrary units normalized to *GAPDH*, with values of the DMSO+PEx group set to 1. Comparison was performed by a Student's t test: for DMSO vs. DMSO+PEx, $t=3.22$, 3.55, 2.89, 2.09, 5.75, 0.64, and for DMSO+PEx vs. SL327+PEx, $t=2.23$, 0.89, 1.81, 4.58, 3.62, 0.27, respectively, from the left; $df=8$. *Significantly different from DMSO+PEx ($P<0.05$). *SPRY1* upregulation did not reach statistically significance in DMSO+PEx group compared with that of DMSO group, possibly because of technical difficulties of this particular experiment (see Experimental methods).

tion by intraperitoneal injection of 100 mg kg^{-1} SL327 was confirmed in the MPOAd of pup-exposed parous females (Fig. 3B). Then we examined the effect of SL327 on pup retrieval tests in both virgin and parous female C57BL/6J mice. Although virgin female mice are spontaneously maternal on the first day of pup exposure, it took more than several minutes for virgins to start retrieving (average latency of the first retrieval was 8.8 min for vehicle-injected virgin females; Fig. 3E). In contrast, post-weaning parous females, which had successfully raised at least one litter, started retrieving immediately after the sniffing of a pup (average 19.6 s) perhaps because of a sustained maternal memory comparable to that reported in rats (Bridges, 1996; Bridges and Scanlan, 2005). Concerning the time course of pERK described above, we hypothesized that inhibition of ERK phosphorylation by SL327 might influence the retrieval behavior of virgin, but not parous females.

Indeed, SL327 administration significantly attenuated retrieving behavior of virgin female mice (Figs. 3C and E). Sixty percent of

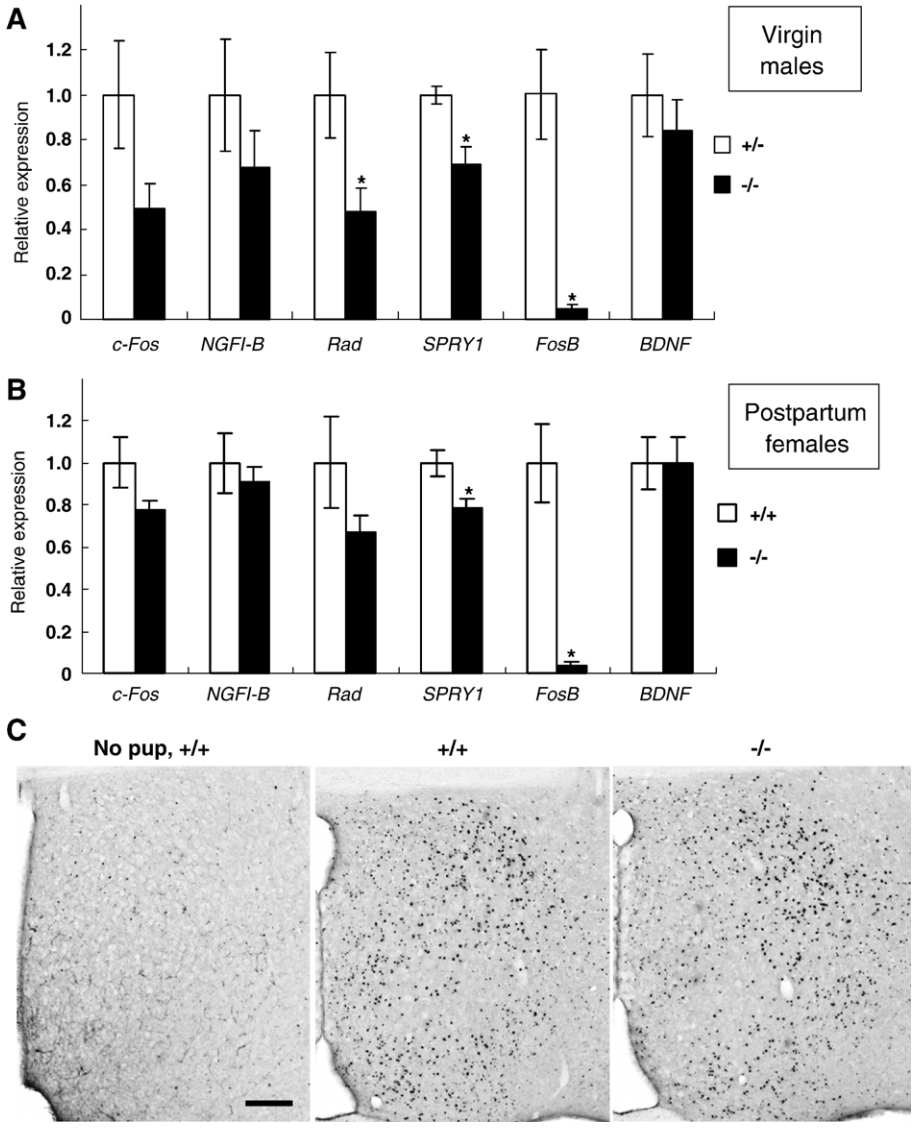

Fig. 4. *SPRY1* induction is impaired in FosB−/− mice. (A, B) Mean±SEM relative expression levels of genes listed in Table 1 plus *FosB* and *BDNF* examined by qRT-PCR. Data are expressed as arbitrary units normalized to *GAPDH*, with values of *FosB*+/− (A) or +/+ (B) group set to 1. *Significant difference ($P<0.05$) from *FosB*+/− (A) or +/+ (B) group. (A) *FosB*+/− parental (open bars, $n=4$) or and *FosB*−/− nonparental males (solid bars, $n=5$); Student's t test: $t_7=2.20, 1.15, 2.64, 2.86, 6.13, 0.69$, respectively, from the left. (B) *FosB*+/+ (open bars, $n=5$) and −/− (solid bars, $n=4$) postpartum females. Since many of *FosB*−/− mothers did not nurture the pups, the protocol was modified from that of the Experiment 1 as follows; *FosB*+/+ or −/− virgin females were mated with *FosB*+/− or *FosB*+/+ males, respectively, and housed singly at late gestation. Between 12:00 and 15:00 on the day of parturition, the mothers were sacrificed. MPOA tissue preparation was performed as in experiment 2. Mothers who had not finished delivery by 9:00 and who had four pups or less were excluded from this study, because the small amount of sensory stimuli from small litters has been shown to interfere with the initiation of maternal behavior (Stern and Johnson, 1990). Comparison was performed by a Student's t test: $t=1.58, 0.51, 1.43, 2.65, 4.49, 0.01$, respectively, from the left; $df=7$, except for *Rad* $df=5.01$. (C) c-Fos IHC in MPOA from the experiment (B). *FosB*+/+ or −/− postpartum day 0.5 mothers kept exposed to their own pups, with a negative control of *FosB*+/+ postpartum mother separated from the pups for 2 days (No pup, +/+). No pup, −/− were indistinguishable from No pup, +/+ (data not shown). The anterior commissure at the top, the third ventricle to the left. Scale bar, 150 μm.

vehicle-treated virgins retrieved all three pups within a 30-min assay period, but only 7% (1 of 15) of SL327-treated virgin females did so. However, SL327 did not affect the retrieving behavior of parous females or the latency of pup sniffing of both virgins and parous females (Figs. 3D and E). Therefore, the retrieving impairments in virgin females could not be attributed to nonspecific effects of the drug such as drowsiness or sickness. These findings suggest that ERK phosphorylation is necessary for the onset of retrieving of virgin females exposed to pups for the first time, but not for the maintenance of well-established retrieving behavior of parous females. We investigated the gene expression profiles in MPOAd of SL327- or vehicle-treated virgin females by qRT-PCR (Fig. 3F). SL327 treatment significantly attenuated the pup exposure-dependent upregulation of *FosB*. *SPRY1* expression was also significantly lower in the SL327+PEx group compared with that of the DMSO+PEx group. Reduction of *c-Fos*, *NGFI-B*, and *Rad* expression by SL327 did not reach statistical significance.

What might be the upstream signals that activate the ERK signaling and subsequent transcriptional cascades in MPOAd neurons? One possibility is Ca^{2+} signaling. Neuronal activity accompanied by transcriptional activation is known to be mediated by cytosolic Ca^{2+} elevation that, in turn, can activate ERK (Mellstrom and Naranjo, 2001; West et al., 2001). The other candidates are growth factors that activate RTKs. Because Sprouty proteins are established ligand-inducible feedback regulator of RTK signaling, the RTK ligand(s) might also be increased in the MPOAd during the expression of parental behavior. We investigated our DNA microarray data of 83 probes for major RTK ligands, including *EGF*, *FGFs*, *VEGF*, *HGF*, *PDGF*, *IGFs*, *BDNF*, *NGF*, *NT3*, and *GDNF*. Forty-three probes were significantly expressed (i.e., they fulfilled criterion 3) in both experiments 1 and 2. Among these, only two probes were upregulated (FC >1.2) in parental groups of both experiments, although the differences did not reach statistical significance. Both of them were the probes for brain-derived neurotrophic factor, *BDNF*. Therefore, of the RTK ligands, BDNF is most likely associated with the signaling pathways of parental behavior. The same trend also was found by qRT-PCR (Figs. 2A and B).

Reduced induction of SPRY1 during parenting in FosB−/− mice

We further tested if the gene expression profiles listed in Table 1 were altered in pup-exposed *FosB*−/− mice, which are known to be defective in parenting (Brown et al., 1996). First, we confirmed the previously reported normal parturition and abnormal nurturing behaviors of *FosB*−/− postpartum females in our experimental condition (data not shown). In addition, we found that, in the experimental conditions used, 90% of *FosB*−/− males were neither infanticidal nor parental, but ignored pups or were only very poorly parental (Fig. 1). Such "irresolute" behavior was rare in *FosB*+/+ or +/− males of this strain.

qRT-PCR analyses for the MPOAd of *FosB*−/− virgin males and postpartum females that were pup-exposed but did not behave parentally, showed that there were significant impairment in *SPRY1* mRNA induction (Figs. 4A and B). In contrast, mRNA induction of *c-Fos* and *NGFI-B* in the MPOAd of *FosB*−/− animals was not significantly smaller than that of +/+ or +/− animals (Figs. 4A and B). c-Fos protein expression, as measured by the number of c-Fos immunopositive neurons, showed a similar result (Fig. 4C) ($t=1.23$, $df=12$). Reduction of *Rad* expression in −/− mice reached statistical significance ($P<0.05$) in virgin males but not in postpartum mothers. These results suggested that FosB plays a pivotal role in transcriptional regulation in MPOA neurons after pup exposure, together with other transcription factors.

Discussion

We examined the gene expression changes in the MPOAd during parental care. The candidate genes were selected based on the results of two separate experiments using postpartum females and virgin males. The advantage of this strategy is to narrow down the candidates and to eliminate the changes caused by specific factors other than parental behavior *per se*. For example, gene expression changes simply associated with pup exposure, and caused by suckling stimulus and subsequent hormonal changes should be included in the list of candidate transcripts of experiment 1. However, such changes were excluded from a list obtained from experiment 2 because both parental and nonparental males were exposed to pups, and males were never suckled even during parenting. All of the transcripts upregulated in both experiments 1 and 2 were previously implicated in ERK MAP kinase signaling. We found that in the MPOAd of postpartum mothers, pup exposure induced rapid phosphorylation of ERK, followed by termination of activation even though the animals were kept with pups. This time course is comparable to that of pERK in the suprachiasmatic nucleus after light exposure (Butcher et al., 2003). Such a narrow time window of ERK phosphorylation, however, made it difficult to analyze pERK increases in virgin males and females, since the individual variation in the onset of parental care was much larger than that of postpartum mothers.

The blood–brain barrier penetrating MEK1/2 inhibitor SL327 is known to block ERK phosphorylation in the hippocampus by systemic administration and to attenuate fear conditioning (Atkins et al., 1998). Similar effects of SL327 have been reported for pERK and concomitant spatial learning in a Morris water maze (Selcher et al., 1999) or psychostimulant administration (Valjent et al., 2000; Ferguson and Robinson, 2004; Shi and McGinty, 2006). Furthermore, SL327 prevents IEG induction such as c-Fos and FosB after cocaine administration in striatum (Zhang et al., 2004; Radwanska et al., 2005). On the other hand, SL327 does not alter baseline synaptic transmission in the hippocampal CA1 region or performance in the visible platform task (Atkins et al., 1998; Selcher et al., 1999). Here we found that the ERK phosphorylation and induction of *FosB* and *SPRY1* in MPOAd were significantly attenuated by SL327 administration. Moreover, SL327 inhibited the initiation of retrieving in virgin females. However, SL327 did not alter well-established maternal care in parous females, nor the latency of pup sniffing of both virgins and parous females (Figs. 3D and E). These findings suggest that SL327 blocks the onset of retrieving specifically, without causing sluggishness or inability of detecting sensory cues emanating from pups. Four of 15 virgins injected with SL327 attacked pups. Although we cannot exclude the possibility that the drug treatment increased aggressiveness nonspecifically, it seems unlikely because no SL327-injected parous females committed infanticide, and virgins did not show any obvious aggressive or irritable behaviors other than this infanticidal attack (data not shown). pERK might be necessary to overcome the initially aversive sensory stimuli from pups reported in rats (Fleming and Luebke, 1981). This issue should be addressed in future studies. Collectively, these findings suggest that the initiation of retrieval behavior for the first time requires the ERK signaling. In parous females, maternal memory

might have been consolidated and thus might not require this signaling pathway.

Finally, we tested if the gene expression profiles listed in Table 1 were altered in pup-exposed *FosB*−/− mice, which are defective in parenting. Fig. 4 shows that the induction of *SPRY1* and *Rad* were reduced to some measure. Therefore, these two genes were suggested to be downstream targets of *FosB*. On the other hand, we did not find statistically significant alteration in the induction of *c-Fos* and *NGFI-B* in *FosB*−/− mice, although their expressions tended to be slightly decreased. The peak of FosB expression after pup exposure comes later than that of c-Fos expression in the MPOA (Numan et al., 1998). Moreover, postseizure *FosB* expression is diminished in conditional *c-Fos*-knockdown mice (Zhang et al., 2002). Therefore, *FosB* induction might be partly c-Fos dependent, but the opposite was rather unlikely. The slight decrease of *c-Fos* in *FosB*−/− mice could be attributed to less sensory input in nonparental−/− animals as these animals stayed away from pups while parental +/+ or +/− animals contacted and licked pups actively.

These findings prompt a working model of the signaling pathway in MPOAd neurons responsible for the initiation of parental behavior (Fig. 5). Sensory stimuli (olfactory, auditory, and somatosensory) from pups activate MPOAd neurons possibly through Ca^{2+} and BDNF. Both BDNF protein and mRNA are shown to be abundant in MPOA (Conner et al., 1997). Then in MPOAd neurons, ERK is phosphorylated and induces transcription factors of *c-Fos*, *NGFI-B*, and *FosB*. FosB induces SPRY1 and Rad, both of which provide feedback regulation of Ca^{2+}/RTK-ERK signaling. One major role of FosB as an IEG might be to deactivate the neuron immediately after the activation. *FosB*−/− mice are more sensitive to cocaine (Hiroi et al., 1997) and develop less tolerance to repeated electroconvulsive shocks (Hiroi et al., 1998). These findings suggest that induction of SPRY1 and Rad by FosB may provide negative feedback regulation of the ERK signaling and act against excitotoxicity, as reported for c-Fos (Deng et al., 1999; Zhang et al., 2002). The proposed signaling cascade (Fig. 5) seems to be the most parsimonious interpretation of the molecular events identified in the present study, although this does not exclude additional signaling mechanisms. Indeed, Fig. 3F suggested that pERK is a major, but not a sole, mediator of transcriptional changes in MPOAd induced by pup exposure. One of such transcription factors acting together with ERK should be Ca^{2+}/cAMP-responsive element-binding protein (CREB). It has been reported that phospho-CREB in MPOA is increased nearly three-fold following pup exposure, and *Creb*-$\alpha\Delta^{-/-}$ mutant mice, which express about 10 % of normal CREB levels, exhibit impaired maternal behavior in pup retrieval assay (Jin et al., 2005). Interestingly, basal expression and induction of FosB in response to pup exposure are equivalent between wild-type and *Creb*-$\alpha\Delta^{-/-}$ females (Jin et al., 2005), suggesting that the pERK-FosB signaling and pCREB signaling are both necessary for retrieving behavior, and they are mutually independent.

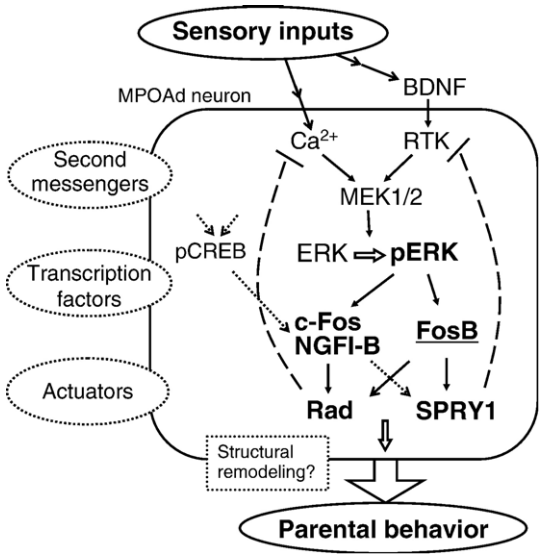

Fig. 5. Proposed intracellular signaling pathway in MPOAd neurons for the initiation of parental behavior. See Discussion for details.

The ERK signaling pathway is known to be important for activity-dependent synaptic plasticity and memory formation (Sweatt, 2004; Thomas and Huganir, 2004). Evidence is accumulating that memories are created by alterations in excitatory synaptic transmission, which are then stabilized by structural changes at postsynaptic sites on dendritic spines (Lamprecht and LeDoux, 2004). For example, induction of long-term potentiation resulted in a sequence of morphological changes including spine swelling (discernible as early as a few minutes after stimulation), formation of perforating synapses (about 30 min), and increase in multiple spine boutons (after 30 min) in hippocampus (Fifkova and Van Harreveld, 1977; Toni et al., 1999). *In vivo* associative learning also increases multiple spine boutons in hippocampus, without changing the total number of synapses (Geinisman et al., 2001). Actin cytoskeleton and its regulator Rho-family small G proteins are involved in such spine morphogenesis (Fifkova and Delay, 1982; Luo, 2002; Van Aelst and Cline, 2004). In this context, Rad is of special interest in our model because the RGK-family small G proteins is implicated in structural remodeling, such as neurite extension by regulating Rho-dependent actin reorganization (Leone et al., 2001). If Rad acts on structural plasticity downstream of BDNF, it might be able to mediate long-lasting behavioral changes such as parental memory induced by parental experience. MPOA neurons undergo morphological remodeling during parturition (Keyser-Marcus et al., 2001). Whether such structural remodeling can be induced in MPOA neurons by experience alone, in the absence of hormonal changes, remains to be clarified.

In summary, the dual roles of FosB in MPOAd neurons could be hypothesized as (1) the deactivation of the neurons by providing negative feedback to the upstream Ca^{2+}/RTK-ERK signaling, and (2) possible remodeling of neuronal microcircuit through Rad to enhance the response of MPOAd neurons to pup exposure in future. The signaling cascade proposed in Fig. 5 at least partly overlaps with the pathway involved in activity-dependent synaptic plasticity, such as associative learning, suggesting that parental memory formation may also involve synaptic plasticity in the MPOAd.

Experimental methods

Animals

All animal experiments were approved by the Institutional Animal Experiment Judging Committees of the McGill University and RIKEN. Wild-type C57BL/6J mice were purchased from Japan SLC (Hamamatsu, Japan). A *FosB*−/− mouse strain

(C;129S-*Fosb*^{tm1Meg}/J; stock number 3077) was obtained from the Jackson Laboratory (Bar Harbor, ME). For initial characterization, we used F2 generation obtained from intercross of F1 generation of the *FosB*−/− original strain and C57BL/6J. The results of Fig. 4C were confirmed using the FosB mutant mice backcrossed at least five times to C57BL/6J. The mice were housed in ventilated shoebox cages (267 mm × 483 mm × 152 mm height) and were maintained under 12:12 h light:dark cycle with lights on from 08:00 to 20:00. We found that the parental behaviors of male mice of this strain were influenced by the type of cage bedding; males showed increased parental behavior when the bedding was purified paper chips (Alpha-Dri, Shepherd Specialty Papers, Watertown, TN) compared to autoclaved wood chips (Beta Chip, NEPCO, Warrensburg, NY; unpublished observation). A similar phenomenon was reported for *Fyn*−/− mice (Hamaguchi-Hamada et al., 2004). In this study, we used the data obtained from animals assessed with Beta Chip bedding.

Pup retrieval assay

To quantify parental responsiveness, pup retrieval assays were performed essentially as described previously (Rosenblatt, 1967; Brown et al., 1996). Briefly, male or female mice were individually housed for at least 1 day prior to the experiment. 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 more than 1 min. After 30 min of observation, the pups were removed in the case of normal pup retrieval assays. The pups were left in the subject's cage for the studies in which c-Fos induction was assessed by means of immunohistochemistry (IHC) to maximally induce c-Fos in MPOA.

For pup retrieval assays using MEK inhibitor SL327 (Calbiochem, San Diego, CA) (Figs. 3B–E), C58BL/6 females were injected intraperitoneally with either 50% DMSO or 40 mg ml^{-1} SL327 in 50% DMSO (2.5 ml kg^{-1} body weight) 45 min before pup exposure. The pups were removed after 60 min of exposure (105 min after the injection). This procedure was set according to the previous report showing that SL327 transiently inhibits ERK phosphorylation levels in hippocampus for both the p42 and the p44 isoforms from 30 to 100 min after intraperitoneal injection (Selcher et al., 1999). Our preliminary study revealed that injection of less than 4 ml kg^{-1} of 50% DMSO did not affect the latency of sniffing the donor pup or of retrieving by adult female C57BL/6J mice (data not shown). For Fig. 3F, to maximize the pup exposure duration under the SL327 effect, each time period had to be further trimmed as follows; ten virgin female mice were injected with 50% DMSO (vehicle) and five virgin females were injected with 100 mg kg^{-1} SL327. Five females injected with DMSO were sacrificed 120 min after injection without pup exposure (DMSO). The rest of females were injected with DMSO or SL327, and 15 min after injection, they were exposed to donor pups for 90 min. They were separated from the pups 105 min after the injection and sacrificed 120 min after the injection (DMSO + or SL327 + PEx). Since SL327 is a transient, reversible MEK inhibitor, the duration of pup exposure and the time of sacrifice after the onset of pup exposure had to be shortened to 90 min and 105 min, respectively, while in other experiments they were 120 min. Although the upregulation of immediate-early genes such as *c-Fos*, *FosB*, *NGFI-B*, and *Rad* reached to statistical significance as in the other experiments, the upregulation of *SPRY1* did not in this protocol. DMSO injection might also have affected the result. Numbers of animals used in each experiment were described in the legend of Fig. 3.

DNA microarray analyses

Two sets of experiments were conducted to compare parental and nonparental groups of mice in both females and males: Experiment 1 (females): C57BL/6J pregnant females (mated at 10 weeks of age, $n = 12$) were purchased and housed singly at late gestation. All such females gave birth and nursed the pups normally. These females were then separated from the pups on postpartum day (PPD) 5. Six females were re-exposed to three donor pups on PPD7 and sacrificed at either 2 h or 6 h ($n = 3$ each) after pup exposure. These two time points were selected to correspond to the peak of c-Fos and FosB immunopositive cells in the MPOA (Calamandrei and Keverne, 1994; Numan and Numan, 1994; Li et al., 1999). All of the pup-exposed females retrieved and nursed the pups within 3 min (data not shown). The remaining six females were sacrificed without pup re-exposure (0 h).

Experiment 2 (males): Adult virgin males (10–16 weeks of age) were housed singly and exposed to three unfamiliar pups for 30 min during which time pup-directed behaviors were scored. Both infanticide and parenting are observed in adult male mice depending on their mating experience, social status, and developmental environment such as intrauterine positioning (vom Saal and Howard, 1982). If a pup was attacked during the test, all the pups were immediately removed. Wounded pups were euthanized, and the others were returned to their home cage. The following day males were again subjected to the pup retrieval assay in the same way as the first day, except that this time the pups were left in the cage until the subject males were sacrificed after 2 h of pup-exposure. Behaviors observed during the first 30 min of the second day were used for behavioral classification. Their second day behaviors were the same as that of the first day, except that one nonparental and one infanticidal male were parental on the second day. All of these males used in experiment 2 were heterozygote (*FosB*+/−) controls from our *FosB*−/− colony. We used these mice because we originally included the DNA microarray analysis of *FosB*−/− mice in this study. However, these data included issues tangential to the central thesis of this study and were omitted from the present study. *FosB*+/− mice were indistinguishable from +/+ littermates with regard to their parental behavior (data not shown).

In both experiments 1 and 2, mice were sacrificed by cervical dislocation at the end of the pup exposure. The brains were immediately removed, soaked in RNAlater (Ambion, Austin, TX) at 4 °C overnight and stored at −20 °C. In experiment 1, 300-μm coronal sections containing the posterior half of the anterior commissure were micropunched (Palkovits micropunch, 0.8 mm; Fine Science Tools, Foster City, CA) to include the entire MPOA. In experiment 2, the dorsal parts of the MPOA from 200-μm sections were taken using a 0.35-mm micropunch. This approach was used because the c-Fos positive neurons were less numerous in virgin parental males than in postpartum females and were concentrated to the MPOAd (cf. Fig. 2D). Indeed, the MPOAd was shown to be more relevant to parental care than the ventral part of the MPOA in previous lesion studies (Jacobson et al., 1980;

Numan et al., 1990). The bilateral tissues from each animal were collected and subjected to total RNA isolation (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 an RNA 6000 Pico LabChip kit (Agilent, Santa Clara, CA). Either 50 ng (experiment 1) or 10 ng (experiment 2) of total RNA were then processed for biotin-labeled cRNA using Two Cycle cDNA Synthesis and IVT labeling kit (Affymetrix, Santa Clara, CA). The resultant cRNA samples were verified using Test2chip (Affymetrix). Samples were excluded if their 3′/5′ ratio of β-*actin* and *GAPDH* genes were larger than 5 and 3, respectively. Each biotin-labeled cRNA sample from one animal was hybridized to a single Affymetrix GeneChip Mouse Genome 430A 2.0 Array (Affymetrix) that contained over 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) and processed using GeneSuite software (GeneMachines, San Carlos, CA), with analysis performed using GeneSpring software (Silicon Genetics, Redwood, CA).

The normalized values of gene expression were subjected to Student's *t* test, and transcripts satisfying the following three criteria were regarded as significantly changed; (1) expression was significantly different between the parental and nonparental groups by a Student's *t* test ($P<0.05$); (2) the fold change (FC = average expression in the parental group divided by the average expression in the nonparental group) was higher than 1.2 or lower than 0.83; and (3) 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, followed by statistical analysis to confirm whether, based on 11 independent measurements, the difference between the signals of the matched and mismatched probes are statistically significant, using a *t*-test-like measure. The System uses these measures to label the genes P for present, M for marginal, or A for absent.) Not all animals observed behaviorally were utilized for the final DNA microarray analyses. The numbers of samples actually used for the statistical analysis are $n=3$ per group in experiment 1 and $n=3$ or 4 for the parental or infanticidal group in experiment 2, respectively.

qRT-PCR detection of mRNA

For Fig. 2, the total RNA samples isolated for DNA microarray analyses were used for qRT-PCR analyses. For Fig. 4, the total RNA preparation was performed following the same protocol as described for the experiment 2 of DNA microarray analysis. For Fig. 3F, which were performed about 2 years after Figs. 2 and 4, the protocol was the same except that the brains were not soaked in RNAlater but were directly frozen using liquid nitrogen and Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan), and that the MPOAd from 150-μm sections were taken using a 0.35-mm micropunch. These slight modifications were applied to further improve the accuracy of micropunch. In all cases, the total RNA isolated from micropunches was linearly amplified through cDNA synthesis and *in vitro* transcription as described for the DNA microarray. The second round first-strand cDNA was synthesized with SuperScript II reverse transcriptase using oligo (dT) primers (Invitrogen Japan, Tokyo). qRT-PCR was performed with an ABI Prism 7000 Sequence Detector Systems and TaqMan 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 $2^{[Ct(\text{each gene})-Ct(GAPDH)]}$ in triplicate. The average Ct(*GAPDH*) did not differ between groups (data not shown). Data were also normalized by β-actin expression to show similar results (data not shown). The TaqMan probe IDs used in this study were Mm99999915_g1 (*GAPDH*), Mm00439358_m1 (*NGFI-B*), Mm00451053_m1 (*Rad*), Mm00500401_01 (*FosB*), and Mm00432069_m1 (*BDNF*). We used custom-made primers for *SPRY1* and for *c-Fos*. Primer design for *SPRY1* and *c-Fos* was accomplished with PrimerExpress software (Applied Biosystems): c-Fos primer f, GGAATGGTGAAGACCGTGTCA; r, CCGCTTGGAGTGTATCTGTCA; Spry1 primer f, ACCCAGCACAAGTTCATCTGC; r, TCCCGTATTCCACCATGCTCT.

Immunohistochemistry

Adult mice were anesthetized and perfused with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB) at pH 7.4. Sodium orthovanadate (1 mM) was added to the fixative when the brain was used for pERK detection. The brains were removed, immersed in the same fixative at 4 °C overnight, and then in PBS containing 30% (w/v) sucrose and cryosectioned coronally at 40 μm. Every third section from the serial sections were washed with PBS, bleached with 0.3% H_2O_2 in methanol, incubated overnight with rabbit primary antibodies against c-Fos (Ab-5, 1:10000, EMD Bioscience, San Diego, CA); FosB (Sc-48, 1:10000 or Sc-7203, 1:1500, Santa Cruz); or Active MAPK (recognizing dual phosphorylated pERK, 1:2000, Promega, Madison, WI). Sections were then washed and incubated with biotin-conjugated secondary Ab, and the signal was intensified and visualized using a Vectastain Elite ABC kit and a DAB substrate kit (Vector, Burlingame, CA). One section from each animal, containing the posterior end of the midline anterior commissure, was used for quantification of c-Fos or pERK positive cells. Following IHC, these sections were mounted on glass slides, lightly counterstained with hematoxylin, 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, c-Fos or pERK positive cell nuclei within the area of the MPOAd (marked on a transparent sheet) and within the same-sized area in lateral preoptic area (for background staining) in the same photograph were counted manually. The numbers of immunopositive cells in the MPOAd minus that in the lateral preoptic area were used as the net numbers of cells. The experimenter was blind to the experimental groups.

Statistical analyses

The data were analyzed either by Student's *t* tests or by one-way analysis of variance followed by Tukey post hoc comparisons using SPSS 10.0 for Windows (SPSS Japan, Tokyo). Significance levels were set at $P<0.05$ (two-tailed).

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