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A Defect in Nurturing in Mice Lacking the Immediate Early Gene *fosB*

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Summary

Although expression of the Fos family of transcription factors is induced by environmental stimuli that trigger adaptive neuronal responses, evidence that Fos family members mediate these responses is lacking. To address this issue, mice were generated with an inactivating mutation in the *fosB* gene. *fosB* mutant mice are profoundly deficient in their ability to nurture young animals but are normal with respect to other cognitive and sensory functions. The nurturing defect is likely due to the absence of FosB in the preoptic area, a region of the hypothalamus that is critical for nurturing. These observations suggest that a transcription factor controls a complex behavior by regulating a specific neuronal circuit and indicate that nurturing in mammals has a genetic component.

Introduction

The central nervous system of higher organisms mediates complex behaviors and adapts rapidly to changing environmental conditions. Central to human life is the capacity to learn and store information. For animals, survival depends on learning and remembering where food can be found and where dangerous predators may await. The birth of young mammals requires the appropriate parental nurturing responses to ensure the survival of the species. Still other adaptations occur without conscious awareness, as when the changing length of the day causes the hypothalamic control of circadian rhythms to shift. In all these situations the nervous system must identify the relevant stimuli, and the organism must then adapt appropriately.

Efforts to characterize the molecular basis of certain adaptive neuronal responses have suggested that the induction of new gene expression may be critical to the response (Goelet et al., 1986). Environmental stimuli such as light or visual images trigger programs of gene expression that begin within minutes of stimulation and continue for many hours in relevant brain regions. The first genes to be activated are immediate early genes (IEGs). The transcription of IEGs is induced rapidly without a requirement for new protein synthesis (for reviews, see Herschman, 1991; Segal and Greenberg, 1996). Over the course of several hours the induction of IEGs is followed by the induction of delayed response genes, whose induction requires new protein synthesis. Many immediate early genes encode transcription factors that are thought to induce the appropriate late response genes to act as effectors of the long-term cellular response.

As molecules that initiate cellular responses to environmental stimuli, IEGs have been the focus of considerable attention, with a particular emphasis on the *fos* family. The *fos* family consists of four genes, c-*fos*, *fosB*, *fra*-1, and *fra*-2 (Herschman, 1991). The *fosB* gene gives rise to both a full-length protein and a truncated form called ΔFosB, derived from an alternatively spliced mRNA (Mumberg et al., 1991; Nakabeppu and Nathans, 1991; Yen et al., 1991). Each of these five Fos-related proteins is a transcription factor that can dimerize with Jun family proteins through a leucine zipper domain and bind to DNA through a conserved basic region. The DNA element recognized by all of these heterodimers is an AP-1 site, with core sequence TGACTCA (Franza et al., 1988; Rauscher III et al., 1988). Although sequences surrounding the core AP-1 site influence the affinity of specific Fos/Jun family heterodimers for particular sites (Hai and Curran, 1991), the function of individual Fos and Jun family members has proven difficult to elucidate because these different transcription factors share many properties.

fos family genes are activated during a wide variety of adaptive neuronal responses. Fos or Fos-like proteins are expressed in the brain regions required for nurturing behavior (Calamandrei and Keverne, 1994; Fleming and Walsh, 1994; Fleming et al., 1994; Luckman, 1995; Numan and Numan, 1995). *fos* family genes are also induced in the hippocampus by long-term potentiation (Demmer et al., 1993), a process that results in a sustained increase in postsynaptic potential following stimulation of a synapse and which may be part of the cellular basis of learning and memory. In addition, light stimulation that results in the shifting of circadian rhythms induces *fos* family genes in the suprachiasmatic nucleus of the hypothalamus (Kornhauser et al., 1992). Many of these effects may involve synaptic release of the neurotransmitter glutamate, which then induces *fos* family genes in the postsynaptic neuron (Bading et al., 1993; Lerea and McNamara, 1993). Despite this extensive body of work correlating the induction of *fos* family mRNAs with adaptive neuronal responses, proof is still lacking that *fos* family genes are critical for nervous system development or function.

The advent of gene targeting in mice has made it possible to demonstrate a functional requirement for a particular immediate early gene in the nervous system and to identify the molecules required for specific behavioral responses. Although animals have been generated that carry mutations in particular IEGs, including c-*fos*, presently little is known about the importance of these genes in adaptive neuronal responses. c-*fos* mutant mice are viable but suffer from osteopetrosis and are of reduced body size (Johnson et al., 1992; Wang et al., 1992). Although kindling development and

kindling-induced sprouting are reduced in c-*fos* mutant mice (Watanabe et al., 1996), these animals do not show any gross behavioral abnormalities, and their skeletal problem and small size have precluded a definitive behavioral analysis. Thus the importance of *fos* family genes in the development and normal function of the nervous system remains to be demonstrated.

Here we report the generation of mice in which the *fosB* gene has been disrupted. These animals develop normally, yet display a profound defect in reproduction. The reproductive failure of *fosB* mutant mice is due to a specific behavioral defect that results in an inability to nurture young. This nurturing defect is seen not only in postpartum females but also in young females and males. Together, these findings provide evidence that nurturing behavior in mammals is genetically controlled and that an immediate early gene, *fosB*, is critical for an adaptive neuronal response.

Results

Generation of *fosB* Mutant Mice

A 7.4 kilobase *fosB* genomic clone obtained from a mouse 129Sv library was used to construct a targeting vector for homologous recombination by positive–negative selection (Mansour et al., 1988), as shown in Figure 1A. A *PGK-neo* cassette was inserted in inverse orientation into the second exon of the *fosB* gene, 5′ to the basic region and leucine zipper and adjacent to a 30 amino acid segment essential for transactivation (Zerial et al., 1989; Wisdom and Verma, 1993). J1 embryonic stem cells were electroporated with the *fosB–neo* construct (Robertson, 1987) and single clones resistant to G418 and FIAU were screened by Southern blot hybridization using a *fosB* probe from the promoter region 5′ to the homology contained in the targeting vector. Restriction enzyme digestion of the wild-type *fosB* locus with BglII and PstI generates a 4.1 kb fragment, while the correctly targeted locus generates a 3.5 kb fragment (Figure 1B). Seven correctly targeted clones were identified among 244 resistant colonies, yielding a targeting frequency of approximately 1 in 35.

Chimeras derived from two heterozygous clones of normal karyotype transmitted the embryonic stem cell DNA through the germline to generate heterozygous offspring. Germline transmission was demonstrated by Southern blot analysis of tail DNA, illustrated in Figure 1C for a representative litter born to two heterozygous parents. The results described below were the same for animals derived from each clone. The *fosB* mutation was bred into both the 129Sv inbred and 129Sv × BALB/c mixed background, with similar results in each background.

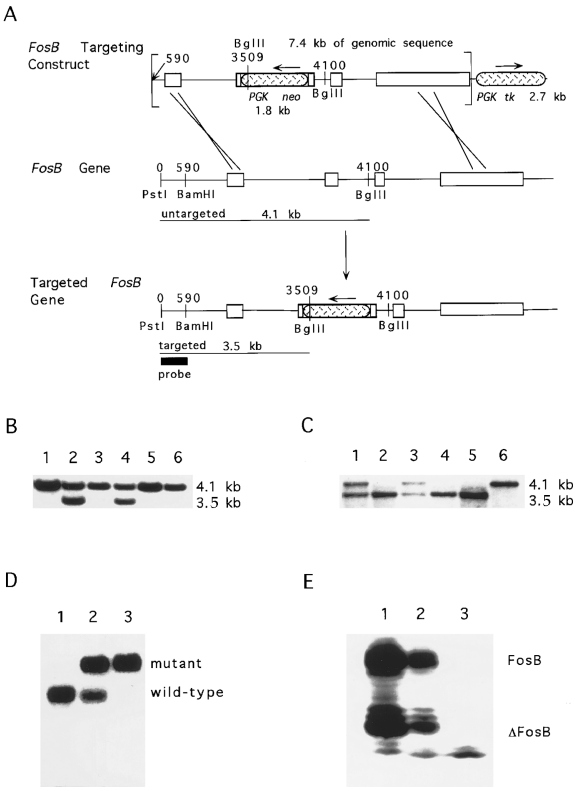

Figure 1. Targeting of the *fosB* Locus by Homologous Recombination

(A) Targeting vector for positive–negative selection of homologous recombinants at the *fosB* locus, with partial restriction map and the structure of the targeted *fosB* locus. The 4 exons of *fosB* are represented by open rectangles and *PGK-neo* and *PGK-tk* are represented by hatched ovals. A BglII/PstI double digest will generate a 4.1 kb fragment from the untargeted allele and a 3.5 kb fragment from the targeted allele, both of which are recognized by the probe shown just 5′ to the sequence contained within the targeting vector.

(B) Southern blot of genomic DNA isolated from individual embryonic stem cell clones. Purified DNA was digested with BglII and PstI and probed as described (Ausubel et al., 1995). Lanes 2 and 4 show digests of correctly targeted clones, while lanes 1, 3, 5, and 6 show digests of wild-type clones.

(C) Southern blot of genomic DNA isolated from each pup in a litter born to two $fosB^{+/-}$ parents. Purified DNA was digested with BglII and PstI. Results from homozygous mutants are shown in lanes 2, 4, and 5; heterozygous mutants in lanes 1 and 3; and one wild-type animal in lane 6.

(D) Northern blot of total cellular RNA isolated from wild-type (lane 1), heterozygous (lane 2), and homozygous *fosB* mutant (lane 3) primary embryonic fibroblasts following serum starvation and stimulation with 20% fetal bovine serum for 45 min. The wild-type *fosB* mRNA is 4.5 kb and the mutant *fosB* mRNA is 6.5 kb.

(E) Western blot using anti-FosB antibody (Santa Cruz sc-48 at 1:1000 dilution). Primary embryonic fibroblasts were serum-starved for 72 hr, then stimulated for 2 hr with 20% fetal bovine serum prior to extraction in boiling SDS. Fibroblasts were wild-type, lane 1; heterozygous *fosB* mutant, lane 2; homozygous *fosB* mutant, lane 3.

Analysis of the *fosB* Mutation

To facilitate the characterization of the *fosB* mutation, primary embryonic fibroblasts from wild-type, heterozygous, and homozygous mutant embryos were derived and cultured at embryonic day 15. When these cells were serum-starved and then stimulated with 20% fetal bovine serum, the expected 4.5 kb *fosB* mRNA was synthesized from the wild-type *fosB* allele but was completely undetectable in the *fosB* mutant fibroblasts (Figure 1D). However, in serum-stimulated cells the *fosB* mutant allele did generate a larger mRNA of 6.5 kb whose size suggests that its transcription initiates within the *fosB* promoter, reads through the *PGK-neo* insertion,

and continues to the end of the *fosB* gene. The nature of the mutant *fosB* transcript was confirmed by hybridization to probes specific to exons 1 and 4 of the *fosB* gene and to *neo* (data not shown). Reverse transcription–polymerase chain reaction (RT–PCR) analysis confirmed the structure of this large mutant mRNA and also identified a small amount of another related but aberrantly spliced transcript. In this transcript the 3′ end of the first exon of *fosB* is joined to the 5′ end of the inverted *PGK-neo* insertion. This splicing event deletes a portion of the 2nd exon of *fosB* but leaves *neo* intact. If a protein were translated from either of these mutant mRNAs, it would be terminated prematurely by nonsense codons in *PGK-neo* and would therefore be nonfunctional.

Western blot analysis of extracts from serum-stimulated wild-type fibroblasts using several different FosB-specific antibodies revealed multiple variants of two different proteins, a larger 45 kDa band and a smaller 35 kDa band (Figure 1E). The larger bands represent full-length FosB while the shorter species are ΔFosB (Mumberg et al., 1991; Nakabeppu and Nathans, 1991; Yen et al., 1991). In the heterozygous fibroblasts a clear reduction in both full-length and ΔFosB proteins is evident, while in the homozygous mutant fibroblasts neither FosB protein is detectable. Some of the anti-FosB antibodies also detect very low levels of a 30 kDa protein (Figure 1E, lower bands) that is variably induced in the heterozygous and homozygous *fosB* mutants. Whether this 30 kDa protein is derived from the *fosB* locus or is a related protein is unclear.

Phenotypic Characterization of *fosB* Mutant Mice

fosB mutant homozygotes were born at the normal Mendelian frequency from heterozygote crosses in both the 129Sv and the 129Sv × BALB/c backgrounds. The mice are healthy and viable but are ~10% smaller than wild-type mice (data not shown). A routine histologic survey of organs has revealed no abnormalities, indicating that *fosB*-derived proteins are not required for the development of a normal adult mouse.

However, upon mating homozygous *fosB* mutant females to homozygous *fosB* mutant males, a dramatic abnormality was observed. Although the rate of pregnancy was normal and the pregnancies were carried to term, the majority of pups died within 1–2 days of birth. This difference in reproductive performance was quantitated as shown in Figures 2A and 2B.

Pup lethality in the early postnatal period could be due to a defect in the *fosB* mutant mothers, the *fosB* mutant pups, or both. To ascertain the nature of the defect in the *fosB* mutants, crosses were established between female *fosB* mutants and male wild types or heterozygotes and between male *fosB* mutants and female wild types or heterozygotes. As shown in Figures 2A and 2B, the number of surviving pups per pregnancy correlates strongly with the genotype of the mother, and not at all with the genotype of the father or the pups. Although the data appear to suggest that pup survival is reduced for female heterozygotes crossed to male mutants, this is believed to be an artifact of small numbers and small litters. Of particular note are eight crosses between *fosB* mutant mothers and wild-type fathers, in which all pups are heterozygous but no pups survived. Conversely, heterozygous females have given birth to and successfully raised four litters composed entirely of homozygous *fosB* mutant pups (data not shown). In addition, five litters of *fosB* mutant pups born to *fosB* mutant mothers have been successfully fostered to wild-type or heterozygous females (data not shown). These data establish that the pup survival defect lies entirely in the *fosB* mutant mothers and is not affected by the genotype of the pups.

Lactation and Parturition in *fosB* Mutant Females

An obvious mechanism by which a maternal mutation could lead to postnatal pup death is via a lactation defect in the mothers. However, histological analysis of the mammary glands of *fosB* mutant females has revealed no abnormality in their development or function. The mammary glands of wild-type and mutant females were indistinguishable 24 hr prepartum (Figure 3A and 3B). Initial observations of the mammary gland 24 hr postpartum suggested a possible defect in the mutant (Figure 3C and 3D), which failed to undergo the transition from a presecretory to a secretory state at parturition. This transition is characterized by secretion of milk into the lumens of the gland with consequent flattening of the epithelial cells. Since this transition normally occurs postpartum when pups are nursed, we were concerned that the apparent failure of mammary development in the *fosB* mutant might be secondary to the failure of the *fosB* mutant mothers to nurse their young. To control for this possibility, the pups of wild-type mothers were removed shortly after birth to prevent nursing and the mammary glands of the mother examined 24 hr later (Figure 3E). Under these circumstances, the wild-type mammary gland was indistinguishable from the mutant mammary gland. This result demonstrates that the difference initially observed between the wild-type and mutant mammary glands was due to the failure of the *fosB* mutant mothers to nurse. This possibility is corroborated by the finding that the mammary glands of mutant females who do nurse some pups resemble those of wild-type nursing mothers (Figure 3F). Together, these findings indicate that the defect in reproductive performance of *fosB* mutant mothers is not due to a developmental defect of the mammary gland that prevents lactation.

Having established that the mammary glands of *fosB* mutant females develop normally, we next examined the anatomy of the reproductive tract before and after birth and found it to be normal. The hormonal status of *fosB* mutant and wild-type females both before and after parturition was also compared, since changing levels of estrogen, progesterone, prolactin, and oxytocin receptor at parturition have all been implicated in nurturing (Rosenblatt et al., 1988; Bridges, 1994). The *fosB* mutants were indistinguishable from wild types in their serum estradiol and progesterone levels, oxytocin mRNA levels in hypothalamus, growth hormone and prolactin mRNA levels in pituitary, and immunostaining for oxytocin and prolactin in the pituitary (data not shown). The

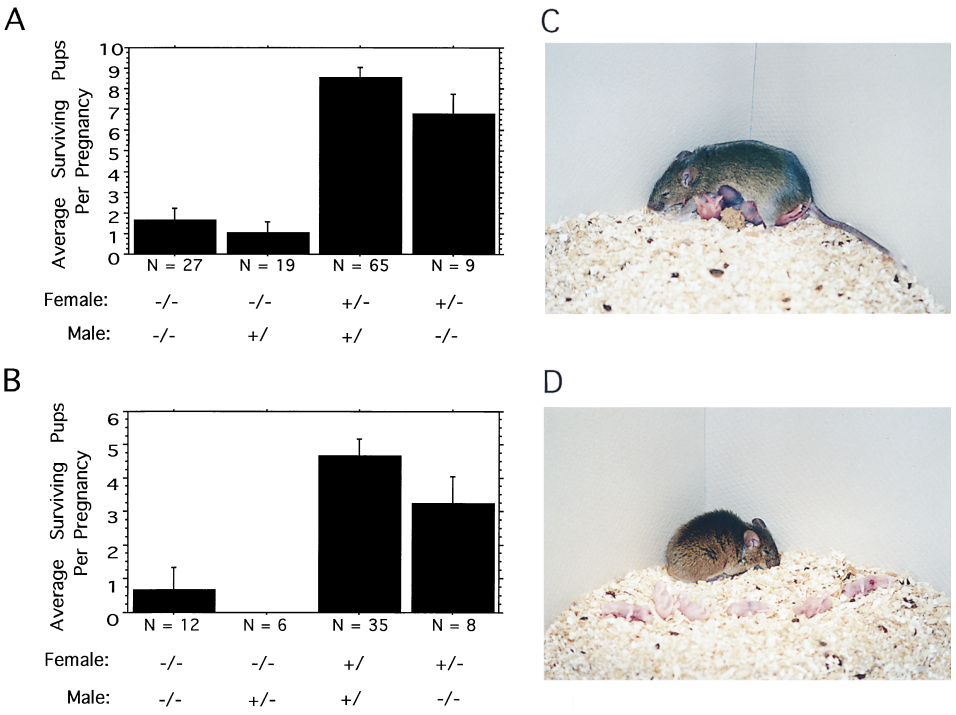

Figure 2. Reproductive Performance Is Impaired in *fosB*$^{-/-}$ Mothers

(A and B) Average number of pups surviving to weaning per pregnancy, in the indicated number of crosses (N) of the indicated parental genotypes. The percentage of pregnancies with surviving pups varies with the background. In the 129Sv × BALB/c mix, 12 of 46 pregnancies had some surviving pups (26%). However, in the 129Sv inbred background, only 1 of 19 pregnancies (5%) had any survivors. +/ means +/+ or +/−.

(A) 129Sv × BALB/c background. All $p < 0.0001$ between different female genotypes (paired t test).

(B) 129Sv inbred background. Comparing −/− × −/− and +/ × +/, $p = 0.0001$ (Welch ANOVA); −/− × −/− and $F^{+/-} \times M^{-/-}$, $p = 0.02$ (Welch ANOVA); $F^{-/-} \times M^{+/-}$ and +/ × +/, $p = 0.0008$ (Kruskal-Wallis/Wilcoxon rank sums); $F^{-/-} \times M^{+/-}$ and $F^{+/-} \times M^{-/-}$, $p = 0.01$ (Kruskal-Wallis/Wilcoxon rank sums).

(C) Typical nursing posture of wild-type female shortly after giving birth. The picture was taken ~6 hr after transferring the mother and her litter to the new environment.

(D) Typical behavior of *fosB* mutant female shortly after giving birth. In her home cage her pups were scattered and neglected, hence their sickly appearance. The picture was also taken ~6 hr after transferring the mother and her litter to the new environment.

gross anatomy of the pituitary and hypothalamus, as well as other brain regions, was indistinguishable between wild-type and *fosB* mutant mothers. Thus, there are no obvious anatomic or physiologic abnormalities at parturition that could help explain the postnatal lethality of pups born to *fosB* mutant mothers.

Defining a Nurturing Defect

Having ruled out obvious anatomic or physiologic causes as an explanation of postnatal pup lethality in the *fosB* mutant mothers, we considered the possibility of a behavioral abnormality, namely a defect in the proper care of newborn pups. Normal nurturing behavior in rodents is quite stereotyped and includes creating a nest, cleaning the pups, retrieving them to the nest, and crouching over them for warmth and nursing (Barnett and Burn, 1970). That the *fosB* mutant mothers have a defect in this behavior was obvious from the outset, as the pups of the *fosB* mutant females are typically scattered around the cage while the mother sits off to the side and ignores them (Figure 2D). In contrast, wild-type or heterozygous females usually have all their pups gathered in the nest and are crouching over them to keep them warm and to nurse (Figure 2C).

To characterize the nurturing defect of the *fosB* mutant mothers, the animals were subjected to behavioral assays of nurturing (adapted from Calamandrei and Keverne, 1994; Fleming et al., 1994). These experiments revealed a dramatic defect in mutant nurturing behavior. Initially, the behavior of newly postpartum females was observed for 20 min before their cage was disturbed. Although wild-type females spent the majority of this period crouching over their pups, most mutants did not crouch at all and their pups were usually found scattered around the cage ($p = 0.01$; Figure 4A).

Following this initial observation, each mother's pups were removed for a 1 hr period. The mother was then challenged with three of her own pups, with one in each corner of the cage away from the mother's nest. The typical wild-type mother retrieved the first pup to the nest within 20 s and all three pups within 4 min. In contrast, the typical *fosB* mutant mother took more than 50 times as long to retrieve the first pup ($p < 0.0001$) and only one mutant retrieved all three of her pups in

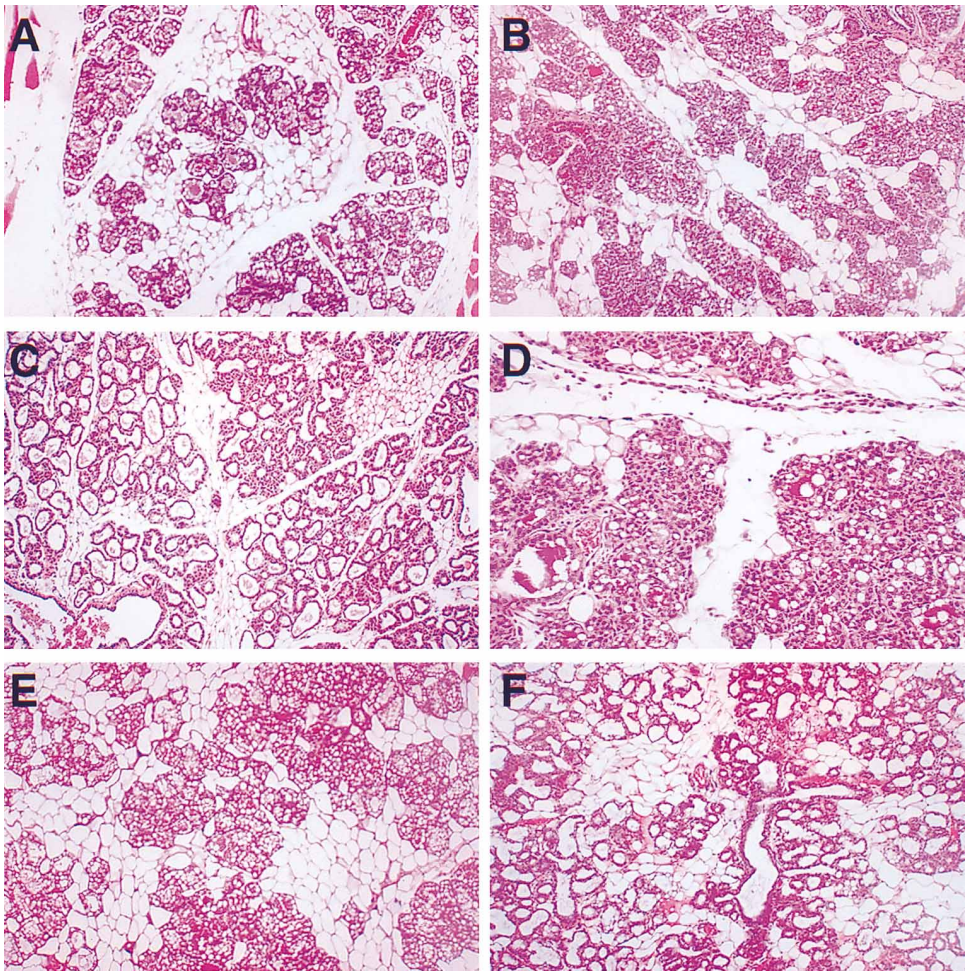

Figure 3. Mammary Gland Development Is Normal in *fosB*$^{-/-}$ Females

H&E-stained sections of inguinal mammary glands.
(A) Wild-type female, 24 hr prepartum.
(B) *fosB* mutant female, 24 hr prepartum.
(C) Wild-type female, 24 hr postpartum. She was nursing her litter.
(D) *fosB* mutant female, 24 hr postpartum. She was ignoring her litter.
(E) Wild-type female, 24 hr postpartum. Her litter was removed shortly after birth, preventing nursing.
(F) *fosB* mutant female, 24 hr postpartum. She was caring for and nursing part of her litter.
Magnification approximately 40×.

the observation period ($p < 0.0001$; Figure 4B). On average only half the *fosB* mutants retrieved even one pup, whereas all of the wild-types retrieved all of their pups ($p = 0.0004$; Figure 4B). This failure of retrieval was not a failure of the mother to observe the pups, since all the females approached their offspring and sniffed them. *fosB* mutant mothers did not crouch over their pups at all, whereas wild-type mothers spent about half the observation period crouching over their pups ($p = 0.0002$; Figure 4B). The nurturing behavior of *fosB* mutant mothers was not improved after multiple pregnancies or after sharing a cage with a wild-type mother who gave birth and raised her pups for one week in the presence of the mutant. The deficiencies therefore cannot be overcome by learning and experience. Thus, these defects in nurturing are significant in magnitude and easily account for the reduced survival of offspring of *fosB* mutant mothers.

Additional Analysis of Nurturing Behavior

Although no gross hormonal abnormalities were found in the *fosB* mutants at parturition, a subtle regulatory abnormality specific to parturition could have been missed and could potentially explain the nurturing defect of *fosB* mutants. We therefore examined whether a defect in nurturing could be demonstrated outside the context of pregnancy and parturition. Young female mice that have never been pregnant, as well as young males, can be induced to engage in nurturing behaviors by repeated exposure to newborn pups (Noirot, 1969a, 1969b). Therefore we undertook an analysis in which each young female was exposed to newborn pups for

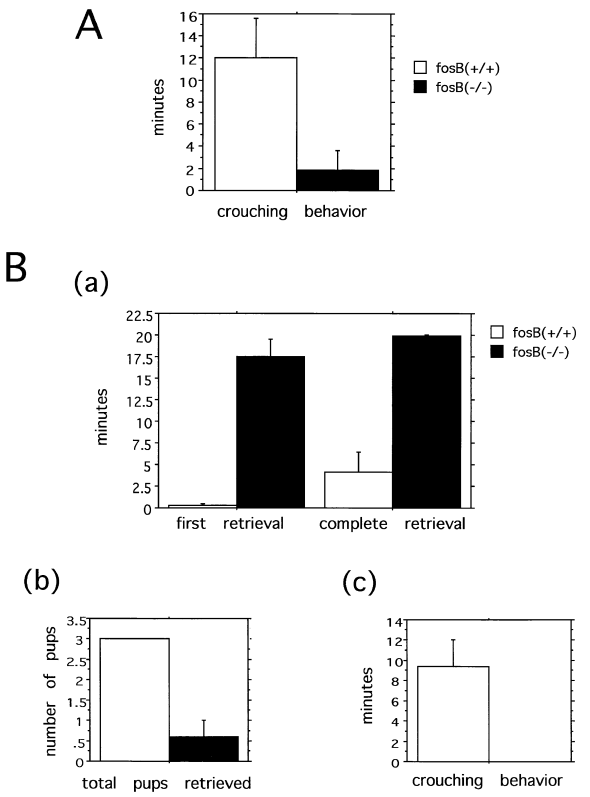

Figure 4. Nurturing Behavior Is Impaired in *fosB*$^{-/-}$ Mothers

(A) Initial observation of newly postpartum females. Each animal was observed for 20 min with minimal disruption of her cage, and the amount of time spent crouching in a nursing posture was recorded. N = 8 wild-types and N = 11 mutants. p = 0.01 by t test. (B) Nurturing analysis of postpartum females that were deprived of their pups for 1 hr and then rechallenged with three pups placed individually in the corners of their cage. N = 6 wild-types and N = 10 mutants. (a) Latency to retrieve the first and third pup. Failure to retrieve was assigned as 20 min, the length of the observation period. Both p < 0.0001, by t test. (b) Total number of pups retrieved. All wild-types retrieved all three of their pups. p = 0.0004, by t test. (c) Time crouching over all three pups in the nest. p = 0.0002, by Kruskal-Wallis/Wilcoxon rank sums.

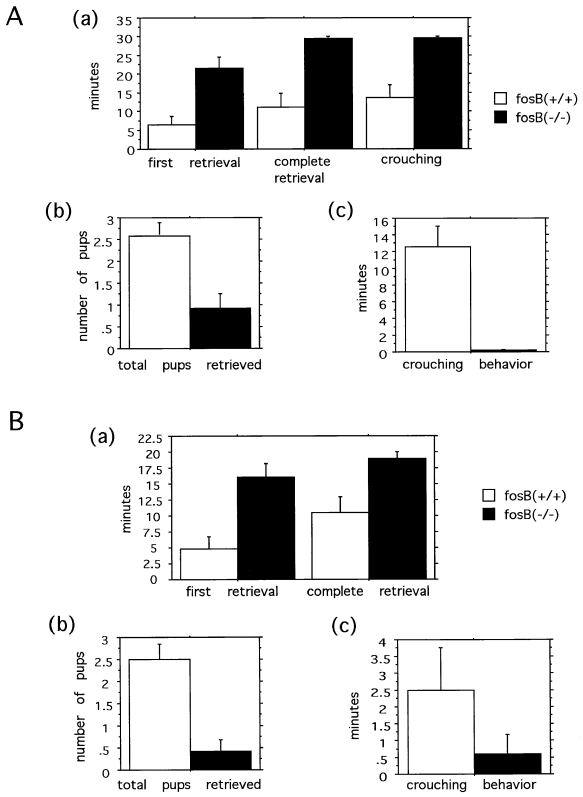

Figure 5. Nurturing Behavior Is Impaired in Young *fosB*$^{-/-}$ Females and Males

(A) Young female nulliparous mice were exposed to three young pups for 30 min on two consecutive days. Their behavior was scored on day two. N = 14 for both mutants and wild-types. (a) Latency to first retrieval, to third retrieval, and to initiation of crouching behavior. Failure to retrieve or crouch was assigned as 30 min, the length of the observation period. Latency to first retrieval, p = 0.0004 by t test (equal variances); latency to third retrieval, p = 0.0006 by Welch ANOVA (unequal variances); latency to crouching, p = 0.0008 by Welch ANOVA (unequal variances). (b) Total number of pups retrieved. p = 0.001 by t test (equal variances). (c) Time crouching over all three pups in the nest. p = 0.0003 by Welch ANOVA (unequal variances).

(B) Young male mice were exposed to three young pups for 20 min on four consecutive days. Their behavior was scored on day four. N = 10 wild-types and N = 14 mutants. (a) Latency to first retrieval and latency to third retrieval. Latency to first retrieval, p = 0.001 by t test (equal variances); latency to third retrieval, p = 0.009 by Welch ANOVA (unequal variances). (b) Total number of pups retrieved. p < 0.0001 by t test (equal variances). (c) Time crouching over all three pups in the nest. p = 0.15 by t test (equal variances).

a defined period on 2–4 consecutive days before scoring nurturing behavior on the final day as described above. This analysis revealed that the wild-type females achieved high levels of nurturing behavior whereas the *fosB* mutants showed little or none (Figure 5A). Most wild-type animals retrieved all three of the pups and crouched over them for more than a third of the observation period. In contrast, the majority of *fosB* mutant females failed to retrieve any pups in the allotted time, and no *fosB* mutant spent appreciable time crouching over the pups (Figure 5A). This result clearly establishes that *fosB* gene function is required for nurturing responses to pups outside the context of pregnancy.

The *fosB*-dependent nurturing response was not specific to female mice. Young wild-type male mice tested as described above for the young females were also induced to display a high level of nurturing behavior (Figure 5B). The *fosB* mutant males displayed a statistically significant impairment in retrieval responses. Crouching behavior was less common and more variable in the wild-type males than in the females and a statistically significant difference in crouching behavior between wild-type and *fosB* mutant males was not observed. Nevertheless, a defect in nurturing behavior is present in males as well as females and can be isolated from the hormonal patterns of parturition. These results suggest that the nurturing defect reflects a function of *fosB* in the brain circuitry involved in the response to pups and provide evidence for a genetic basis for nurturing behavior that is present in both females and males and involves the immediate early gene *fosB*.

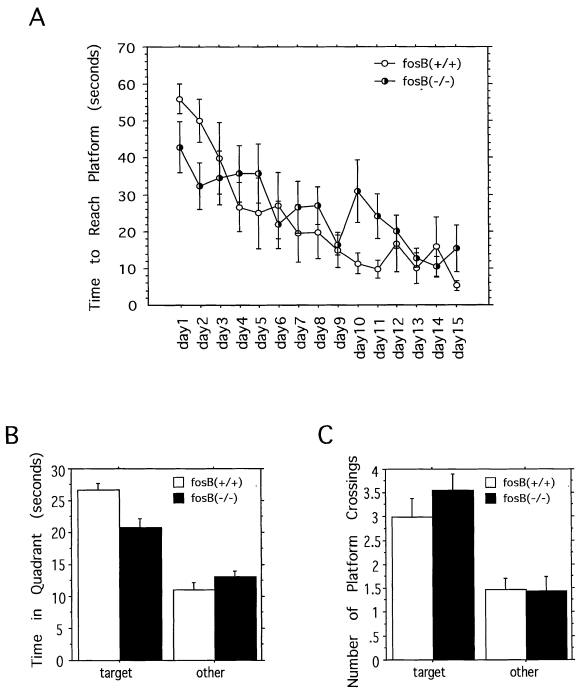

Figure 6. *fosB*$^{-/-}$ Mice Learn Normally in the Morris Water Maze

(A) Latency to reach the hidden platform, by day of training. Mice were given one trial per day for fifteen days. ANOVA: effect of genotype, $p = 0.5373$; effect of training day, $p < 0.0001$; effect of interaction, $p = 0.1123$. $N = 7$ wild-types and $N = 9$ mutants.

(B) Time spent in the trained target quadrant versus each of the other three quadrants, during the probe test on day 15. Paired t test, $p = 0.0004$ for *fosB*$^{-/-}$ and $p < 0.0001$ for *fosB*$^{+/+}$.

(C) Number of crossings of the target platform location versus the other three platform locations, during the probe test on day 15. Paired t test, $p = 0.0001$ for *fosB*$^{-/-}$ and $p = 0.0004$ for *fosB*$^{+/+}$.

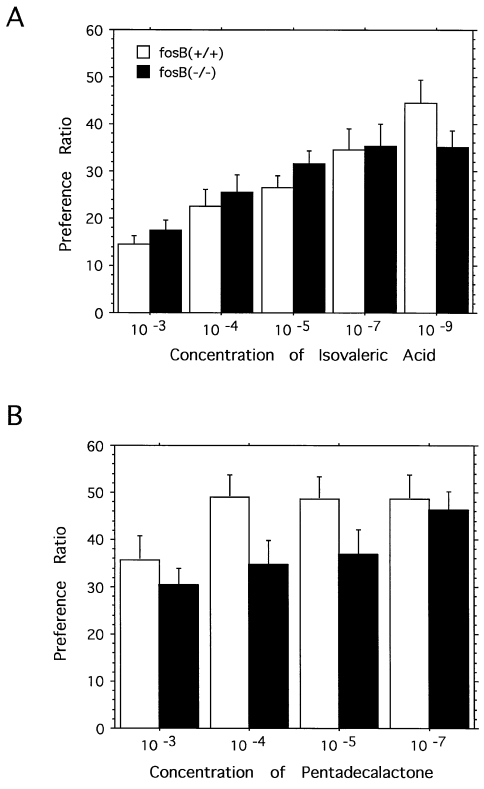

Figure 7. *fosB*$^{-/-}$ Mice Show Normal Olfactory Discrimination

The preference ratio shown on the y axis is the percentage of total liquid consumed over 48 hr that contained the odorant.

(A) Aversion conditioning to isovaleric acid. ANOVA: $p = 0.8972$ for effect of genotype; $p < 0.0001$ for effect of isovaleric acid concentration; $p = 0.1175$ for effect of interaction.

(B) Aversion conditioning to pentadecalactone. ANOVA: $p = 0.1155$ for effect of genotype; $p = 0.0002$ for effect of pentadecalactone concentration; $p = 0.3554$ for interaction.

Mechanism of the Nurturing Defect

These results demonstrate a genetic requirement for *fosB* in nurturing behavior but leave many mechanistic questions unanswered. Although the function of FosB in nurturing behavior could be very specific to the particular neural circuitry involved, the data so far do not rule out a more general defect. For example, our data could conceivably result from either a cognitive or a sensory abnormality that also has other important behavioral effects. To address the question of cognitive impairment, the mice were trained on the hidden platform test of the Morris water maze. The *fosB* mutants were able to learn the platform location as well as wild-type animals and were indistinguishable in the probe test (Figure 6). Since they perform normally on this complex test of spatial learning, the *fosB* mutants do not suffer from global cognitive dysfunction.

A second possible explanation for their nurturing defect is a sensory abnormality. An olfactory abnormality seemed particularly likely, given that olfactory cues have been shown to be critical to pup retrieval (Smotherman et al., 1974). The olfactory sense of *fosB* mutants was assayed in an aversive conditioning paradigm in which the mice learn to avoid a distinctive odor, in this case isovaleric acid or pentadecalactone (Wysocki et al., 1977; Griff and Reed, 1995). *fosB* mutants were indistinguishable from wild-type animals in their ability to detect and avoid isovaleric acid (Figure 7A). Although *fosB* mutants appear to be better able to detect pentadecalactone than wild-type animals (Figure 7B), this difference is not statistically significant. Although these results clearly establish that the *fosB* mutants are not anosmic, they do not rule out a more subtle regulatory defect, for example in the correct interpretation and response to odor.

Since nurturing behavior is known to involve the hypothalamus, the defect in *fosB* mutants could be limited to the hypothalamus but might affect several of its functions. However, preliminary results suggest that *fosB* mutant animals are normal in a number of different processes regulated by the hypothalamus, including adaptation to cold, male aggression against an intruder, locomotor activity, and exploration of a foreign object. Eating and sexual behavior also appear to be intact, given the essentially normal body weight and normal pregnancy rate of the mutants. These results suggest that the *fosB*-dependent defect in nurturing behavior is quite specific and may be mediated by brain regions whose primary function is to control the nurturing response.

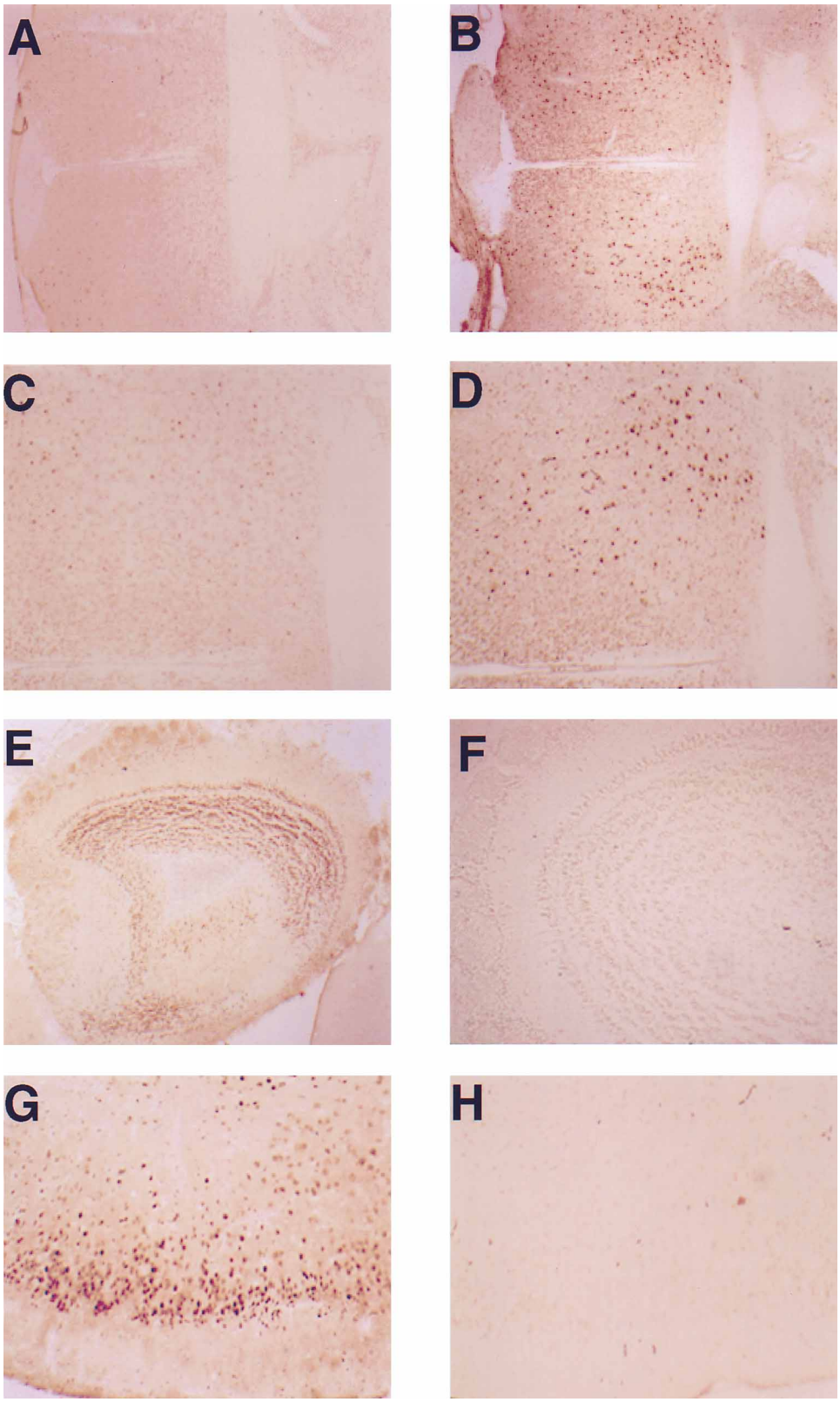

Figure 8. Expression of FosB in the Adult Mouse Brain

FosB-specific immunostaining of paraformaldehyde-fixed frozen coronal sections of brains from young female animals either without or with six hours of exposure to newborn pups

In (C) and (D), the anterior commissure is located at the right side and the third ventricle at the bottom of the picture.

(A) Preoptic area of hypothalamus from *fosB*$^{+/}$ female without exposure to pups (18×).

Expression of FosB in the Brain

The presence of a specific nurturing defect in *fosB* mutant mice suggests that FosB normally functions in the brain within regions that control nurturing. Since the presentation of pups elicits a nurturing response in a protein synthesis–dependent manner (Fleming et al., 1990; Malenfant et al., 1991), it seemed likely that exposure to pups triggers the induction of *fosB* in a region of the brain that is critical for nurturing. The FosB protein might then activate late genes that are required to effect the nurturing response. To test this possibility, we examined the effect of pup presentation on FosB expression in brain regions identified by lesion studies to be critical for nurturing. Brain sections prepared from mice before and 2 and 6 hr after pup presentation were stained with anti-FosB antibodies. This experiment demonstrated a clear induction of FosB in the preoptic area of the hypothalamus (POA) after 6 hr of exposure to pups (Figures 8A–8D). As reported previously (Calamandrei and Keverne, 1994; Fleming and Walsh, 1994; Fleming et al., 1994; Numan and Numan, 1995), induction of c-Fos was also observed in the POA at this time, although at lower levels than FosB (data not shown). Since the disruption of *fosB* function leads to a nurturing defect and the POA is known from lesion studies to be critical for nurturing behavior, these FosB expression studies indicate that FosB may act specifically within POA neurons to induce nurturing behavior.

The immunohistochemical analysis also demonstrated that FosB is constitutively expressed in several brain regions, including the main and accessory olfactory bulbs and pyriform cortex (Figures 8E–8H). Expression in the accessory olfactory bulb paralleled expression in the main olfactory bulb and no further induction of FosB was seen in either region upon presentation of pups. Since ablation experiments have clearly shown that olfactory information is critical for the nurturing response (Gandelman et al., 1971; Fleming and Rosenblatt, 1974; Fleming et al., 1992), the observation that FosB is constitutively expressed within olfactory bulb and pyriform cortex suggests that FosB may function in the integration or modulation of multiple olfactory inputs that regulate the nurturing response. Expression of FosB but not c-Fos was also variably detected in the cerebral cortex, striatum, hippocampus, amygdala, and other hypothalamic regions (data not shown), suggesting that FosB may be uniquely responsive to subtle environmental perturbations. Given this wide expression in the brain, FosB may have functions in the central nervous system in addition to its role in the nurturing response.

Discussion

In this study we describe the effect of a knockout mutation of the *fosB* gene on mouse development and on adaptive neuronal responses of mature mice. Mutating the *fosB* gene results in a nurturing defect in *fosB* mutant mothers that severely impairs the survival of their offspring. The nurturing defect is not limited to the context of pregnancy, but is also observed in young male and female mice. Since *fosB* mutant mice show no other obvious cognitive or olfactory abnormalities and do not suffer from general hypothalamic dysfunction, their defect in nurturing may be quite specific. The *fosB* mutants do show normal exploratory and investigative behavior toward pups, yet seem unable to effectively process pup-induced cues that normally elicit a nurturing response.

Previous studies of nurturing in mice provide insight into how FosB regulates this response. In mammals, the birth of young must trigger an immediate nurturing response if the survival of the species is to be ensured. This postpartum nurturing response is thought to have two components, one experiential and one hormonal (Rosenblatt, 1994). Both components appear to require the same brain regions. The experiential component, which is induced in responding animals over several days of exposure to pups, requires new protein synthesis and probably new gene expression within the critical brain regions (Orpen and Fleming, 1987; Fleming et al., 1990; Malenfant et al., 1991; Numan, 1994). Our finding that exposure to pups induces FosB expression within POA neurons of mice that nurture effectively is consistent with a role for FosB in the experiential component of nurturing. Furthermore, this experiential component of nurturing can be induced not only in postpartum females, but also in young males and nulliparous females (Noirot, 1969a, 1969b), both of whom also show impaired nurturing when *fosB* is mutated. In contrast, *fosB* mutants are unlikely to be defective in the second component of the nurturing response, hormonal priming at parturition, because our results indicate that the hormonal milieu at parturition is normal in the *fosB* mutants.

Previous analysis of the neuronal circuitry responsible for nurturing has provided a foundation for understanding how FosB regulates nurturing and more generally how immediate early genes might regulate other adaptive neuronal responses. The initial stimulus for nurturing is the interaction of a mature animal with newborn pups, which results in multisensory stimulation of the adult animal. The processing of olfactory cues is particularly critical for pup recognition and presumably activates the neuronal circuit that mediates the nurturing response (Smotherman et al., 1974). In the postpartum animal the olfactory signals appear to be mediated by the main olfactory bulb, as lesion of the accessory olfactory system has no effect on postpartum maternal behavior (Jirik-Babb et al., 1984; Lepri et al., 1985). Additional lesion studies have shown that the nurturing response involves the transmission of olfactory information to the

(B) Preoptic area of hypothalamus from $fosB^{+/}$ female following 6 hr exposure to pups (18×). Note inducible staining throughout region (N = 8).
(C) Preoptic area of hypothalamus from $fosB^{+/}$ female without exposure to pups (47×).
(D) Preoptic area of hypothalamus from $fosB^{+/}$ female following 6 hr exposure to pups (47×).
(E) Olfactory bulb of $fosB^{+/}$, unexposed female, demonstrating constitutive FosB expression (18×).
(F) Olfactory bulb of $fosB^{-/-}$, unexposed female, demonstrating loss of FosB expression (47×).
(G) Pyriform cortex of $fosB^{+/}$, unexposed female, demonstrating constitutive FosB expression (47×).
(H) Pyriform cortex of $fosB^{-/-}$, unexposed female, demonstrating loss of FosB expression (47×).

amygdala and from the amygdala to the preoptic area of the hypothalamus (POA), particularly the medial region (Fleming et al., 1980, 1983; Numan et al., 1988). The preoptic area, which probably integrates the animal's response to multiple external stimuli from newborn pups, appears to function as the primary regulator of nurturing behavior (Numan et al., 1988). Lesion studies have also established that the lateral efferent projections of the POA are required for the nurturing response and may directly trigger retrieval and crouching behavior by signaling to the ventral tegmental area (VTA) of the midbrain or to unknown regions of the brainstem (Numan et al., 1988).

While the neuronal circuit that controls the nurturing response is complex, our observation that exposure to pups specifically induces FosB expression in the preoptic area suggests that FosB may regulate the nurturing response by acting directly within neurons of the POA. However, the finding that FosB is constitutively expressed in the olfactory bulbs as well as pyriform cortex suggests that FosB may also be involved in olfactory processing. Although the *fosB* mutant mice are as effective as wild-type mice in recognizing and efficiently avoiding an odor, it remains possible that a more subtle olfactory processing defect is present in the *fosB* mutant mice and may contribute to their nurturing defect. Our experiments also do not rule out the possibility that neurons required for nurturing behavior fail to develop normally in *fosB* mutant mice. However, neuroanatomic studies, including immunostaining for oxytocin and Fos, have not revealed any structural abnormalities in the hypothalamus (unpublished data).

The activation of FosB in the POA following exposure to pups, taken together with the findings that both the POA and FosB are critical for nurturing, constitutes compelling evidence that FosB acts in the POA to mediate the nurturing response. However, several critical issues remain to be addressed, including the mechanism of FosB induction in the POA, the identification of FosB targets that mediate nurturing, and the elucidation of the specific adaptive changes that enhance the nurturing response following repeated exposure to newborn pups. The activation of *fosB* may be mediated via synaptic stimulation by glutamate, which is known to activate IEGs (Bading et al., 1993; Lerea and McNamara, 1993). Alternatively, *fosB* activation may be triggered by another neurotransmitter, neuropeptide, or hormone. Compelling evidence implicates the hormones oxytocin and prolactin in the regulation of nurturing behavior (Fahrbach et al., 1985; Pedersen et al., 1985; Van Leengoed et al., 1987; Bridges and Mann, 1994). Both oxytocin and prolactin induce nurturing behavior when injected into the POA (Bridges and Mann, 1994; Pedersen et al., 1982; Pedersen et al., 1994), suggesting that these agents act directly on POA neurons. Since in other cell types exposure to prolactin or oxytocin activates signal transduction pathways that can lead to IEG transcription (Sidis and Horseman, 1994; Zhuge et al., 1995), it is possible that in the POA these hormones activate signaling pathways that regulate *fosB* expression. Our findings that oxytocin and prolactin expression are normal in the *fosB* mutant are consistent with the possibility that these hormones function upstream of *fosB* in the nurturing pathway, perhaps by acting on POA neurons.

Once induced, FosB most likely contributes to the activation of a program of gene expression that facilitates the strengthening of the neural circuit and leads to an enhancement of nurturing behavior. The simplest mechanism by which FosB might activate transcription is by dimerizing with Jun family proteins and binding to AP-1 sites within the regulatory regions of target genes. The genes that are activated by FosB are unknown, but one excellent candidate is the oxytocin receptor gene, which contains within its promoter an AP-1 site (Rozen et al., 1995). An interesting possibility is that FosB, which may be induced by oxytocin binding to receptors on POA neurons, then leads to increased expression of the oxytocin receptor gene. Thus, by a feedforward mechanism, POA neurons would express more oxytocin receptors and would therefore have enhanced sensitivity to oxytocin. Consistent with this possibility is the observation that oxytocin receptor binding is significantly increased in the MPOA and the VTA during parturition, compared to mid-pregnancy or mid-lactation (Pedersen et al., 1994). This enhanced sensitivity to oxytocin is one possible molecular mechanism by which the nurturing circuit might be reinforced.

The study of designed mutations in mice promises to provide new insight into the molecular basis of behavior. Our analysis of the *fosB* mutation has demonstrated a requirement for *fosB* in the nurturing response in mice and raises the possibility that *fosB* and other immediate early genes may regulate nurturing in higher mammals as well. The availability of *fosB* mutant mice should provide an opportunity to develop a more detailed understanding of the molecular basis of nurturing and, given the widespread expression of FosB in the brain, may also prove useful for elucidating the mechanisms that control other adaptive neuronal responses.

Experimental Procedures

Generation and Characterization of Knockout Mice

A 7.4 kilobase BamHI/SalI fragment of *fosB* genomic DNA (129Sv) was subcloned into Bluescript KS and linearized with HincII by partial digestion. An EcoRI/HindIII fragment containing *PGK-neo* was blunted and ligated into the HincII-digested genomic fragment in inverse orientation. A 2.7 kilobase EcoRI/HindIII fragment of pPNT (Tybulewicz et al., 1991) which contains *PGK-tk* was subcloned into Bluescript, digested with XbaI and HindIII, blunted, and ligated into the genomic clone containing *PGK-neo*, which had been digested with XhoI and blunted. This targeting vector was linearized with NotI and electroporated into J1 embryonic stem cells (Li et al., 1992). Clones were isolated and genotyped as described (Laird et al., 1991; Li et al., 1992). Injections into BALB/c blastocysts and animal care were performed essentially as described (Robertson, 1987). Male chimeras with 80%–90% agouti coat color were mated to BALB/c females. Germline-transmitting animals were also mated to 129Sv females. Genotyping was performed as described (Laird et al., 1991). The DNA was extracted twice with phenol-chloroform and subjected either to Southern blot analysis or to PCR (Ausubel et al., 1995). PCR and Southern, Northern, and Western transfer were performed as described (Ausubel et al., 1995). Hybridization was performed as described (Church and Gilbert, 1984) using probes labeled by random hexamer priming. Embryonic fibroblasts were cultured as described (Robertson, 1987; Li et al., 1992).

Behavioral Analysis

Postpartum Females

All females were individually housed once pregnant. Births were recorded each morning. Each new mother was observed for 20 min

with minimal disruption. All of her pups were then removed and kept warm for 1 hr. After that hour the mother was briefly removed from her home cage, one pup was placed in each corner of the cage distant from her nest (total of 3), and the mother was returned to her nest, facing the wall. During the next 20 min she was continually observed, and the following data points were recorded: latency to retrieve each pup, total number of pups retrieved, and time spent crouching over all three pups in the nest. Retrieval was defined as the mother picking up a pup in her mouth and transporting it to the nest. If she picked up and dropped the same pup more than once en route to the nest, the retrieval was not scored until the pup was in the nest. Crouching was defined as the mother arching her back and assuming the nursing posture with all 3 pups under her ventral surface in the nest, although the pups did not actually have to be nursing. These procedures were adapted from Calamandrei and Keverne, 1994, Fleming et al., 1994, and Van Leengoed et al., 1987.

Nulliparous Females

Females aged 28–45 days were individually housed for at least one day prior to the experiment. All experiments were performed blind to genotype. On day 1, each female was exposed to three 1- to 3-day-old pups, which were placed in her cage as described above. After 30 min, the pups were returned to their mother. On day 2, ~24 hr after the first exposure, each female was again exposed to three 1- to 3-day-old pups. For the succeeding 30 min she was continually observed and the following endpoints were recorded: latency to retrieve each pup, total number of pups retrieved, and total time spent crouching over all the pups in the nest. In two out of four experiments, this paradigm produced a high level of performance in at least half the animals by the second day. In the third and fourth experiments, little nurturing occurred on day two of this paradigm, so several days were allowed to elapse and the identical experiment was repeated, this time with nurturing behavior achieved. All the data were pooled.

Males

Experiments on the males were performed essentially as above, except that the experiment time period was 20 min and exposure was for three consecutive days before testing on day four.

Statistical Analysis

Data analysis was performed with the programs StatView and JMP. The reproductive performance and nurturing behavior data were analyzed by t test unless the variances of the two genotype groups were significantly different by the Brown–Forsythe test, in which case the Welch ANOVA was applied if defined. If not defined, the nonparametric Kruskal–Wallis/Wilcoxon rank sums test was applied. The water maze and olfaction data were analyzed by repeated measures ANOVA.

Histology and Immunohistochemistry

Mammary glands were stored in Bouin's fixative overnight, embedded in paraffin, and stained with hematoxylin and eosin. For brain histology and immunohistochemistry, the mice were perfused with 4% paraformaldehyde and the brains removed, postfixed overnight, equilibrated in 30% sucrose in water, and frozen in liquid nitrogen. The brains were then sectioned at 20 μm on a cryostat. Immunostaining employed a polyclonal rabbit antibody that was raised against an N-terminal (amino acids 79–131) FosB fusion protein (courtesy of Y. Nakabeppu [Nakabeppu and Nathans, 1991]). The primary antibody was used at 1:250 and incubated at 4°C overnight. Staining was developed using the Vectastain Elite ABC kit.

Morris Water Maze

The Morris water maze testing was performed essentially as described (Abeliovich et al., 1993). Two- to three-month-old male mice were trained in multiple paradigms, including 1 trial per day for 15 days (data shown), 4 trials (one per hour) per day for 9 days, and 12 trials (four per hour for three hours) per day for 3 days. On the day following the conclusion of training, the learning of the mice was assessed using a probe test, in which the platform is removed and the mice swim around the pool for one minute. The amount of time spent in the trained vs. all other quadrants and the number of times the mice crossed the location of the trained platform vs. the other platform locations is recorded. Male mice, who do show a demonstrable defect in nurturing, were used in this analysis because behavior in female mice can vary with the estrus cycle. All experiments were performed blind to the genotype of the mice.

Olfaction

Olfactory discrimination was assessed essentially as described (Wysocki et al., 1977; Griff and Reed, 1995). In brief, male mice were housed individually with access to a saccharin solution restricted to one hour per day for 5–7 days. On the first day of the experiment, each mouse was given a saccharin solution containing either a 10^{-3} dilution of isovaleric acid or a 10^{-3} dilution of pentadecalactone for 10 min. Each mouse was then injected intraperitoneally with 15 λ/g body weight of 0.6 M LiCl. Twenty-four hours later each mouse was given two bottles, one with saccharin solution and one with saccharin solution plus odorant. Each bottle was weighed before being placed in the cage and again after 24 and 48 hr. After 48 hr the odorant concentration was reduced and the experiment continued. Bottle leakage was prevented by using sipper tubes containing ball bearings. Male mice, who do show a defect in nurturing, were used in this analysis because olfactory discrimination in female mice varies with the estrus cycle.

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