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The winged helix gene, *Mf3,* is required for normal development of the diencephalon and midbrain, postnatal growth and the milk-ejection reflex

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SUMMARY

The mouse *Mf3* gene, also known as *Fkh5* and *HFH-e5.1*, encodes a winged helix/forkhead transcription factor. In the early embryo, transcripts for *Mf3* are restricted to the presomitic mesoderm and anterior neurectoderm and mesoderm. By 9.5 days post coitum, expression in the nervous system is predominantly in the diencephalon, midbrain and neural tube. After midgestation, the highest level of mRNA is in the mammillary bodies, the posterior-most part of the hypothalamus. Mice homozygous for a deletion of the *mf3* locus on a [129 × Black Swiss] background display variable phenotypes consistent with a requirement for the gene at several stages of embryonic and postnatal development. Approximately six percent of the *mf3*–/– embryos show an open neural tube in the diencephalon and midbrain region, and another five percent show a severe reduction of the posterior body axis; both these classes of affected embryos die in utero. Surviving homozygotes have an apparently normal phenotype at birth. Postnatally, however, *mf3*–/– pups are severely growth retarded and approximately one third die before weaning. This growth defect is not a direct result of lack of circulating growth hormone or thyrotropin. Mice that survive to weaning are healthy, but they show an abnormal clasping of the hindfeet when suspended by the tail. Although much smaller than normal, the mice are fertile. However, *mf3*–/– females cannot eject their milk supply to feed their pups. This nursing defect can be corrected with interperitoneal injections of oxytocin. These results provide evidence that *Mf3* is required for normal hypothalamus development and suggest that *Mf3* may play a role in postnatal growth and lactation.

Key words: winged helix gene, fork head gene, gene targeting, forebrain patterning, midbrain patterning, hypothalamus, pituitary, growth regulation, oxytocin, lactation, mammillary bodies

INTRODUCTION

Over the past few years there has been much progress in our understanding of the genetic regulation of the early patterning, growth and differentiation of the vertebrate central nervous system (CNS). While many studies have focused on the role of Hox family members in the rhombomeric organization of the hindbrain, more recent work has addressed the genetic regulation of forebrain and midbrain development. According to one model, the forebrain is divided transversely into six prosomeres (p1 through p3 make up the diencephalon, and the telencephalon comprises p4 through p6) that are further subdivided longitudinally into basal and alar subregions (reviewed by Puelles and Rubenstein, 1993; Lumsden and Krumlauf, 1996). Most of the evidence supporting this model is based on the expression patterns of molecular markers, in addition to morphological data. The patterning of the midbrain is less complex and seems to be regulated by extracellular signals emanating from the isthmic constriction between the mesencephalon and the hindbrain (reviewed by Lumsden and Krumlauf, 1996).

Members of diverse families of transcription factors exhibit temporally and regionally restricted patterns of expression in the anterior CNS, and in some cases genetic analysis has demonstrated a role for these proteins in forebrain and midbrain development. For example, homeobox genes of the *orthodenticle* family, *Otx1* and *Otx2*, and the *distal-less* family, *Dlx1* and *Dlx2*, are expressed in nested domains in the anterior embryonic CNS. *Otx2* null mutant embryos have severe deletions of the forebrain and midbrain regions, while inactivation of the *Dlx2* locus does not affect gross regional subdivisions of the forebrain but does result in the abnormal differentiation of cells within the olfactory bulb (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996 for *Otx2*; Qui et al., 1995 for *Dlx2*). *Lim1*, a LIM class homeobox genes that is expressed in the anterior of the embryo, is essential for development of head structures (Shawlot and Behringer, 1995). Several transcription factors of the POU family, for example

Brn1, *Brn2* and *Brn4*, are expressed in the anterior CNS (Alvarez-Bolado et al., 1995), and *Brn2* has recently been shown to function in one of the last steps of development of the hypothalamus and the pituitary (Schonemann et al., 1995; Nakai et al., 1995).

Our lab has focused on the role of the winged helix (WH) family of transcription factors during mouse embryogenesis (Sasaki and Hogan, 1993; Labosky et al., 1996) (for review of the WH family see Kaufmann and Knochel, 1996). Members of this evolutionarily conserved family are known to affect cell fate, proliferation and tissue-specific gene expression in several different organisms, and a number, including *Hnf3β, Hnf3α, Bf1*, *Bf2*, *Fkh4* and *Mf2* and *Mf3*, are expressed, among other places, in the CNS of the embryo. Mutational analyses have shown that several mouse WH genes are essential for normal development. For example, embryos homozygous for a null mutation in *HNF3β* die at the neurula stage and lack a floorplate and notochord, structures that both normally express *HNF3β* (Ang and Rossant, 1994; Weinstein et al., 1994). *Nude* mice, which are hairless and lack a thymus, are homozygous for a spontaneous mutation that generates a truncated product of the *winged helix nude (whn)* gene (Nehls et al., 1994). Embryos homozygous for a targeted deletion of the *brain factor 1 (Bf1*) locus die around birth, with a dramatic reduction in the size of the cerebral hemispheres. Analysis of these *Bf1–/–* embryos showed a reduction in cell proliferation in the telencephalic neuroepithelium and premature differentiation of cells in the cerebral cortical neuroepithelium. This suggests that *Bf1* may be critical not only for dorsal-ventral patterning of the telencephalon but also for the correct differentiation of specific cell lineages (Xuan et al., 1995). Mutations in WH genes do not always affect all tissues in which they are expressed. For example, although *Bf2* is expressed in both kidney and forebrain, a targeted null mutation in *Bf2* grossly affects morphogenesis of the kidney but causes only subtle abnormalities in the forebrain (Hatini et al., 1996), suggesting that the expression of other WH genes in the diencephalon may compensate for the absence of *Bf2*.

The *Mf3* gene (Sasaki and Hogan, 1993), which has been identified elsewhere as *HFH-e5.1* (Ang et al., 1993) or *Fkh-5* (Kaestner et al., 1996), maps to mouse Chromosome 9 near several well characterized mouse mutations including *prenatal lethal factor 1* (*pnlf1*), and *small thymus* (*sty*) (Labosky et al., 1996; Kaestner et al., 1996). The expression pattern of *Mf3* has been well characterized (Ang et al., 1993; Kaestner et al., 1996; and this manuscript). Transcripts are first localized in the early embryo in anterior neurectoderm and mesoderm and in the presomitic mesoderm. By 9.5 days p.c., a band of expression is seen in the developing diencephalon and midbrain region. However, late in gestation the predominant region of expression is the most caudal region of the hypothalamus, within the mammillary bodies, raising the possibility that, like *Bf1*, *Lim1*, *Otx2* and *Dlx2*, *Mf3* may play a role in the growth and differentiation of a specific segment of the anterior CNS. To address the function of *Mf3*, we have used homologous recombination in ES cells to delete the protein coding region of the gene. On a [129 × Black Swiss] genetic background, 11% of the homozygous null embryos die in utero. Approximately half of these show reduction of the posterior diencephalon and midbrain regions, while the other half are severely developmentally delayed, with striking posterior deficiencies. Surviving *mf3–/–* pups appear normal at birth. However, all show reduced postnatal growth and approximately half die before weaning. Additionally, adult *mf3–/–* mothers are unable to let down milk in response to suckling, and all tested *mf3–/–* animals display an abnormal clasping together of the hindlimbs along the ventral midline. This variable phenotype, presumably due to differences in genetic background, provides evidence for multiple roles for *Mf3* during embryogenesis and adult life.

MATERIALS AND METHODS

Northern analysis and in situ hybridization

Embryos were from crosses of ICR (Harland) mice. Noon on the day of appearance of the vaginal plug is 0.5 days post coitum (p.c.). RNA samples from ES cells and embryos of different stages were prepared by the LiCl/urea method (Auffrey et al., 1980). 10 μg of total RNA was analyzed on formaldehyde gels, blotted and probed by standard methods (Sambrook et al., 1989). Whole-mount in situ hybridization was performed essentially as described by Winnier et al. (1995). Modifications were made in the processing of 18.5 days p.c. embryonic heads and dissected brains; in both cases Proteinase K treatment was lengthened from 5 minutes to 30 to 60 minutes. Antisense riboprobes for *Mf3* span nucleotides 30 to 666 of the cDNA as reported in Kaestner et al. (1996) which includes the 5′ UTR, amino terminus and the WH domain of the protein. This probe was shown to be specific for *Mf3* as it hybridized to one band on northern blots and failed to hybridize to *mf3–/–* embryos. Section in situ hybridization was performed essentially as described by Zhao and Hogan (1996). The riboprobes for *Bf2, Islet1, Msx1, Pax3* and *Sonic hedgehog* (*Shh*), were gifts from Drs E. Lai, S. Thor, B. Hill, M. Goulding and A. McMahon, respectively (Hatini et al., 1994; Ericson et al., 1995; Hill et al., 1989; Goulding et al., 1991; Echelard et al., 1993).

Gene targeting construct

Genomic DNA clones for *Mf3* were isolated from a genomic 129/SvJ mouse library (Stratagene) using a partial cDNA for *Mf3* (designated *c43* in Sasaki and Hogan, 1993). Restriction mapping and partial sequencing was used to determine the structure of the locus as shown in Fig. 1A. A targeting vector was constructed in pPNT (Tybulewicz et al., 1991; gift from A. Joyner and J. Rossant). The 5′ region of homology is 2.5 kilobase pairs (kb) and includes mostly intron sequence and 13 base pairs (bp) of the 5′ end of exon 2. The 3′ arm is a 4.5 kb *Bam*HI-*Asp*718 fragment. The deletion construct results in the replacement of the entire protein coding region of *Mf3*, including the winged helix domain, with the PGKneo^r cassette. The targeted allele is designated *mf3*^{tm1blh}, according to the guidelines of the International Committee on Standardized Genetic Nomenclature for Mice (The Jackson Laboratories). The name *Mf3* has been approved by the International Committee for Mouse Nomenclature (Labosky et al., 1996).

Electroporation and selection of targeted ES cells

TL1 cells from an ES cell lined derived by PAL from 129/SvEvTac blastocysts (mice purchased from Taconic Farms), were used for targeting. Electroporations were carried out as described by Winnier et al. (1995). In two separate electroporations, DNA from 155 G418 and gancyclovir double resistant clones was screened with the 5′ internal probe shown in Fig. 1A. Four targeted lines were identified, giving a targeting frequency of 1 in 39.

DNA analysis

Analysis of ES cell lines and genotyping of animals was performed by Southern blotting as described by Hogan et al. (1994). DNA was

restricted with *Xba*I, and Southern blot analysis was performed essentially as described by Church and Gilbert, (1984). The 5′ external probe illustrated in Fig. 1A was used to screen the initial ES cell colonies. Integrity of the 3′ end of the locus was confirmed by probing DNA samples restricted with *Spe*I, *Hin*dIII and *Pst*I with the external probe indicated in Fig. 1A. Examples of these Southern blots are shown in Fig 1B. In addition, Southern analysis with a neo probe confirmed the absence of additional random integrations (data not shown).

Generation of chimeras and mutant animals

ES cells from two independently targeted cell lines (1G and 8C) were injected into C57BL/6 blastocysts and transferred into pseudopregnant (C57BL/6 × DBA)F_1 females as described by Hogan et al. (1994). Male chimeras were bred to either Black Swiss or 129/SvEvTac females. Agouti offspring were analyzed by Southern blot and heterozygous animals were interbred to obtain homozygous animals. Mice derived from both cell lines showed the same phenotype, and most of the analysis was performed with animals generated from the cell line 1G on the mixed 129/Black Swiss background. Mutants derived from both cell lines also showed a similar postnatal growth retardation phenotype on an inbred 129/SvEvTac background.

Histochemistry and immunohistochemistry

Samples for histochemistry and immunostaining were prepared according to standard techniques. Primary antibodies to the following antigens were used at the following dilutions: ACTH (gift from Dr David Orth), 1:500; antibodies against human FSH (Zymed Laboratories, Inc., South San Francisco, CA), prediluted; antibodies against mouse GH (gift from NIDDK), 1:1000; antibodies against human prolactin (Zymed), prediluted; antibodies against human TSH (Zymed), prediluted; oxytocin (Peninsula Labs), 1:500. The polyclonal antibodies generated against human FSH, TSH and prolactin crossreact with mouse FSH, TSH, and prolactin. Immunostained sections of pituitary glands were counterstained with hematoxylin. Oxytocin staining was quantitated by counting positive cells in serial sections. Three serially stained brains of both *mf3*+/+ and –/– animals were counted, and all brains used were from virgin females between 6 and 7 weeks of age. In order to avoid double-scoring of cells, a cell was counted only if the nucleus was visible in that section. Immunostaining for BRN1, 2, 4 and TST1 was performed as described by Schonemann et al. (1995).

Radioimmunoassays

Radioimmunoassays (RIAs) for oxytocin were performed as described by Robinson (1980) with the antiserum R35 from Dr Robinson.

Mouse serum RIAs for GH and TSH were performed by a double antibody method, using the immunoreagents distributed by the NIDDK's National Hormone and Pituitary Program, including highly purified mouse GH antigen for iodination, AFP10783B, mouse GH Reference Preparation AFP-10783B, anti-rat GH (monkey) serum NIDDK anti-rGH S5, highly purified rat TSH antigen AFP-7308C for iodination, anti-TSH (guinea pig) #AFP98991, and mouse TSH Reference Preparation AFP51718MP, a partially purified extract of mouse pituitary glands. For GH, results were expressed as nanogram-equivalents of AFP-10783B per ml of serum, and TSH results are expressed as nanogram-equivalents of the crude Reference Preparation per ml of mouse serum.

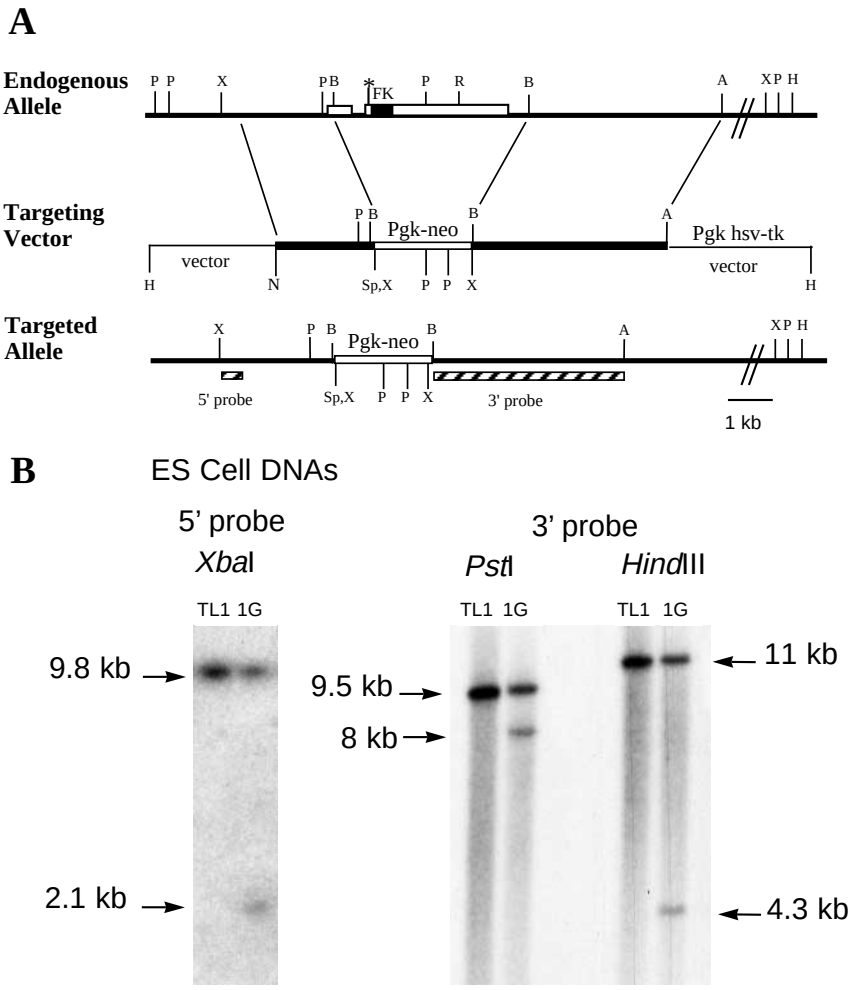

Fig. 1. Targeted mutagenesis of the *mf3* locus. (A) The endogenous *mf3* locus, shown on the top line, is composed of at least two exons with the winged helix domain shown in the black box. The targeting vector has 2.5 kb of DNA in the 5′ arm and 4.5 kb in the 3′ arm with the PGKneo^r cassette between the two arms and a PGK-HSV-thymidine kinase cassette at the 3′ end of the construct. The targeted allele replaces most of the region represented by the cDNA including the region encoding the WH domain with the neo^r cassette. The 5′ and 3′ probes are indicated by bars shown below the targeted locus. The start methionine as reported by Kaestner et al., (1996) is indicated with an asterisk. (B) Southern analysis of ES cell DNAs with the external 5′ probe and the internal 3′ probe. Homologous recombinants are detected with the 5′ probe using an *Xba*I digest. The endogenous allele is indicated by a 9.8 kb hybridizing band and the targeted allele is indicated by a 2.1 kb hybridizing band. The integrity of the targeted locus was verified using the 3′ probe to hybridize to ES cell DNAs digested with several restriction enzymes. Using *Pst*I, the endogenous allele is represented by a 9.5 kb hybridizing band and the targeted allele by an 8 kb band. Using *Hin*dIII, the endogenous and targeted alleles are represented by 11 kb and 4.3 kb hybridizing bands, respectively. The position of endogenous *Hin*dIII, *Pst*I, and *Xba*I restriction sites in the normal allele were determined by Southern blot analysis. Lanes labeled TL1 are untargeted ES cells, while those labeled 1G represent a correctly targeted cell line. A, *Asp*718; B, *Bam*HI; H, *Hin*dIII; N, *Not*I; P, *Pst*I; R, EcoRI; S, *Spe*I; X, *Xba*I.

RESULTS

Expression of *mf3* in the mouse embryo

An *Mf3* cDNA was originally isolated from an 8.5 days p.c. cDNA library and designated *c43* (Sasaki and Hogan, 1993). Northern analysis reveals *Mf3* transcripts of 3.1 kb in embryos from 10.5 days p.c. to 15.5 days p.c (data not shown).

Analysis of *Mf3* expression by whole-mount and section in situ hybridization shows that *Mf3* transcripts are first detected at 7.0 days p.c. in the posterior of the embryo (Fig. 2A). At 7.5 days p.c. this expression is maintained, and an additional domain is seen in the anterior neurectoderm and mesoderm (yellow arrowhead; Fig. 2A). By 8.0 days p.c., *Mf3* transcripts are localized to the neural tube and presomitic mesoderm (Fig. 2B). This localization is maintained at 9.5 days p.c., and a prominent band of expression is now apparent in the prospective forebrain and midbrain (Figs 2C, 3A). By 14.5 days p.c., the strongest neural expression is in the posterior of the hypothalamus, with a weaker hybridizing region in the thalamus (Fig 2D). At 15.5 days p.c., the only expression detectable by whole-mount in situ hybridization is in the mammillary region of the posterior hypothalamus (Fig. 2E). All somite-derived structures are negative for *Mf3* expression, but presomitic mesoderm staining is maintained in the tail bud (data not shown). By 18.5 days p.c., and in newborn mice, *Mf3* expression is most highly expressed in the mammillary bodies (Fig. 2F,G). This overall expression pattern is consistent with, and confirms, earlier studies by Ang et al. (1993) and Kaestner et al. (1996), and further identifies the mammillary bodies of the hypothalamus as the major site of *Mf3* expression in the newborn mouse.

Targeting of the *Mf3* locus

As shown by Kaestner et al. (1996), the *Mf3* locus comprises two exons, the second of which encodes the MF3 protein. Based on this information, our targeting construct (shown in Fig. 1A) deletes the entire protein coding region of *Mf3*, including some 5′ untranslated sequences. The accurate replacement of the *Mf3* coding region by the PGKneo^r cassette was confirmed by Southern analysis with a 5′ external and a 3′ internal probe (Fig. 1B). Whole mount in situ hybridization of *mf3*–/– 18.5 days p.c. embryonic heads with an antisense RNA probe for *Mf3* shows no signal, verifying the specificity of the probe and the targeted inactivation of the locus (data not shown). For the sake of brevity, the *mf3*^{tm1blh}–/– animals will be referred to here as *mf3*–/–.

Four ES cell lines with a correctly targeted allele were identified and cells from two of these were injected into C57BL/6 blastocysts. The resulting male chimeras were crossed with Black Swiss females. Both cell lines transmitted the mutation into the germline. The heterozygotes appear normal in all respects, and were intercrossed to obtain homozygous null animals.

Phenotype of *mf3*–/– embryos

The genotypes, as determined by Southern blotting, of embryos from heterozygous crosses approached normal Mendelian ratios (Table 1). However, there were significant numbers of resorbed, dead and abnormal embryos at 13.5-14.5 days p.c. We therefore examined embryos at earlier stages of development from homozygous crosses in order to obtain a greater number of *mf3*–/– embryos. This analysis confirmed that the large majority of *mf3*–/– embryos are morphologically and histologically normal throughout gestation. However, 11% of the embryos from these double homozygous crosses displayed abnormalities (Table 2 and Fig. 3B-D). The severe class of affected embryos (6 of the affected 13 total, an example in Fig. 3C, left) was substantially developmentally delayed, with large reductions of the posterior of the embryo

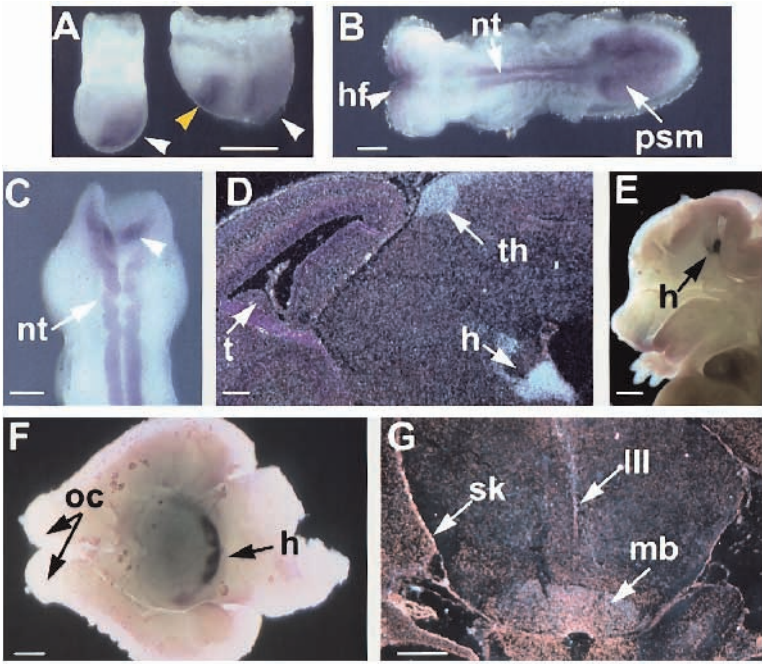

Fig. 2. Localization of *Mf3* transcripts by in situ hybridization. A-C, E, and F are of embryos subjected to whole-mount in situ hybridization using digoxigenin-labelled RNA probes for *Mf3*. D and G are sections subjected to in situ hybridization with ^{35}S-labelled RNA probes. Samples in A,B,D,E and F are oriented with anterior towards the left. (A) *Mf3* transcripts are first detected in the posterior (white arrowhead) of a 7.0 days p.c. embryo (left embryo). In a 7.5 days p.c. embryo (right) *Mf3* transcripts are still detected in the posterior of the embryo (white arrowhead) and also in the anterior neurectoderm (yellow arrowhead). (B) At 8.5 days p.c. *Mf3* transcripts are detected in the presomitic mesoderm, the neural tube and the head folds. (C) At 9.5 days p.c. a band of *Mf3* expressing cells is seen in the future diencephalon (white arrowhead). (D) ,A sagittal section of an embryo at 14.5 days p.c. reveals two domains of *Mf3* expression in the embryonic forebrain, one in the thalamus and one in the hypothalamus. (E) At 15.5 days p.c. the only *Mf3* transcripts in the embryo detected by whole-mount in situ hybridization are in the hypothalamus. (F) At 18.5 days p.c., a ventral view of the embryonic brain shows the location of *Mf3* transcripts in the posterior of the diencephalon. (G) A coronal section through an 18.5 days p.c. head shows *Mf3* transcripts in the mammillary bodies of the hypothalamus. The signal associated with the bones of the skull is non-specific. Scale bars, 150 μm in A, 300 μm in B, 250 μm in C, 100 μm in D, and 500 μm in E-G. h, hypothalamus; hf, headfolds; III, third ventricle, oc, occipital lobes; nt, neural tube; mb, mammillary bodies; psm, presomitic mesoderm; sk, skull; t, telencephalon; th, thalamus.

in the region of the presomitic mesoderm (asterisk) and an open neural tube along two thirds of the body axis (between arrowheads). These embryos had up to 15 somites, although the presomitic mesoderm was reduced when compared to unaffected littermates. The other half of the affected mutants (7 out of 13) showed an open neural tube (indicated by arrowheads) in the region between the posterior forebrain and the midbrain hindbrain junction, where *Mf3* transcripts are seen at high levels at 9.5 days p.c. (Table 2, and Fig. 3B left, C right and D left). Many tissues of the forebrain such as the eyes (Fig. 3D) and Rathke's pouch (Fig. 3E) appeared normal. To more precisely define the disruption caused by the lack of MF3 in

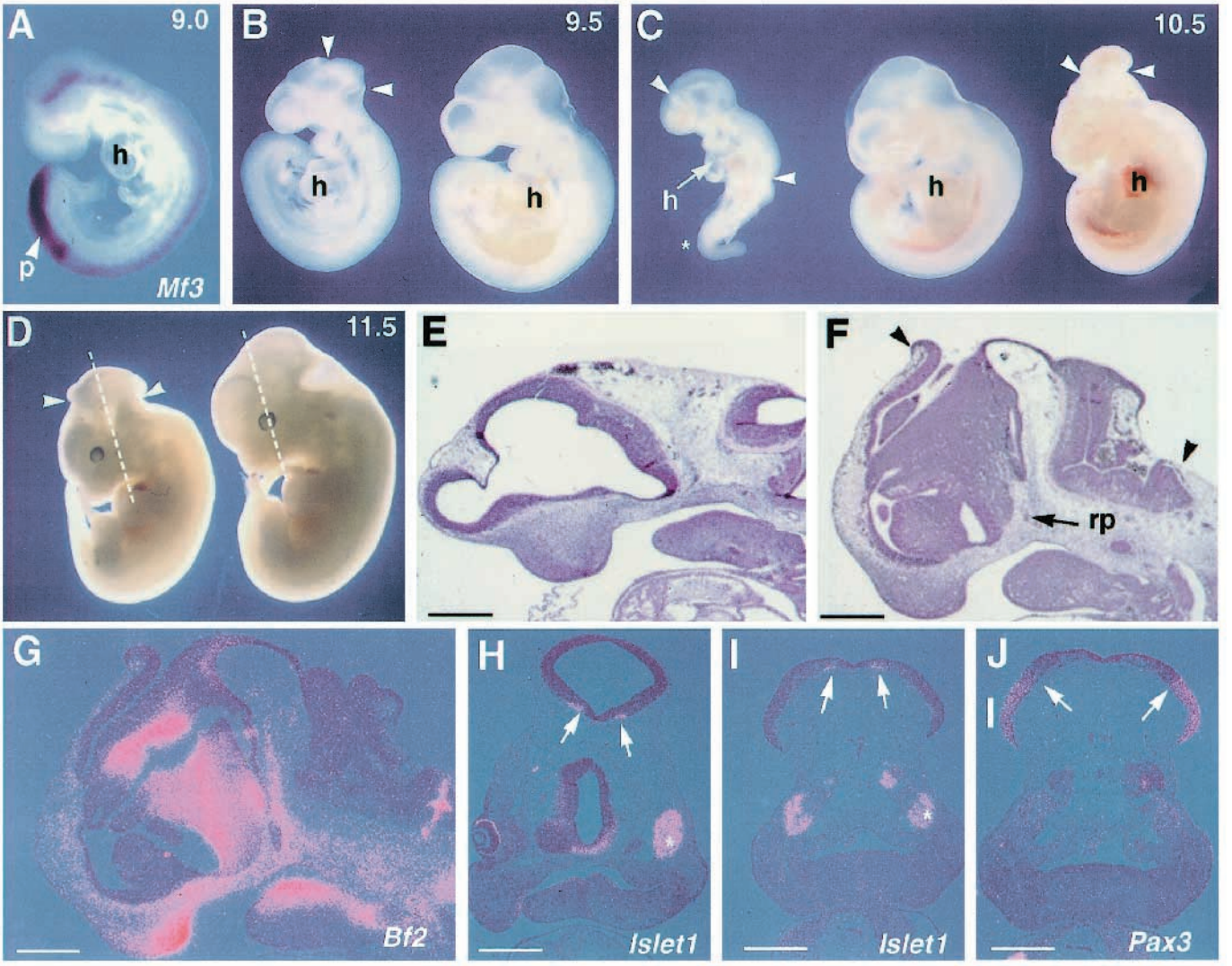

Fig 3. Embryonic consequences of the lack of MF3 in a minority of null mutant embryos. (A) Localization of *Mf3* transcripts by whole mount in situ hybridization at 9.0 days p.c. shows that in the wild type embryo *Mf3* is expressed in the neurectoderm of the neural tube and the future diencephalon and midbrain and the presomitic mesoderm (p). (B-D) Phenotype of *mf3*–/– embryos at 9.5-11.5 days p.c. All embryos are derived from crosses between *mf3*–/– males and females. (B) Typical affected *mf3*–/– embryo at 9.5 days p.c. (left) compared to an unaffected *mf3*–/– embryo (right). Note the open neural tube in the midbrain region (arrowheads). (C) Embryos at 10.5 days p.c. showing, on the left, an example of the most severely affected class of *mf3*–/– embryos with an extensive open neural tube (arrowheads) and reduction of posterior tissue (asterisk). An unaffected *mf3*–/– littermate is shown in the center. On the right is an example of the less severely affected class with an open neural tube in the midbrain region (arrowheads). (D) Example of a *mf3*–/– at 11.5 days p.c. (left) with an open neural tube in the midbrain region (arrowheads) and an unaffected littermate (right). (E) Sagittal section through the midbrain of the unaffected littermate, shown in D, stained with hematoxylin and eosin. (F) Sagittal section stained with hematoxylin and eosin illustrating the reduction in neural tissue in the midbrain region. Arrowheads indicate the anterior-posterior boundaries of the open neural tube. The forebrain and midbrain are collapsed, presumably due to the encephaly. (G-J) Radioactive in situ hybridizations of sections of *mf3*–/– embryos at 11.5 days p.c. (G) Sagittal section of the embryo illustrated in F, hybridized with *Bf2* riboprobe. The pattern of *Bf2* expression is unaltered in the affected *mf3*–/– embryo compared with an unaffected *mf3*–/– embryo, and shows a clear posterior boundary in the posterior diencephalon. (H-J) Frontal sections of the embryos as shown in D with the dashed lines. (H) An unaffected *mf3*–/– embryo showing the normal expression of *Islet1* in the motorneurons of the neural tube (arrows) and the midbrain. (I) An affected *mf3*–/– embryo hybridized for *Islet1* illustrates that the motorneurons adjacent to the floorplate are present, as well as the neurons in the midbrain region. (J) An affected *mf3*–/–embryo showing *Pax3* expression in the lateral neurectoderm. This region would have been dorsal if the neural tube had closed normally. h, heart; p, presomitic mesoderm; rp, Rathke's pouch. Scale bars, 200 μm in E-G, and 270 μm in H-J.

Table 1. Genotypes of embryos and mice generated from *Mf3* heterozygous crosses

Age	+/+	+/–	–/–	Dead	N.D.
11.5-18.5 days p.c.	54 (22%)	117 (47%)	54 (22%)	19 (8%)	4 (<1%)
3 weeks	71 (27%)	148 (56%)	43 (16%)		

Genotypes of embryos from 11.5 days p.c. to 18.5 days p.c. were determined by Southern blots of yolk sac DNA. Some embryos were dead at the time of dissection and intact DNA samples for genotyping could not be obtained. Genotypes from mice at 3 weeks of age were determined by Southern blot of tail DNA. The numbers in parentheses indicate embryos that appeared abnormal. ND, not determined.

these embryos, we examined the expression of other anterior neural markers. In situ hybridization for *Bf2* and *Fgf8* (Fig. 3G and data not shown) confirmed that the area affected in these mutants lies between the diencephalon and the midbrain-hindbrain junction, since the expression of both of these markers is unchanged in the affected *mf3–/–* embryos. Transcripts for *Islet1*, *Pax3, Shh,* and *Msx1* are also unchanged in their expression patterns in the affected mutant embryos (Fig. 3H-J). For example, *Islet1* transcripts are localized to motorneurons adjacent to the floorplate while *Pax3* transcripts are seen in the lateral neural tube which would have been dorsal if the neural tube had closed.

Reduced postnatal growth and neurological phenotype of *mf3–/–* animals

At birth, there are no gross external anatomical differences between the surviving *mf3–/–* pups and their littermates. However, by weaning, *mf3–/–* animals are considerably smaller than their siblings. Additionally, as shown in Table 1, approximately one third of the homozygous mutant animals die before weaning. In order to determine when the postnatal growth deficiency first occurs, pups from heterozygous matings were weighed daily from postnatal day 2 (Fig. 4). These data show that the growth differences between mutant animals and their littermates are discernible as early as 2-3 days after birth, but after weaning the growth curves are parallel.

In addition to the growth retardation and the lactation defect described below, it was noted that 17 of 17 *mf3–/–*, 2 of 4 *mf3+/–*, and none of 30 *mf3–/–* mice, regardless of sex, showed an abnormal contraction and clenching together of the hindlimbs (but not the forelimbs) toward the ventral midline when suspended by their tail. This behavior resembled that shown by mice transgenic for Exon 1 and an expanded CAG repeat of the *Huntington's Disease* (*HD*) gene. In those mice, this clenching posture was one of the first symptoms of a progressive neurological phenotype similar to the symptoms displayed in Huntington's disease (Mangiarini et al., 1996). Mice carrying a null mutation in the cyclin D1 gene also showed growth retardation, this abnormal clenching behavior, and lactation defects (Fantl et al., 1995; Sicinski et al., 1995).

Table 2. Genotypes of embryos generated from *Mf3* homozygous crosses

Age (days p.c.)	Total	Normal	Abnormal	Resorbed
9.5	32	27	4	1
10.5	57	49	5	3
11.5	22	19	3	0
12.5	6	5	1	0
	117	100	13	4
		86%	11%	3%

Genotypes were determined by Southern blots of yolk sac DNA. Embryos classified as abnormal displayed defects in the region of the diencephalon and midbrain.

Table 3. Levels of serum growth hormone and thyrotropin in *Mf3* mice

	Adult		1 week old pups	
Genotype	GH	TSH	GH	TSH
+/+	14.2±4.1 (*n*=9)	303.5±11.2 (*n*=8)	21.6	247
+/–	13.4±5 (*n*=21)	307±14.3 (*n*=13)	13.5	192
–/–	23.1±4.8 (*n*=26)	263.5±10 (*n*=13)	14.3	205

For adult measurements, males of 7-8 weeks were used. For measurements on one week old pups, serum from 10-15 mice was pooled. All values are in nanograms per milliliter (ng/ml).

Analysis of *mf3–/–* pituitary glands

The reduced growth rate of the *mf3–/–* mice is similar to that reported for many mutant small mice (see Voss and Rosenfeld, 1992 for review). However, the observation that the *mf3–/–* animals are smaller as early as 2-3 days after birth is markedly different from most of these classic examples. Both the Ames dwarf (*Prophet of Pit-1*^{df}), Snell and Jackson dwarf (*Pit1*^{dwSn} and *Pit1*^{dwJ}) and the *little* (*Grfr*^{lit}) mice are not noticeably smaller than their wild-type littermates until approximately 2 weeks after birth (Sornson et al., 1996; Li et al., 1990; Lin et al., 1993). All of these mutations (in the homeodomain encoding genes *Prophet of Pit-1* and *Pit1*, and the *Growth hormone releasing factor receptor* gene *(Grfr)*, respectively) affect the cells of the anterior pituitary, most notably the somatotrophs – cells that secrete growth hormone (GH). Additionally, mice homozygous for a null mutation in the glycoprotein hormone α gene are growth retarded due to a deficiency in TSH (Kendall et al., 1995). Given the similar reduced growth rate of these other small mice and of the *mf3–/–* mice, and the observation that *Mf3* is expressed in the hypothalamus, a part of the brain intimately connected to the pituitary, we analyzed the functional status of the principal cell types present in the anterior pituitary gland of adult animals. Fig. 5 shows examples of pituitary glands immunohistochemically stained with antibodies to GH and thyrotropin (TSH). In all cases, the staining was done in parallel and the color reaction developed for the same time to compare directly wild-type and mutant animals. The gross morphology of *mf3–/–* pituitary glands was normal, as shown in Fig. 5A,B. Somatotrophs, identified by GH staining, were present at normal levels in the *mf3–/–* pituitary (Fig. 5C,D), as were thyrotrophs stained for TSH (Fig. 5E,F), corticotrophs stained for adrenocorticotropin, lactotrophs by prolactin staining and gonadotrophs which are positive for follicle stimulating hormone (data not shown). The absence of a significant difference between normal and *mf3–/–* animals is in sharp contrast to the Ames, Snell and Jackson dwarfs, which lack mature somatotrophs, lactotrophs and thyrotrophs, and the *little* mice which lack somatotrophs.

To quantitate the differences in circulating pituitary hormones in these animals, radioimmunoassays (RIA) were performed for GH and TSH (Table 3). In the case of adult mice, male animals were used to avoid any hormone fluctuations that occur in ovulating females. The levels of GH, although highly variable due to the pulsatile nature of GH secretion and the animal's response to factors such as stress (see Harvey and Daughaday, 1995 for review), showed no correlation with the weight of the mouse, and neither GH or TSH levels indicated differences between genotypes. We also measured the levels of circulating GH and TSH in 1 week old pups. Due to the difficulty of obtaining sufficient quantities of blood from a single pup, these readings were obtained from a pool of 10-15 pups, and again these measurements showed no significant differences between genotypes.

mf3–/– mothers lack a milk-ejection reflex

Although approximately one third of the *mf3–/–* animals die before weaning, the surviving mice were healthy, although small, and have lived to over one year of age. Matings between null mutant males and females resulted in normal sized litters of as many as 9 pups. However, none of the *mf3–/–* mothers fed their pups (*n*=12). *mf3–/–* mothers made nests and suckled their pups. The pups were often seen attached to the nipples, and the mothers nipples appeared red and distended. However, milk was never seen in the pups' stomachs, and they died after 2 days.

To determine more precisely the nature of the nursing defect in the *mf3–/–* mutant mothers, histological analysis of mammary glands was performed 1 day after the birth of the pups. Wild type mammary glands showed ductal branching and lobuloalveolar formation characteristic of a nursing mother (Fig. 5G). Mammary glands from *mf3–/–* females 1 day after giving birth showed similar morphological development to wild type, including the accumulation of milk in ducts of the glands (Fig. 5H).

During suckling-induced lactation, the myoepithelial cells surrounding the terminal ducts and alveoli in the mammary glands contract in response to oxytocin (OT), causing the release of milk (or let down) through the mother's nipple. Since *mf3–/–* mothers displayed defects in nursing, and *Mf3* is expressed in the posterior hypothalamus, we reasoned that OT levels might be affected in these animals. In fact, when *mf3– /–* mothers were injected with 600 mU/kg of synthetic OT twice a day, starting 1 day after the birth of their litters, they were then able to feed their pups until weaning (*n*=2). Fig. 6 shows the weights of the pups suckled from an *mf3–/–* mutant mother injected with OT compared to pups fostered onto a wild-type ICR mother. In both cases, all the pups were derived from matings between *mf3–/–* females and males. In this side-by-side comparison we did not observe significant weight differences between *mf3–/–* pups fed by different mothers. This is in contrast to the data shown in Fig. 4 where we observed distinct weight differences between pups of different genotypes when all pups were nursed by a *mf3*+/– mother. Two distinct conclusions came from this experiment. First, the growth retardation of *mf3–/–* pups was unaffected by nursing from a wild-type mother. Second, the *mf3–/–* mutant mothers injected with OT could effectively nurse their pups to weaning age, although near the end of the nursing period the health of the *mf3–/–* mothers had deteriorated as estimated by observing their behavior and appearance.

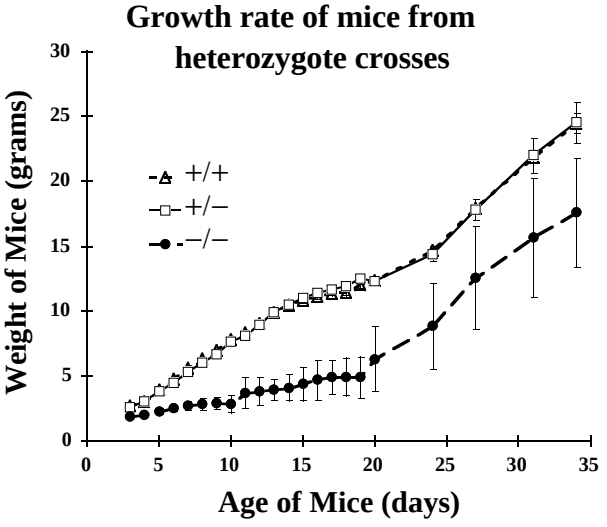

Fig. 4. Growth curves from a representative litter from a double heterozygote cross show that *mf3*+/+ (open squares, dashed line) and *mf3*+/– (open triangles) pups have the same growth rate while *mf3– /–*mice (closed circles, dashed line) show a slower growth rate from day 2 until 3 weeks after birth.

Because *mf3–/–* females appeared deficient in generating an OT surge, we examined the synthesis of OT in the *mf3–/–* mice. OT is secreted into the blood in response to many different stimuli, including the suckling of pups, hemorrhage, or other stresses such as the anesthesia required to collect blood (Forsling, 1986). Despite these caveats, we measured OT levels in the serum of *mf3*+/+, +/– and –/– females 1 day after they gave birth to a litter of pups. Average OT levels were 80.9±14.4 pg/ml (*n*=7) in a *mf3–/–* mother, 217.9±97.7 (*n*=8) in a +/– mother and 47.6±14.4 (*n*=7) pg/ml in a wild-type mother 1 day after giving birth. These data were highly variable, but showed no correlation with the nursing ability of different *mf3* genotypes. However, they illustrate that there is no inherent defect in the secretion of OT into the bloodstream of *mf3–/–* animals.

Cells in the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) of the hypothalamus synthesize OT (Cunningham and Sawchenko, 1991; Swanson and Sawchenko, 1983) and immunohistochemistry revealed that wild-type and *mf3–/–* animals make immunoreactive OT in both the SON and the PVN (Fig. 7 and data not shown for the SON). Since we were unable to determine if the serum RIA variations were due to sample collection, we quantitated the number of cells making OT in both the SON and the PVN wild-type and *mf3– /–* animals by immunohistochemistry. Serial sections from three adult female brains of each genotype were stained for OT, and immunopositive cells were counted. The number of cells synthesizing OT was highly variable, especially in the *mf3–/–* animals, but the numbers were reduced by about 40% in the *mf3–/–* females (2667±310.4 OT positive cells for *mf3*+/+ mice and 1475±387.8 for *mf3–/–* mice). This reduction in cell number could be accounted for by a proportional reduction of the hypothalamus with the size of the mice. Taken together, these data suggest that the cells making OT are present and can synthesize and secret OT, but that the OT surge

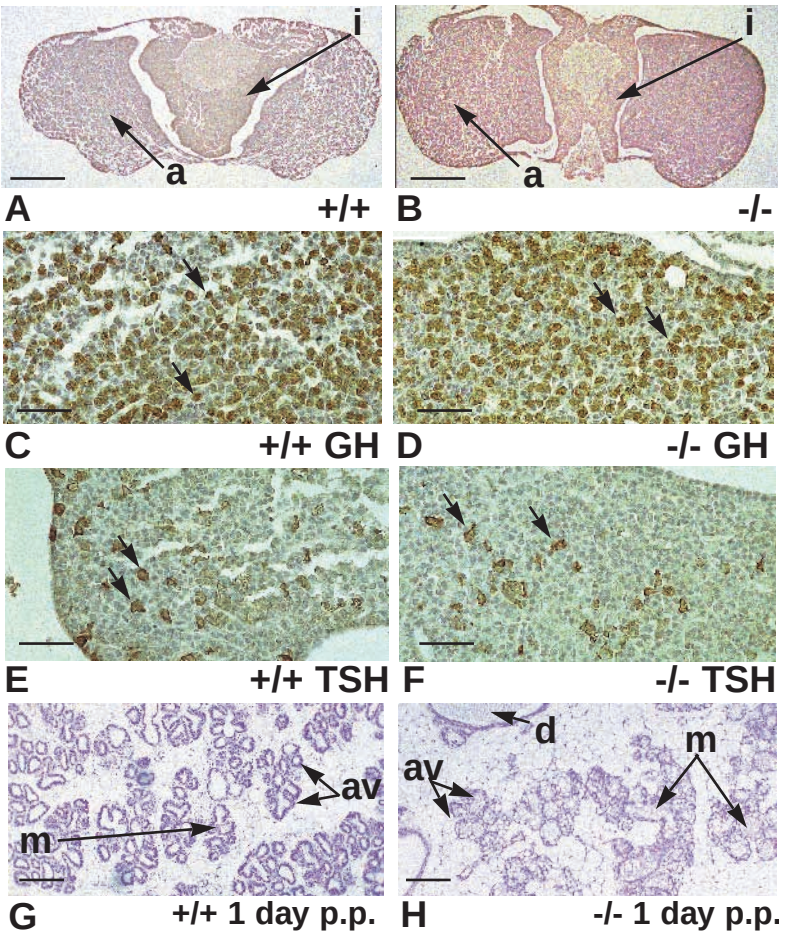

Fig. 5. Pituitary and mammary glands from *mf3*–/– adult animals have an apparently wild-type morphology and histology. (A,B) Low magnification views of *mf3*+/+ and – /–pituitary glands stained for ACTH, showing that the overall structure of the pituitary is normal. The anterior and intermediate lobes are indicated. (C,D) Higher magnification of *mf3*+/+ and –/– pituitary glands stained for GH illustrates that both pituitaries contain somatotrophs (representative positive cells indicated by arrows). (E,F) *mf3*+/+ and –/– pituitary glands stained for TSH illustrates that both pituitaries contain thyrotrophs (representative positive cells indicated by arrows). Histological analysis of *mf3*–/– mammary glands by hematoxylin and eosin staining. Despite the observation that *mf3*–/– mothers do not let down their milk, their mammary glands show normal differentiation during pregnancy. One day after giving birth, both *mf3*+/+ (G) and –/– (H) mammary glands show the highly branched morphology characteristic of pregnancy, with many well developed alveoli and an accumulation of milk in the ductules. The *mf3*–/– mammary gland contains more alveoli that are full of milk, presumably because the milk cannot be ejected to the pups. Scale bars, 500 μm in A and B; 50 μm in C-F; 200 μm in G and H. a, anterior lobe; ad, adipose tissue; av, alveoli; d, ducts; GH, growth hormone; i, intermediate lobe; m, milk; TSH, thyrotropin; 1 day p.p., one day post partum.

necessary to induce milk ejection is either not generated at all, or it is not of sufficient amplitude to be functional.

The hypothalamus of *mf3*–/– animals is histologically normal

Gross morphological examination of the brain and pituitary of normal appearing *mf3*–/– embryos and adult mice revealed no differences compared to wild type. Fig. 8A and B illustrates this in coronal sections of 15.5 days p.c. wild-type and *mf3*– /– mutant embryos. The adult brain was also histologically normal at the level of hematoxylin and eosin staining; Fig. 8C,D show coronal sections through an adult wild-type and *mf3*–/– brain at the level of the mammillary bodies, the site of the strongest *Mf3* expression.

Because the injection of OT allowed *mf3*–/– mothers to nurse their pups, we examined other hypothalamic markers in the *mf3*–/– mutant animals to see if the lactation defect might be explained by inappropriate cell differentiation in regions of the hypothalamus outside the mammillary region. Many molecular markers are expressed in specific regions of the hypothalamus during pre- and postnatal development. We used immunohistochemistry to examine the expression pattern of several POU domain proteins in the brains of 5 day old pups to see if their expression patterns were affected in the *mf3*–/– animals (Fig. 9). BRN-1 and TST-1 are expressed in the ventromedial nucleus (VMH) of the hypothalamus of wild-type and *mf3*–/– brains. BRN-2 and BRN-4 are expressed in the PVN of the hypothalamus, although it should be noted that BRN-2 and BRN-4 are often, but not always, co-expressed in the same cells of the PVN (Schonemann et al., 1995). The

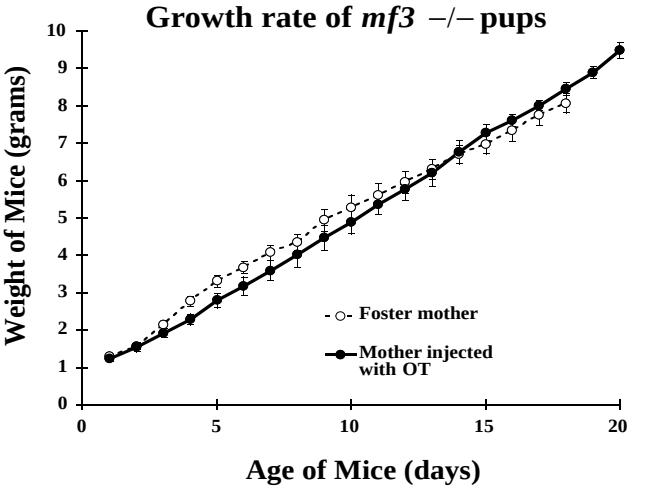

Fig. 6. Growth curves for *mf3*–/– pups fostered onto an ICR mother (open circles, dashed line), or fed by their *mf3*–/– birth mother injected with 600U/kg OT 2-3 times per day (closed circles, solid line), show that the growth retardation is not corrected by a wild-type foster mother's milk. Additionally, this shows that a *mf3*–/– mother can effectively nurse her pups when injected with OT, and that there are no nutrient defects in the *mf3*–/– mother's milk.

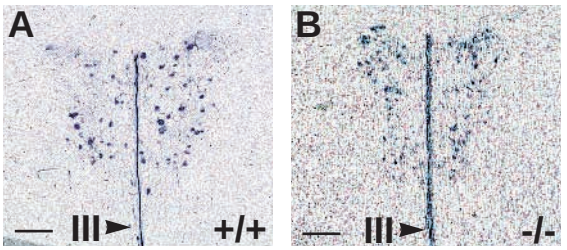

Fig. 7. Immunohistochemical staining of coronal sections of brains from a wild-type (A) and *mf3*–/– (B) animal for oxytocin shows that animals of both genotypes are making oxytocin in the paraventricular nucleus. Scale bars are equal to 50 μm. III, third ventricle.

expression of these hypothalamic markers was unchanged between wild-type and *mf3*–/– animals, suggesting that not only the gross morphology of the *mf3*–/– hypothalamus is normal, but that many aspects of the overall differentiation of specific cell types is also normal.

DISCUSSION

The winged helix gene, *Mf3*, is expressed at high levels in two distinct domains in the embryo; the posterior presomitic mesoderm and the neurectoderm (Fig 2). The neuroectodermal expression is most prominent in the neural tube and future diencephalon and midbrain, eventually becoming restricted to a strong region of hybridization in the mammillary bodies of the hypothalamus.

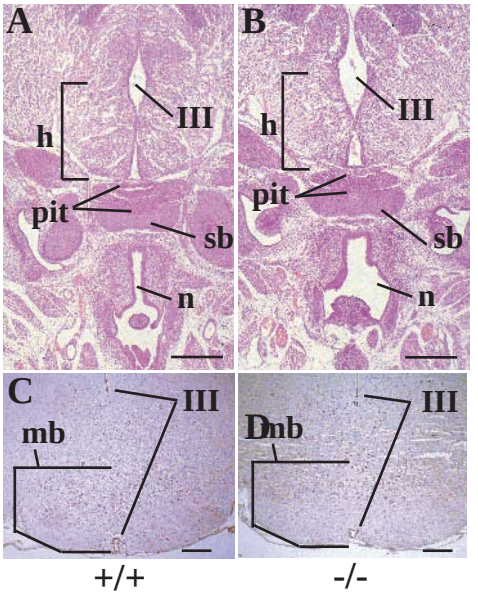

Fig. 8. Histological examination by hematoxylin and eosin staining of coronal sections of 15.5 days p.c. embryos (A shows a wild-type and B is a *mf3*–/– embryo) and adult mice (C shows a wild-type and D is a *mf3*–/– brain). The hypothalamus and pituitary (denoted by brackets) are unaffected in the *mf3*–/– 15.5 days p.c. embryos. The mammillary bodies (bracketed on the left side of the brain) of a adult *mf3*–/– mouse are also histologically normal. Scale bars in A and B are equal to 500 μm, while scale bars in C and D are equal to 100 μm. h, hypothalamus; mb, mammillary bodies; n, nasopharynx; pit, pituitary gland; sb, sphenoid bone, III, third ventricle.

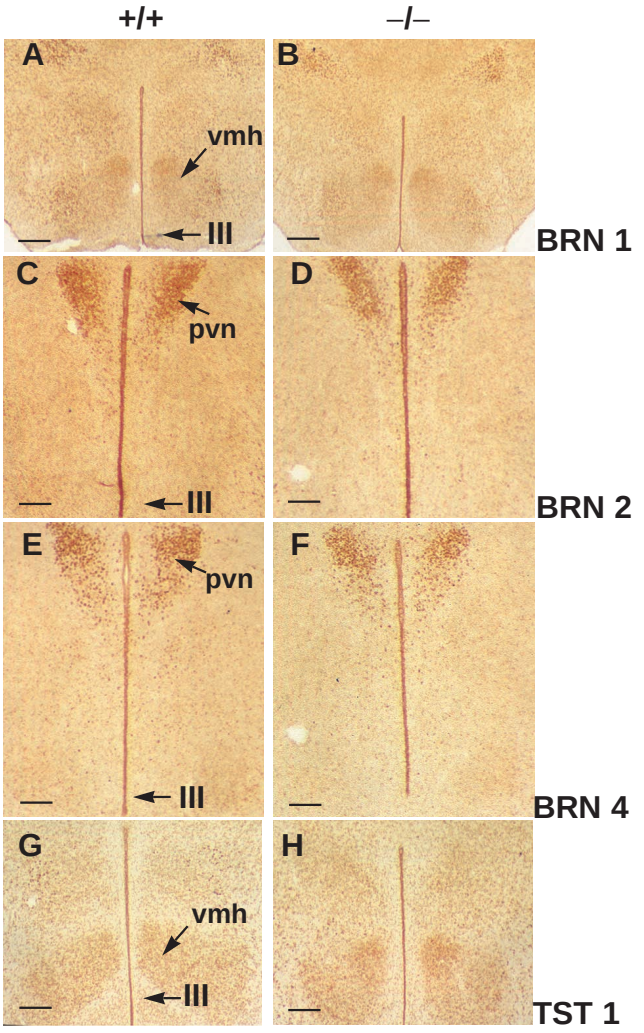

Fig. 9. Immunohistochemical analysis of the hypothalamus of pups 5 days after birth for proteins in the POU transcription factor family. The localization and gross numbers of cells staining for BRN1 (A,B), and BRN2 (C,D) are not altered in *mf3*–/– animals (B,D) when compared to wild type (A,C). BRN1 is expressed in the ventromedial nucleus of the hypothalamus while BRN2 is expressed in the periventricular nucleus. Similar results were seen for BRN4 (E,F) and TST1 (G,H). Scale bars, 50 μm. III, third ventricle; pvn, paraventricular nucleus; vmh, ventromedial nucleus.

We have used targeted mutagenesis in ES cells to generate animals lacking functional MF3. The mutation was maintained in [129/SvEvTac × Black Swiss] mice, and on this mixed genetic background the phenotypes of *mf3*–/– homozygotes fall into several classes, presumably due to the effect of different combinations of modifier genes. First, 11% of *mf3*– /– embryos die in utero by about 14.5 to 15.5 days p.c., and are either very small with multiple developmental abnormalities (Fig. 3C, left and Table 2), or show specific defects in the growth and/or development of the diencephalon and midbrain (Fig. 3C right and B and D left, and Table 2). Second, the *mf3*– /–pups that survive to birth have a reduced pre-weaning growth rate and approximately one third die before weaning (Fig. 4 and Table 1). Surviving animals are smaller than their

wild-type and heterozygous siblings, but are generally healthy and fertile, and some have lived for over a year. The other postnatal functional defect we have detected is the inability of *mf3–/–* mothers to respond to suckling by ejecting milk to feed their pups. Although the *mf3–/–* mice make OT and secrete it into the bloodstream, they are apparently unable to produce a functional surge of OT in response to suckling, thus disrupting the milk ejection reflex necessary for nursing.

Prenatal lethal phenotypes

Although only a small proportion of *mf3–/–* embryos die in utero, those affected consistently show one of two phenotypes. The most severe, involving reduced size and an open neural tube along two thirds of the embryo (Fig. 3C left) is consistent with defects in both the presomitic mesoderm and the anterior neurectoderm and spinal cord, all tissues in which *Mf3* is normally expressed. The second phenotype, involving a more specific reduction of tissue in the forebrain and midbrain region, might be explained by the expression of another WH protein compensating for the lack of MF3 in both the presomitic mesoderm and most of the spinal cord, but not in the developing brain.

It has been shown by our lab and others that several WH genes are expressed in the presomitic mesoderm and/or somitic tissues, including *Mfh1, Mf1* and *Mf2* (Miura et al., 1993, Sasaki and Hogan, 1993). Moreover, there are at least four WH genes in addition to *Mf3* expressed in the developing mammillary region of the embryonic hypothalamus: *Bf1, Bf2, Fkh4* and *Mf2* (Hatini et al., 1994; Kaestner et al., 1996; and data not shown for *Mf2*). It is therefore likely that the expression of these WH genes, or others, partially or completely compensates for the lack of MF3 in the presomitic mesoderm and neurectoderm in the less affected prenatal lethal and surviving *mf3–/–* embryos. Experiments are in progress to test this hypothesis by generating embryos carrying mutations in both the *mf3* and *mfh1* loci.

In situ hybridization analysis of the *mf3–/–* embryos with abnormal forebrain and midbrain development showed that the affected region extends between the posterior limit of the diencephalon, marked by *Bf2* expression, and the midbrain-hindbrain junction, marked by *Fgf8* expression (Fig. 3 and data not shown). Moreover, there is no obvious defect in dorsal-ventral patterning as judged by the expression of *Pax3, Islet1, Shh* and *Msx1* (Fig. 3), suggesting that the defect most likely involves a reduction in cell proliferation in the regions where *Mf3* is expressed, rather than an overall change in neural patterning. A neurectodermal proliferation defect has been seen in the telencephalon of *Bf1–/–* mutant embryos, although in this case there was also precocious differentiation of the cells in the telencephalon (Xaun et al., 1995). More studies will be needed to test the possibility that cell proliferation is affected similarly in *mf3–/–* embryos. However, these experiments must await placing the *mf3* mutation on a uniform genetic background to increase the penetrance of the embryonic lethal phenotype.

While it is possible that the overlapping expression of other WH genes may compensate for the lack of MF3 in most (89%) of the *mf3–/–* embryos, allowing the diencephalon and midbrain to develop relatively normally and the mice to survive to birth, there must nevertheless be subtle defects in the surviving *mf3–/–* mice that are responsible for the postnatal phenotypes of growth retardation and lactation defects.

Growth regulation in *mf3–/–* animals

All surviving *mf3–/–* mice are smaller than their siblings. The only behavioral difference between *mf3–/–* animals and wild-type mice is the observation that all *mf3–/–* mice exhibit an abnormal clasping together of their hind legs toward the ventral midline when suspended by the tail. The cause of this behavior is unknown although it is similar to the first neurodegenerative symptoms in a mouse model for Huntington's disease (Mangiarini et al., 1996) and is observed in mice carrying a null mutation for the cyclin D1 gene (Sicinski et al., 1996). One third of *mf3–/–* pups die before weaning. These are severely runted and dehydrated at the time of death, and even the early removal of competing siblings does not improve their health status (data not shown). The growth difference between wild-type and *mf3–/–* animals is especially prominent during the first three weeks of life, and the growth curves after this time show that *mf3–/–* mice and wild-type siblings grow at the same rate after weaning. An additional observation is that unlike wild-type mice, *mf3–/–* mice aged to over one year never become fat. This growth deficiency is independent of the milk supply and does not seem to be a consequence of nurturing differences.

It is well known that growth hormone (GH) regulates growth. However, although highly variable, the level of circulating GH in *mf3–/–* animals is not appreciably different from that in *mf3+/+* or *+/–* animals, at either 1 week of age or in adults. Additionally, GH immunostaining shows no major differences in the overall population of somatotrophs in the *mf3– /–* pituitary, a result in sharp contrast to other dwarf mutants such as the Snell, Jackson and Ames dwarf mutants, and *little* mice (Li et al., 1990; Lin et al., 1993). The *mf3–/–* mice synthesize GH in their pituitaries, and secrete it into the bloodstream, but still fail to reach normal weight. Although *Mf3* is expressed in the hypothalamus, the proteins that regulate GH, somatostatin and growth hormone releasing factor, are expressed in the arcuate and periventricular nuclei of the hypothalamus, regions further rostral than the mammillary bodies that express *Mf3* (Epelbaum, 1992). Other mice with a growth deficiency that also do not have a hypothalamic-pituitary defect are those carrying a null mutation for Cyclin D1 (Fantl et al., 1996; Sicinski et al., 1996). These mice show the abnormal clenching behavior that we have observed in the *mf3–/–* mice and also have a lactation defect. However, in the case of *cyclin D1–/–* mice the lactation defect is due to failure of the mammary glands to undergo branching and produce milk rather than a defect in the milk ejection reflex. Taken together, these data suggest that *Mf3* may not be involved with growth regulation at the level of GH production and secretion, but instead may be important in the physiology of feeding or nutrition in juvenile mice. It is therefore possible that neurosecretory mechanisms regulating feeding behavior and satiety setpoint, such as those mediated by neuropeptide Y (NPY) or leptin are defective in these mice.

Regulation of milk ejection in *mf3–/–* animals

Unlike mice mutant for the *FosB* gene (Brown et al., 1996), *mf3–/–* mothers have a normal nurturing response. They make nests and allow their pups to suckle; notably, they even keep

the dead babies in the nest, arguing against hormonal or behavioral defects in nurturing. The mammary glands of *mf3–/–* mothers develop normally during pregnancy and contain milk (Fig. 5H). Most importantly, when these mothers are injected with oxytocin twice a day, they then let down their milk. This defective suckling phenotype is identical to that seen in *oxytocin–/–* mice (Nishimori et al., 1996). However, because the *mf3–/–* animals can make OT in the correct cells and secrete it into the blood, we conclude that it is the inability to generate the normal 100–150 fold surge in plasma OT in response to suckling that prevents milk let down (see Robinson, 1986; Cunningham and Sawchenko, 1991, for review of the milk-ejection reflex and the role of oxytocin).

Role of the mammillary bodies

The mammillary region is the caudal-most part of the hypothalamus. Unlike other nuclei in the hypothalamus, there are no specific functional cell populations identified in the mammillary bodies, although much is known about their neuroanatomy and interneuronal connections made between them and other regions of the brain. For example, there is evidence that axons originating in, or passing through, the premammillary region are intimately and functionally connected to many parts of the hypothalamus (Zaborszky and Makara, 1979). More recent analyses of the connections within the hypothalamus showed no direct link between the OT expressing cells of the PVN or SON with the mammillary bodies, but did reveal connections to the supramammillary nucleus and the premammillary nucleus (Sawchenko and Swanson, 1983). It is possible that either the supramammillary nucleus, the premammillary nucleus, or both, serve as a relay connection involved in transmitting the milk-ejection reflex. Because of the lack of antibodies to detect them, it is not yet known which specific neurons express *Mf3* and might therefore be directly affected in the *mf3–/–* mice. Although much remains to be learned about the function of this region of the hypothalamus, we have uncovered a link between a transcription factor expressed in the mammillary bodies of the hypothalamus and a growth regulatory/milk ejection response, raising important questions about the organization and function of the hypothalamus.

In conclusion, we have provided evidence for multiple roles for the WH gene, *Mf3*. First, the early embryonic phenotype of some of the *mf3–/–* embryos illustrates that *Mf3* is involved in the normal development of the diencephalon and midbrain, and the normal proliferation of the presomitic mesoderm. Second, postnatal growth regulation is severely affected in all surviving *mf3–/–* mice, and they show neurological defects characterized by abnormal hindlimb clenching. Finally, *mf3–/–* mothers are unable to eject their milk supply to their pups. Presently it is not clear whether these postnatal defects are a result of subtle defects in the patterning of the hypothalamus and/or other regions of the brain caused by a lack of *Mf3* during embryogenesis, or if the postnatal expression of *Mf3* plays a role in growth regulation and/or the milk-ejection reflex. Future experiments will address these issues.

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